JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12597

Strong and parallel salinity-induced phenotypic plasticity in one generation of threespine stickleback

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Keywords:

adaptive radiation;
allometry;
morphometrics;
phenotypic plasticity;
threespine stickleback.

Abstract

Phenotypic plasticity is a major factor contributing to variation of organisms in nature, yet its evolutionary significance is insufficiently understood. One example system where plasticity might have played an important role in an adaptive radiation is the threespine stickleback (*Gasterosteus aculeatus*), a fish that has diversified after invading freshwater lakes repeatedly from the marine habitat. The parallel phenotypic changes that occurred in this radiation were extremely rapid. This study evaluates phenotypic plasticity in stickleback body shape in response to salinity in fish stemming from a wild freshwater population. Using a split-clutch design, we detected surprisingly large phenotypically plastic changes in body shape after one generation. Fish raised in salt water developed shallower bodies and longer jaws, and these changes were consistent and parallel across families. Although this work highlights the effect of phenotypic plasticity, we also find indications that constraints may play a role in biasing the direction of possible phenotypic change. The slopes of the allometric relationship of individual linear traits did not change across treatments, indicating that plastic change does not affect the covariation of traits with overall size. We conclude that stickleback have a large capacity for plastic phenotypic change in response to salinity and that plasticity and evolutionary constraints have likely contributed to the phenotypic diversification of these fish.

Introduction

Phenotypic plasticity, when one genotype is able to produce different phenotypes in different environments, is a universal property of organismal life (Bradshaw, 1965; West-Eberhard, 2003). Plasticity may be adaptive, neutral or maladaptive with respect to an individual's fitness. Adaptive phenotypic plasticity is expected to enable organisms to better cope with heterogeneous environments (Doughty & Reznick, 2004; Pigliucci, 2005; Fischer *et al.*, 2009), and indeed, this has been documented in numerous different empirical systems (Scheiner, 1993; Schlichting & Pigliucci, 1998; Schlichting, 2004; Pigliucci, 2005). Debate continues about whether phenotypic plasticity can additionally hinder or facilitate genetic adaptation (West-Eberhard, 1989; Losos *et al.*, 2000; Ghalambor *et al.*, 2007); the presence of plasticity can change the phenotypes available for selection after exposure to a new environment, thus there is the possibility that further genetic adaptation would be influenced by previous plastic changes. The potential importance of phenotypic plasticity in diversification and adaptation is not sufficiently understood (West-Eberhard, 1989; Schlichting, 2004; Pigliucci, 2005).

The threespine stickleback, *Gasterosteus aculeatus,* is a fish well known for having undergone adaptive radiation following the end of the last glaciation, 10 000–12 000 years ago, when it repeatedly colonized and adapted to coastal freshwater environments from its saltwater origins (Bell & Foster, 1994). Freshwater populations are comprised of individuals that are smaller and less armoured than those in marine or anadromous populations, and they also have shorter or fewer spines compared with their marine ancestors. These morphological similarities across lake populations are typically interpreted as the result of parallel genetic adaptation to a novel environment (Schluter, 1996; Foster & Baker,

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2004; Colosimo *et al.*, 2005). A selective advantage of having fewer lateral plates in freshwater compared with marine habitats has been detected (Barrett *et al.*, 2008, 2011; Le Rouzic *et al.*, 2011), and it has been shown that this fitness advantage can be related to the differences in predator regimes or intensity between these habitats, for example (Reimchen, 1983, 2000). However, convincing evidence for the many potential selective agents that have been proposed to explain the parallel evolutionary changes in morphology across freshwater stickleback populations is rare (Voje *et al.*, 2013; Smith *et al.*, 2014; MacColl & Aucott, 2014; Spence *et al.*, 2013).

Stickleback are known to have remarkable phenotypic plasticity in their physiology in that they are able to osmoregulate in a wide range of salinities at no apparent cost (Heuts, 1947; Grøtan *et al.*, 2012; Taugbøl *et al.*, 2014). Several experiments have also revealed plasticity in morphology in this species in response to food type (Lucek *et al.*, 2014; Wund *et al.*, 2008; Day *et al.*, 1994; Svanbäck & Schluter, 2012), environmental complexity (Garduño-Paz *et al.*, 2010), and a combination of these two cues (Wund *et al.*, 2012). Transgenerational plasticity has recently been shown to exist in stickleback in response to exposure to elevated temperatures (Shama & Wegner, 2014; Shama *et al.*, 2014). One study has previously tested for effects of salinity on body shape (McCairns & Bernatchez, 2012). However, this study used fish from an open system of varying salinity, and in addition, the morphological changes they found were described exclusively via partial warp scores and not in terms of physical shape differences, making it difficult to interpret these results biologically. Therefore, it remains unclear whether and how stickleback morphology, in addition to physiology, shows a significant plastic effect in response to salinity.

Here, we present an experimental study where we test whether freshwater stickleback are plastic in their morphology in response to developing in different salinities. The purpose of this study was to evaluate if part of the diversity in morphology that is observed in nature could be due to plastic responses. To this end, we used a split-clutch design to control for genetic diversity. To assess differences in morphology, we applied geometric morphometric tools. We also contrast the magnitude of the salinity-induced morphological change and variability of our experimental fish with differences in morphology observed between natural freshwater and marine populations collected from the same general region.

