Salinity-induced phenotypic plasticity in lateral bony plate number of the threespine stickleback (*Gasterosteus aculeatus*)

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Master of Science Thesis

2014

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Acknowledgements

This thesis would not have been possible without the help from Professor Asbjørn Vøllestad, my main supervisor; Anna Black Mazzarella and my additional supervisors; Barbara Fischer and Kjetil Lysne Voje and Annette Taugbøl. These have been a tremendous help and I thank them for their patience with me and for always being understanding and helpful beyond anything I would have expected based on observations of my fellow master students.

I would also like to thank the department engineer, Haaken Hveding Christensen for invaluable assistance and advice in the execution of this experiment.

Furthermore, Jan-Erik Thrane was a great resource in providing assistance and advice on statistics and statistical programing in "R". Thank you for taking time of your work to help me.

Additional people strongly deserving of thanks are;

Anna-Marie Winter who also provided assistance with statistics and "R" programing as well as formatting, writing and general supportiveness.

Tage Bratrud and Anette Øygarden for their help in feeding the sticklebacks, on occasions when I could not.

Synnøve Botnen for taking time to read through and provide input on the formatting of my thesis.

This experiment would not have been possible without the joint funding from the Norwegian Research Counsil and N. C. Stenseth (the Center for Ecological and Evolutionary Synthesis at the University of Oslo).

Finally I would like to thank my family and loved ones for all of their help and support throughout this project. Without them I would not have been where I am today.

Abstract

Phenotypic plasticity allows organisms with identical genotypes to develop different phenotypes in response to different environmental cues. Phenotypic plasticity is a trait in itself, and can be either adaptive or non-adaptive. In this study I test for a potential salinity-induced plastic effect on the development of lateral bony plates in a fresh water population of the threespine stickleback (*Gasterosteus aculeatus*), a fish known for having plastic responses to other environmental cues. In order to do this, I bred male and female sticklebacks, partitioned each family of offspring into two groups, and reared each group in two different environments (salt and fresh water). This kind of experiment is called a split-clutch design and makes it easier to differentiate genetic and plastically induced differences. 16 families survived until the experiment's end, and when the fish had reached the size where all plates were fully developed, plate number was counted for all fish. These data were analyzed using a general linear mixed model to see if plate number was affected by the different environments. The results showed that there is a plastic effect on plate number induced by increased salinity: sticklebacks reared in fresh water developed more plates than those in salt water. This was somewhat contrary to my hypothesis, as wild sticklebacks consistently show a considerably lower number of plates in fresh water populations than in salt water populations. This along with the fact that the observed difference in plate number was relatively small, indicates non-adaptive rather than adaptive plasticity. However further research is needed in order to confirm or deny this.

Introduction

Phenotypic plasticity is the ability of a genotype to produce different phenotypes in different environments. This can be manifested as either distinctly different phenotypes/morphs, or as a continuous spectrum of phenotypes following an environmental gradient (Stearns 1992; Pigliucci 2001). This means that phenotypic plasticity can be a great tool for surviving and adjusting to a rapidly changing environment, which is why it may play an important role and even be selected for in the evolutionary process (West-Eberhard 2003). However, phenotypic plasticity is not necessarily adaptive. There are many examples of non-adaptive plasticity where a trait's plastic response does not yield any fitness advantage (Langerhans & DeWitt 2002; Mazzarella *et al.* 2014, submitted[1]). In the example of Mazzarella *et al.*, non-adaptive plasticity was found in the body shape of sticklebacks induced by salinity. The difference between adaptive and non-adaptive plasticity can be complex to determine; adaptive plasticity can become non-adaptive if the environmental factor it was selected for changes sufficiently (West-Eberhard 2003). Although plasticity can be an advantage in a highly variable environment, it can also be a disadvantage in a stable environment, as it may be costly to maintain the controlling mechanisms of plasticity, potentially leading to a reduction in fitness. The importance of costs of plasticity, however, remains a debated issue. Costs of plasticity are hard to identify empirically and recent research has shown that wherever they are detected, they are usually mild (Van Buskirk & Steiner 2009).

The threespine stickleback (*Gasterosteus aculeatus*, hereafter stickleback) is an ideal model organism for studying phenotypic plasticity in relation to adaptation and evolution. The stickleback is an ancestrally marine species that has repeatedly colonized freshwater after the end of the last glacial period (Bell 1976; Bell & Foster 1994; Johnson & Taylor 2004). Today it can be found in coastal marine, brackish and freshwaters all over the northern hemisphere, from north of the Arctic Circle to as far south as the Mediterranean Sea (Wootton 2009; Bell & Foster 1994). Considering that the stickleback is capable of thriving in such a large spectrum of highly different environments, and that these fish are able to switch between

[1] Mazzarella, A. B., Voje, K. L., Hansson, T. H., Taugbøl, A., Fischer, B. 2014. Strong and parallel salinity-induced phenotypic plasticity in a single generation of threespine stickleback. (submitted and under review at time of writing).