Materials and methods

Sampling

Parental stickleback were collected from lake Glitredammen (59.931767°N, 10.498728°E) in Baerum, Norway, in late June, 2013. Fish were collected using coated metal minnow traps baited with cheese (Breder, 1960, chamber 100 cm long, 40 cm diameter, 1 cm openings). Any nonreproductive fish were released on site, whereas reproductive fish were transported immediately to the laboratory at the University of Oslo where they were kept in fresh water until the crosses were made, which was within one week of the fish being collected.

Breeding and husbandry

A total of nine full-sibling families were created. Parental fish were euthanized using benzocaine solution (one part benzocaine, five parts water). The female's eggs were stripped manually into a petri dish with some embryo medium (15 ppt saltwater solution). The testes were removed from the male surgically, macerated, mixed with embryo medium and placed on the eggs. After 2 min, the liquid was removed, the eggs washed and submerged in clean embryo medium.

One day after fertilization, the eggs were separated using sterile plastic probes to mimic the father's activity in the wild. About 80% of the embryo medium in each dish was changed twice daily, and dead or unhealthy embryos were removed. After hatching (approximately after 5 days), each family of surviving larvae were split into two and transferred to treatments, one of fresh water (0 ppt) and one of salt water (25–30 ppt). In total, 575 live embryos were allocated to the treatments. The initial volume of these tanks was 1 L; water was changed 50% twice daily. After one week, the volume was increased to 2 L and water was changed 50% once each day.

After the yolk sacs of the larvae were consumed, about one week after hatching, they were fed twice a day *ad libitum* with live brine shrimp nauplii (genus *Artemia)*. 32 days after hatching, the feeding was changed to a mix of brine shrimp and chopped frozen blood worms (chironomid larvae). The proportion of worms was increased until all fish were large enough to consume this food, then they were fed only worms. Feedings were increased to three times a day for the final month.

At approximately day 75 after hatching, the fish were transferred to 40-L aquaria with an automated flow-through water system with UV-radiation filters, nitrogen filters and oxygen supply. They were kept until the age of 6 months, when the smallest fish had reached a minimum length of 30 mm to ensure complete armour development (Hagen & Gilbertson, 1973). All major biological markers of development, such as development of the eye, yolk sac depletion, onset of feeding and growth rate, were the same for the two treatments, ensuring that the fish remained at the same life stage. The fish were kept on a summer light schedule (19 : 5 h light : dark).

All surviving fish ($n = 489$) were killed in late December 2013, euthanized in benzocaine solution (one part benzocaine, five parts water). Immediately after euthanization, fish were each assigned an identification number. Imaging was performed using a Canon flat-bed CanoScan 9000F scanner following the methods of (Herler *et al.*, 2007). The left side of each fish was scanned while the fish was submerged in water. The fish were then stored on 96% ethanol.

Shape analysis

We placed 20 landmarks on each picture (Fig. 1) using TPSDIG version 2.16 from the Thin Plate Spline (TPS) software suite (Rohlf, 2005). Morphometric shape analysis was done with MATHEMATICA 8 (Wolfram Research, Inc., Champaign, IL, USA) on the 20 × 2 landmark coordinates. First, a Procrustes superimposition was created for the entire landmark data set of the fish ($n = 469$) to remove variation due to position, size and orientation (Rohlf & Slice, 1990; Mitteroecker & Gunz, 2009). The resulting Procrustes shape coordinates were analysed by principal component analysis (PCA). The effect of body size on body shape was estimated by a shape regression (multivariate regression of the Procrustes coordinates on log body length). This regression was later used to identify which one of the PC axes represented size-dependent shape changes. We used vector plots to visualize the differences between the mean shapes of the treatment groups, and thin-plate spline deformation grids to visualize shape change along particular axes in shape space (Bookstein, 1991). None of the principle component (PC) axes clearly captured the treatment effect; the variation due to the treatment was rather spread across several PCs. To disentangle the variation due to the treatment from variation due to other factors, we projected the Procrustes shape coordinates onto the treatment axis (orthogonal projection). The treatment axis was defined as the axis that connects the mean saltwater treatment shape with the mean freshwater treatment shape across families. The percentage of variance explained by the salinity treatment was calculated as the fraction of the variance explained by this axis divided by total variance, which is defined as the trace of the covariance matrix. Permutation tests using Monte Carlo sampling were applied to test for shape differences in treatment group means with Procrustes distance as a test statistic (Good, 2000).

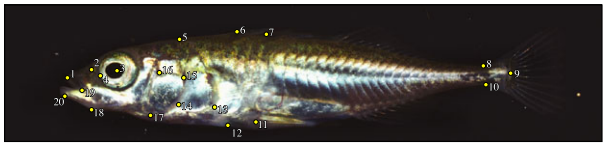

Fig. 1 Positions of the 20 landmarks that were used to characterize body shape and define the linear measurements in the sticklebacks. All landmarks were used in the shape analysis; the 12 linear traits were based on the following pairs of landmarks: body length (L1–L9), eye radius (L3–L4), mouth length (L1–L19), jaw length (L18–L20), distance from snout to eye (L1–L4), head depth (L5–L17), head length (L1–L15), body depth (L7–L11), tail width (L8–L10), length of operculum and pectoral area (L13–L16), pectoral area length (L13–L14) and the distance from snout to start of operculum (L1–L16).

Linear measurements

We extracted 12 linear measurements of morphological traits for each fish using R v.2.10.1 (R Developmental Core Team, Vienna, Austria), see Fig. 1: body length (L1–L9), eye radius (L3–L4), mouth length (L1–L19), jaw length (L18–L20), distance from snout to eye (L1–L4), head depth (L5–L17), head length (L1–L15), body depth (L7–L11), tail width (L8–L10), length of operculum and pectoral area (L13–L16), pectoral area length (L13–L14), and the distance from snout to start of operculum (L1–L16). These traits were picked because variation in these traits may represent ecologically important adaptations to freshwater and marine environments as hypothesized from differences in wild-caught freshwater and saltwater sticklebacks (Taugbøl *et al.*, 2013; Aguirre & Bell, 2012).

We tested for an effect of the salt- and freshwater treatment on log-transformed linear trait measurements using linear mixed effect models as implemented in the package NLME in R.v.2.10.1. Family was treated as a random effect, and treatment was considered a fixed effect. We included body length as a covariate to test for a treatment-specific effect of body length on each linear trait, that is, for a plastic response in the static allometric slope (the slope of the linear relationship between two log-transformed traits of individuals in the same developmental stage) across salt- and freshwater treatment fish. Based on the AIC scores of these models, we decided which allometric model fit each linear trait best, that is whether fish from the two treatments had the same or different intercept and slope parameters.

We additionally included an interaction between family and treatment as a random effect using the package LME4, and we compared these models using cAIC. Such an interaction could potentially indicate genetic variation in plasticity across families. Only for four of 12 of the linear traits, the model with this interaction was marginally better in terms of cAIC than the model that included solely family as a random effect. The intercept and slope parameter estimates for the fixed effects (treatment, body size and the interaction between these two variables), however, did not differ between the models with and without the interaction in the random effects for three of the four traits (change in intercept parameters was < 0.2% for all four models, change in slope parameter was < 1.2% for three of the four models). Only for mouth length, the slope estimate differed

by 7.7%. Because of these minor effects of the additional interaction term on the parameters, we did not include the random effect interaction term between treatment and family in the further analyses.

We mean-centred the log body length of fish within each treatment around zero to make the intercept in the model equal to the trait mean within each treatment. This standardization enabled us to estimate the proportional trait change across treatment as the ratio of the two intercepts if no plastic response in the allometric slope was detected.

We also tested for an effect of the saltwater and freshwater treatment on survival and body length using linear mixed effect models. First, we evaluated the effect of treatment alone on body size and survival, respectively. Subsequently, family was added as a random factor to the two models.

Magnitude of change and variability compared to natural populations

We compared the observed shape differences between our saltwater and freshwater treatments with the shape differences in a large sample of wild-caught freshwater ($n = 74$) and saltwater ($n = 13$) populations across Norway (10 fish per population, data from Voje *et al.*, 2013) to relate the magnitude of induced phenotypic plasticity in our experiment to phenotypic differences from natural populations. For this purpose, we restricted our comparison to the subset of 14 landmarks that were available from both studies (landmarks 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 17, 18 and 19). Shape differences between salt- and freshwater fish were calculated as Procrustes distances between group mean shapes. The shape differences were also visualized by vector plots. To assess whether the plastic shape change in our experiment was in the same direction as the shape differences between wild-caught freshwater and marine fish, we projected the Procrustes shape coordinates of all individuals from both samples onto the wild-caught shape axis. This axis was defined as the axis that connects the mean marine shape with the mean freshwater shape in the wild-caught sample. We further compared the variance in shape between the fish from our experiment and the wild-caught fish (Voje *et al.*, 2013). Total variance was again used as a measure for multivariate variability. We tested for the equality of variances in fresh- vs. saltwater fish populations using a Monte Carlo permutation test with total variance as a test statistic.

Results

From the transfer of the larvae to the treatments until the end of the experiment, the survival rates were 0.88 (standard deviation, SD = 0.05) in salt water and 0.83 (SD = 0.07) in fresh water across the 9 families. Although survival was consistently high in both treatments, fish survived slightly better in salt water (5.2% higher survival in salt water, $P < 0.0001$, Adding family as a random factor did not affect this result; also see Table S1). Our final sample consisted of 96.6% of all the fish that survived until the end of the experiment. The remaining 3.4% were excluded due to physical deformity or because the scans of these fish were of insufficient quality. A total of 469 fish from the nine families, 241 fish raised in salt water and 228 raised in fresh water, respectively, with an average of 52 offspring per family (SD = 12.8, see Table S1 for offspring counts per family) were used for the following analyses.

Variation in the first principal component in our analysis of the shape coordinates was due to a positioning artefact, which happens frequently in shape studies whenever 3D objects are recorded by 2D imaging techniques (Wund *et al.*, 2008; Valentin *et al.*, 2008; Voje *et al.*, 2013; Ramler *et al.*, 2014; Arif *et al.*, 2013). As we used fresh fish that were scanned right after killing, we avoided extreme post-mortem curvature seen in several earlier studies, and the positioning artefact was limited to a relatively mild bending and tilting effect during imaging (compare Wund *et al.*, 2008; Valentin *et al.*, 2008; Ramler *et al.*, 2014). Deformation grids for the shape variation along this principal component and inspection of extreme individuals (see Figs S1 and S2) showed that this was an artificial component of variation due to slight bending of the body and correlated tilted positioning of the fish on the glass surface of the scanner, and not an actual component of shape variation. Inspection of the deformation grids for all other PCs up to PC10 showed that PC1 was the only axis associated with a positioning artefact and variation due to positioning was also independent of the treatment, family and body size. We therefore removed this component of variation from the data by projecting the shape coordinates into a subspace orthogonal to the first PC (Valentin *et al.*, 2008; Ramler *et al.*, 2014). In the joint PCA of the experimental and the wild-caught sample, this artificial component of variation was removed in the same fashion. All the following shape analyses, including the subsequent PCA, were then based on the residual shape data.

Morphological variation

Body length did not differ between treatments (linear model, $P = 0.85$; adding family as a random factor did not affect this result, $P = 0.91$); there was, however, variation in body length within each treatment. On average, fish had a body length of 34.8 mm (SD = 2.8 mm) in the saltwater treatment and 34.8 mm (SD = 2.6 mm) in the freshwater treatment at the end of the experiment.

Although treatment did not affect body length, body shape did change: Fish raised in salt water developed a longer, narrower head and a more slender body

(Fig. 2a). Shape differences due to the treatment, calculated as variation along the treatment axis, amounted to 16.4% of the total body shape variation (Fig. 3). The shape difference between the salt- and freshwater treatments was also confirmed by a permutation test ($P < 0.001$). The shape differences were mostly parallel across families, particularly in the head region where most changes were found (see particularly landmarks 1, 18 and 20 in Fig. 2b).

In the principal component analysis, the first PC explained 26.2% of the overall variation of the Procrustes shape coordinates. The PC scores along this axis had the highest correlation with log body length ($r = 0.31$), compared with all other PC axes scores. Furthermore, the regression scores from a multivariate regression of the Procrustes coordinates on log body length correlated strongly with the PC1 scores ($r = 0.95$). PC1 therefore captured variation in shape due to differences in body length (Table 1 and Fig. 4). PC2 explained 16% of the variation and represents variation in relative tail length (Fig. 4). PC3 explained 13% of the shape variation and represents variation in relative head length (Fig. 4).

We also found significant differences across treatments in the analyses of the linear traits using linear mixed models (Table 2). Treatment led to a statistically significant change in mean trait size in 10 of 12 traits between fish raised in fresh water and salt water, which corresponds to a plastic response in the static allometric intercept across treatments in these traits (Fig. 5a,b show traits where the mean trait size changed, whereas Fig. 5c shows a trait that did not differ between treatments); however, we detected no significant differences in the interaction between trait size and body size (i.e. the allometric slope) in any of the twelve traits between the fresh- and saltwater treatment groups. Fish in the saltwater treatment had a proportional change in eye radius of 2.4% compared with fish in salt water, which means the radius increased on average 0.01 mm in salt water. The eyes are also located further back on the head in fish raised in salt

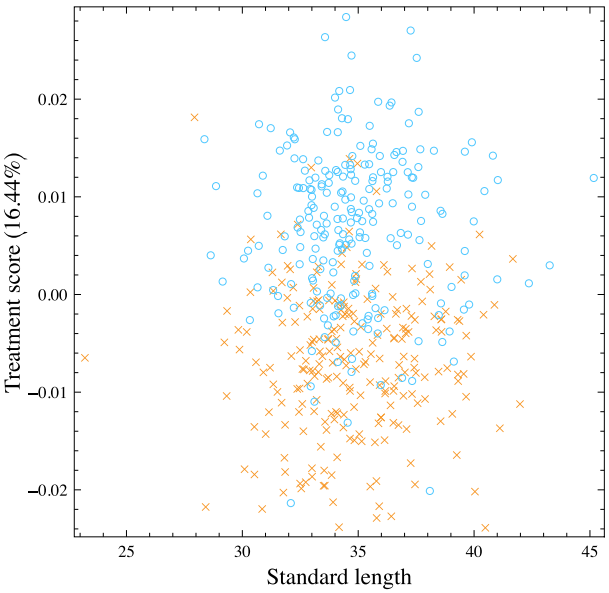

Fig. 3 Treatment effect vs. body length. The freshwater–saltwater treatment axis is based on the vector that points from the mean freshwater body shape to the mean saltwater body shape and it explains 16.44% of the total variation. Shape data of saltwater treatment fish (orange exes) and freshwater treatment fish (blue circles) were orthogonally projected onto this vector. The resulting treatment score is shown together with the body length of the fish. There is a clear difference in mean value caused by treatment but not by body length.

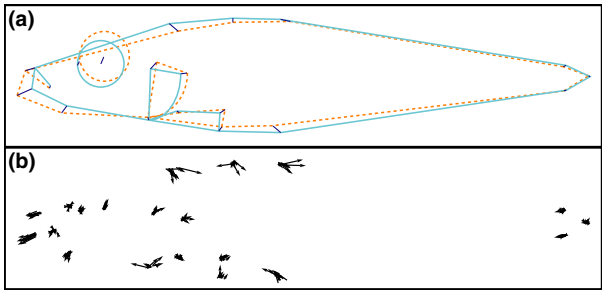

Fig. 2 (a) Vector plot showing change in shape: Vectors point from the mean shape of the freshwater treatment fish (connected by solid blue lines) to the mean shape of the saltwater treatment fish (connected by dashed orange lines). Vector length is extrapolated ×4 to show the change in body shape between treatments more clearly. (b) Joint vector plot showing the change in shape from the mean freshwater treatment fish (beginnings of vectors) to the mean saltwater treatment fish (ends of vectors) for each of the 9 families separately, but layered on top of each other. Although there is some family-level variation (for example, landmark no. 17), shape changes seem parallel across families, particularly landmarks in the head region where most of the change is concentrated. Vector length is extrapolated ×4.

Table 1 Summary of principle component scores from principle component analysis. Shown are the variance and the cumulative variance explained by the first ten principle components.

PC	Expl. Var. (%)	Cum. Expl. Var. (%)
1	26.22	26.22
2	16.04	42.26
3	13.18	55.44
4	8.57	64.01
5	6.81	70.82
6	6.17	76.99
7	3.67	80.66
8	3.20	83.86
9	2.20	86.06
10	1.36	87.42

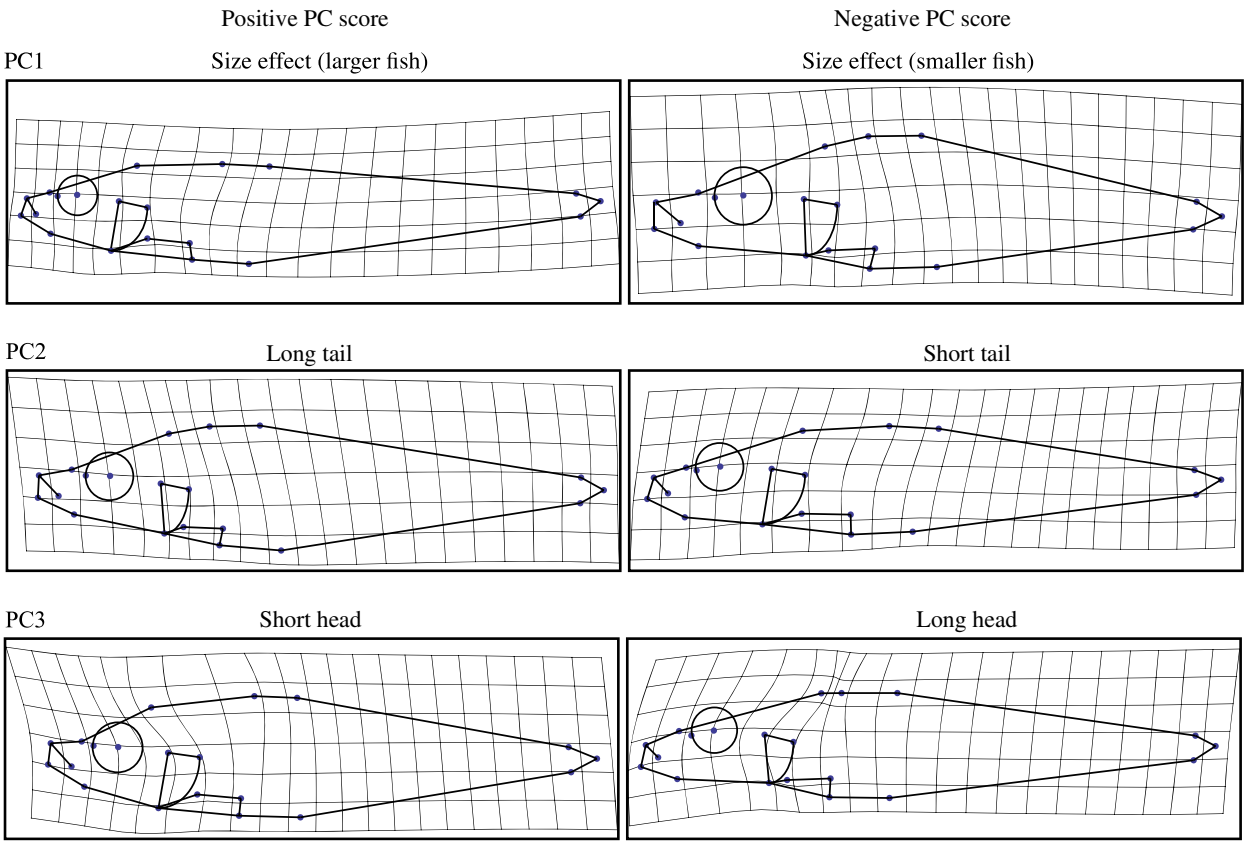

Fig. 4 Deformation grids for the three first principle component (PC) axes of shape change. For each PC, the grid for the positive PC score is shown on the left, the grid for the negative score on the right. The landmarks were connected into outlines to give a better understanding of the fish body shape associated with each of the PCs. The variation shown here is extrapolated and corresponds to ± 6 standard deviations along each PC axis from the mean.

water, as these fish have on average a 7.1% longer snout to eye length than fish raised in fresh water (0.19 mm longer). The biggest proportional trait change across treatments was in mouth length: Fish raised in salt water had a 13.5% larger mouth size compared to freshwater fish, or an average 0.08 mm longer mouths in the saltwater treatment. The lower jaw in the saltwater treatment was also on average 6.0% (0.13 mm) longer than in fish raised in fresh water. The outline made from the vector plot (Fig. 2a) shows that the jaw shape diverges in the two treatments, thereby causing many of the corresponding linear distance measurements to change in concert.

Magnitude of change and variability compared with wild populations

Body shape differences measured as Procrustes distance between saltwater treatment fish and freshwater treatment fish in our experiment amount to 59% of the differences between the freshwater and marine wild-caught fish from Voje *et al.* (2013). The direction of shape change associated with salinity, however, was different in the wild-caught sample and the experimental sample (Fig. S3).

Total shape variation of the experimental fish amounts to 63% of the total variation in body shape of the wild-caught fish. Freshwater and saltwater fish were equally variable in both the experimental and in the wild-caught sample (Permutation test for the equality of variances, $P < 0.0001$). However, fish from the wild-caught sample were larger than the experimentally reared fish, and body size also differed between freshwater and marine populations within the wild-caught sample. Standard length was 40.1 mm (SD = 6.2 mm) in the freshwater group of the wild-caught sample and 45.3 mm (SD = 6.6) in the marine group. As reported above, experimentally reared fish did not differ in standard length between treatment groups.

Discussion

One of the major changes in environment experienced by marine stickleback when they colonized freshwater

Table 2 Allometric parameter estimates from the model that best fitted the 12 linear traits according to AIC, analysed using linear mixed models.

Linear trait (landmarks)	Mean log trait size fresh water (SE)	Mean log trait size salt water (SE)	Proportional mean trait change	Allometric slope (SE)
Eye radius (L3–L4)	0.166 (0.002)	0.170 (0.001)	2.41%	0.542 (0.022)
Mouth length (L1–L9)	0.170 (0.013)	0.193 (0.004)	13.53%	0.728 (0.063)
Jaw length (L18–L20)	0.386 (0.004)	0.409 (0.003)	5.96%	0.812 (0.039)
Distance from snout to eye (L1–L4)	0.424 (0.004)	0.454 (0.003)	7.08%	1.131 (0.046)
Head depth (L5–L17)	0.791 (0.002)	0.780 (0.002)	−1.41%	0.899 (0.026)
Head length (L1–L15)	0.960 (0.002)	0.970 (0.001)	1.04%	0.876 (0.022)
Body depth (L7–L11)	0.850 (0.005)	0.841 (0.002)	−1.06%	0.851 (0.030)
Tail width (L8–L10)	0.194 (0.003)	0.190 (0.002)	−2.06%	0.898 (0.030)
Snout to start of operculum (L1–L16)	0.858 (0.002)	0.872 (0.002)	1.63%	0.871 (0.028)
Length of operculum and pectoral area (L13–L16)	0.493 (0.004)	0.504 (0.003)	2.18%	1.056 (0.039)
Pectoral area length (L13–L14)	0.712 (0.004)	0.712 (0.004)	–	1.085 (0.024)
Distance from snout to start of operculum (L1–L16)	0.433 (0.005)	0.433 (0.005)	–	1.143 (0.038)

Mean trait size refers to the intercept(s) of the models. Mean proportional trait change is calculated as the ratio between the two trait means. Negative values imply that the freshwater fish had the larger trait value compared with the saltwater fish (see the Linear Measurements section in the Methods part for further detail). SE, standard error.

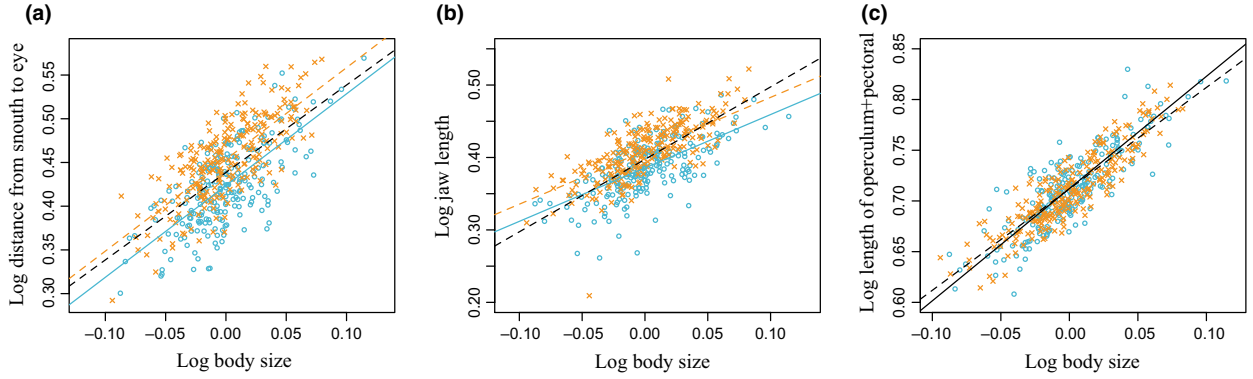

Fig. 5 Allometric scaling relationships for 3 of the 12 investigated linear traits: (a) Log distance from snout to eye (LM1–LM4), (b) Log jaw length (LM18–LM20) and (c) Log length of operculum and pectoral area (LM13–LM16), as functions of log body length. The solid line(s) represents the regression parameters from the best model according to AIC for each of the traits (see Table 2); Orange line represents saltwater treatment, blue line represents freshwater treatment, and the black line in panel c represents a case where neither intercept, nor slope differed across treatments. None of the allometric slopes differed between the treatments in any of the traits (Table 2). Panels (a) and (b) indicate a plastic change in mean trait size (intercept) between freshwater (blue circles) and saltwater raised fish (orange exes), which was detected for most of the investigated linear traits. Panel (c) shows an example where the intercept does not differ between treatments. The dashed black line is added for comparison, it has a slope of 1 and an intercept equal to the median intercept across the two treatments.

lakes was a substantial change in salinity. In this study, we showed that stickleback body shape changes plastically in response to different salinities, with these effects being parallel across families. We also found clear plastic responses in the mean trait size for 10 of 12 linear traits we investigated, with especially large effects detected in the head region across the two treatments.

The direction of these plastically induced phenotypic changes is difficult to interpret, as there is no consensus in the literature as to what phenotypic changes are found when comparing body shape of wild-caught freshwater stickleback to marine/anadromous stickleback. Some studies have found body shape changes that resemble those that we found in our treatments (Taylor & McPhail, 1986; Klepaker, 1993; Kristjánsson, 2005), whereas others found morphological differences in a different direction (Leinonen *et al.*, 2006; Aguirre, 2009; Voje *et al.*, 2013). Shape analysis of the joint landmarks available from the fish used in Voje *et al.* and the fish used here shows that the direction of shape change between the wild-caught freshwater and saltwater fish is different than the direction of shape

change in our experiment (see Fig. S3). However, given the large differences in size and age between the samples from these two papers, this direct comparison is not conclusive. A new analysis using a more homogenous wild-caught sample might yield a different and more informative result. Given that Glitredammen is a shallow and eutrophic lake, it could be argued that the stickleback population in Glitredammen should be benthic-adapted. The predicted direction of adaptive plasticity in a move from fresh water to salt water would then be towards a narrower head and body, in the direction of the marine morphology, and our results resemble this prediction. We cannot conclusively interpret the observed shape changes in response to salinity in our experiment as adaptive plasticity.

If nonadaptive, the plastic effects we detected may be due to expression of an initially hidden part of the reaction norm of the fish. Selection had no opportunity to shape the reaction norm outside the range of the freshwater condition, these fish were exposed to for thousands of years. Mutations might thereby have accumulated over long time spans in the hidden part of the reaction norm due to their neutral effects in a freshwater environment, with effects solely in salt water (Schlichting, 2008). In our study, we detected a phenotypic shift, but no increase in phenotypic variance in the saltwater environment. An increase in phenotypic variance, however, is expected to be likely when cryptic genetic variation is released (Rutherford, 2000; Schlichting, 2004; Le Rouzic & Carlborg, 2008).

The magnitude of the shape differences that arose between the average freshwater and saltwater treatment body shapes in our study amounted to 59% of the shape differences found across natural populations in Norway. The parental fish in our experiment stemmed from a single Norwegian freshwater lake population, whereas the wild-caught fish came from 87 different saltwater and freshwater populations, covered a much broader size range, and their natural habitats differed in many aspects beyond salinity (Voje *et al.*, 2013). This indicates a more genetically heterogeneous sample, which should add to the morphological variability of the wild-caught sample compared with the experimental sample. Yet the plastic shape differences that arose experimentally in one generation are of a similar magnitude as the shape differences between fish that have evolved in different habitats for several thousand years. Additionally, we find that the variation in body shape that emerged in the experimental fish was also large; it amounted to 63% of the variation detected in the wild-caught sample.

Marine stickleback adapted remarkably fast to fresh water after their colonization of these environments (Bell & Foster, 1994; Foster & Baker, 2004; Ostlund-Nilsson *et al.*, 2006). Our results, along with earlier work detecting substantial plastic phenotypic changes in stickleback body shape (e.g. Day *et al.*, 1994; Wund *et al.*, 2008, 2012; Lucek *et al.*, 2014; Garduño-Paz *et al.*, 2010; McCairns & Bernatchez, 2012), suggest that a significant fraction of the observed phenotypic diversity across stickleback populations may be due to plastic responses to similar cues in lake environments. The work of McCairns & Bernatchez (2012) indicates that what we have found here is not limited to Norwegian populations, or even to freshwater populations. Plasticity in body shape in response to salinity is therefore likely to be a general property of this species.

Shared genetic and developmental constraints may also partly explain why stickleback repeatedly developed similar phenotypes (Schluter, 1996), as evolutionary constraints structure the phenotypic variation that natural selection acts upon (Langerhans & DeWitt, 2004). The shape analysis conducted in this study clearly shows that different phenotypic trait combinations can arise in different salinities due to plasticity and that these trait changes are large and parallel across families. Consequently, stickleback seem to have a large capacity for plastic adjustment in response to salinity in many of their morphological traits. But because the allometric slopes did not show a plastic response, the results of the allometric analysis indicates that this capacity is limited to particular directions in morphospace, which is an observation predicted from the allometric-constraint hypothesis (Voje *et al.*, 2014; Pelabon *et al.*, 2014). Thus, the trait differences observed across treatments happen without affecting the covariation between single linear traits and overall size. The allometric slopes remained unchanged for all the 12 linear traits we investigated whereas the static allometric intercepts (mean trait sizes) were plastically adjusted in 10 of 12 traits, although to different extents. This observation is in line with recent evidence that has shown that allometric scaling of traits may have constrained the phenotypic radiation of other freshwater stickleback populations in Norway (Voje *et al.*, 2013).

There is debate about the relevance of 'genetic accommodation' for the stickleback system (Wund, 2012), the process by which environmentally induced phenotypic variation may be subsequently selected to become heritable (Schmalhausen, 1949; Waddington, 1953; West-Eberhard, 1989). There is some empirical evidence that this controversial mechanism may aid diversification (Rutherford & Lindquist, 1998; Ruden *et al.*, 2003; Rutherford, 2003). However, to assess whether genetic accommodation plays a role in body shape divergence in stickleback, and to explore the role that phenotypic plasticity may have played in the colonization history of this species, our experiment must be repeated on fish from native saltwater populations. This experiment, originally designed for a study on plasticity of lateral plates (T. H. Hansson, B. Fischer, A. B. Mazzarella, K. L. Voje, A. Taugbøl and L. A. Vøllestad, unpublished) that necessitated the use of low plated freshwater fish, has given us an indication that this

reverse experiment using saltwater fish has the potential to add significant insight to this system. A plastic response in the direction of a freshwater morphology in marine fish brought up in a freshwater environment would indicate genetic accommodation. Such an experiment may also lead to insights about whether the reaction norms we observe in this study evolved before or after the freshwater colonization by stickleback. A full exploration of the role of adaptive plasticity in the stickleback adaptive radiation should also take into account that plastic effects may be trans-generational. Recent work by Shama and colleagues (Shama & Wegner, 2014; Shama *et al.*, 2014) indicates the need for experiments on stickleback plasticity over multiple generations to assess for how long environmentally induced effects can last.

Local adaptation has played a critical role in enabling stickleback to persist in new freshwater habitats (Jones *et al.*, 2012; Barrett *et al.*, 2008; Schluter *et al.*, 2010), but the magnitude of plastic change detected in this experiment was comparatively large. It remains unclear whether these plastic changes are adaptive, but based on these results, we suggest that plastic effects to similar environmental cues may have played a role in the radiation of freshwater stickleback. We also found that allometric constraints limit the directions in phenotype space in which these plastic phenotypic changes can occur. Similar constraints have also been identified in earlier studies on the stickleback radiation (Schluter, 1996; Hansen & Voje, 2011; Voje *et al.*, 2013). Phenotypic plasticity, evolutionary constraints and genetic adaptation seem to jointly determine the diversification of stickleback. The interactions of these forces and their relative strengths across different populations may be one reason that it has been so difficult to pinpoint the exact selection pressures that have shaped the stickleback adaptive radiation.

Acknowledgments

We would like to thank L. A. Vøllestad for guidance and helpful comments. Comments and suggestions from two anonymous reviewers and associate editor Christoph Haag helped to improve the manuscript. L. A. Vøllestad (Grant Number 16639/F20 from the Norwegian Research Council) and N. C. Stenseth (the Center for Ecological and Evolutionary Synthesis at the University of Oslo) jointly funded the rehabilitation of our fish facility, without which this project would have been impossible. The fish facility was built by Haaken H. Christensen, whom we also thank for valuable advice on fish husbandry. Thanks also to S. Boessenkool and P. Mitteroecker for discussion and comments on the manuscript. We also thank the owners of Glitredammen for granting us access to the lake. University of Oslo experimental permit number for this experiment: 5514.

Data accessibility

Tab separated txt file with fish ID, family, treatment and coordinates of all landmarks for all experimental fish is available on Dryad on 13.8.15.

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Supporting information

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

Figure S1 Bending/tilting digitisation artefact as revealed by the first PC in the initial PCA.

Figure S2 Video showing the variation along the first PC in the initial PCA, which is due to a bending/tilting artefact.

Figure S3 A comparison of shape variation due to salinity between the wild-caught sample from Voje *et al.* (2013) and our experimental sample.

Table S1 Numbers of surviving individuals and survival rates

Data deposited at Dryad: doi:10.5061/dryad.871q1

Received 3 September 2014; revised 23 January 2015; accepted 26 January 2015