these environments with ease, one would expect the stickleback to be a fish with a high degree of plasticity in its traits. And indeed, it has been shown that stickleback traits such as body shape and behavior show plastic responses to several different environmental variables. Sticklebacks are for example capable of osmoregulation over a wide gradient of salinities, which they do without obvious costs (Heuts 1947; Grøtan *et al.* 2012). Plasticity in life history traits such as size of fish at breeding, family size and egg size have been documented as a response to life in stream environments versus pond type environments (Baker & Foster 2002). Furthermore variation in food type, environmental complexity and a combination of the two have been found to cause plastic responses in morphological traits (Wund et al. 2008; Wund *et al.* 2012; Day *et al.* 1994; Svanbäck & Schluter 2012; Lucek *et al.* 2014). Wund *et al.* (2008) found plastic responses in marine ancestral populations that mirror phenotypes of local fresh water populations when reared in environments that simulate those in the lakes. Similarly, Day *et al.* (1994) found that reversal of diets between sister populations of sticklebacks leads to trait switching, meaning that the stickleback developed traits associated with the new diet instead of the diet they ate in the wild. The population originally feeding on a larger variety of food types displayed a larger degree of plasticity. These examples suggest the presence of adaptive plasticity, as the direction of plasticity was in the predicted directions indicated by the phenotypes found in wild populations in those environments. Thus, sticklebacks possess a large repertoire of plastic responses to a wide gradient of environmental factors, making this species a good candidate for studying phenotypic plasticity.

The stickleback evolutionary history is a well-known example of an adaptive radiation, where the fresh water populations have adapted to their new habitats with a surprising pace; the adaptations consist of small but noticeable phenotypic, physiological and behavioral changes, perhaps most noticeable in the number of bony lateral plates (Bell & Foster 1994). The variation in these lateral bony armor plates (hereafter plates) has continued to spark interest and debate, as the underlying selective pressures have eluded explanation for an extended period of time. Based on the number of plates, stickleback are often categorized into 3 plate morphs: Fully plated, partially plated and low plated stickleback. In marine environments, the stickleback usually has 30-36 plates from the head region to the base of its tail (fully plated morph), while in freshwater they normally have less than 10 plates on the anterior part of their body (low plated morph). In brackish and fresh water environments, individuals with intermediate number of plates (partially plated morph) may also be common (Hagen &

Gilbertson 1972; Myhre & Klepaker 2009; Song *et al.* 2010; Bell & Foster 1994). Most theories trying to explain why low and partially plated fish are found in freshwater while full plated fish are found in the ocean claim there is a selective advantage for fish with fewer plates in freshwater environments due to differences in predation level and/or calcium availability (Hagen & Gilbertson 1972; Kitano *et al.* 2008; Spence *et al.* 2012, 2013). However a fully satisfying answer to this question remains to be found and it is still debated whether the selection pressures from these factors are of any importance (MacColl & Aucott 2014; Smith *et al.* 2014). The genetic basis behind plate morph in sticklebacks has been well studied (Colosimo *et al.* 2004, 2005). This involved the discovery of a gene coding for the signaling molecule "ectodysplasin-A" (Eda), which is comprised of one locus with two alleles ("A" and "a"). In this gene model, the AA genotype gives a completely plated phenotype (fully plated morph), aa genotype gives a low plate morph, and Aa usually results in intermediate morphs. This model explains about 70% of the existing plate morph variation. However, the Eda gene fails to explain variation in plate number within each morph, which is likely to be of ecological importance if plate morph itself is. There are also examples showing that the three plate morphs are not always predicted by the Eda gene model (Lucek *et al.* 2012; Taugbøl *et al.* 2014). What has not yet been examined to date is the potential importance of phenotypic plasticity in plate number variation within the plate morphs.

To investigate this question, I have conducted a full sibling split-clutch common garden experiment on fish from a fresh water population of low plated sticklebacks (Figure 1). In performing this experiment, I hope to discover whether phenotypic plasticity induced by different levels of salinity could help explain the variation in the number of lateral bony plates.

Materials and methods

Sample collection

Threespine stickleback were collected from the small (0.5 km^2) lake called Glitredammen (59.931767°N, 10.498728°E) in Bærum, Akershus County in the period 20^{th}-27^{th} of June 2013. This population was chosen because of its proximity to our laboratory and because a steep dam and waterfall prevents migration into the lake from downstream freshwater populations and/or marine populations.

The Glitredammen lies at an altitude of 84 m above sea level around 5 km from the marine Oslo fjord environment. It is a small, artificial, shallow, and relatively eutrophic freshwater lake. There is one large river flowing into the lake as well as a few seasonal creeks. The lake contains several different species of fish, including brown trout (*Salmo trutta*), threespine stickleback, and common minnow (*Phoxinus phoxinus*).

Stickleback were captured using coated metal minnow traps baited with cheese (Breder, 1960, chamber 100 cm long, 40 cm diameter, 1cm openings). The captured fish were transported in 20 L plastic buckets to the lab where they were outfitted with an air pump (Tetra Whisper Air Pumps).

Experimental design & experimental protocol

For this full sibling common garden experiment, 16 families of threespine stickleback were created. Each family was split in two after hatching. One half was reared in salt water and the other half in fresh water. The experiment lasted until all fish had reached 30 mm of length, the length where all plates are fully developed (Bell 1981), at which point the experiment ended with the euthanization of all fish. Figure 1 gives an overview of the experimental setup.

After being captured, the mature males were identified and paired with one gravid female each. Both males and females were euthanized using a Benzocaine solution (1 part benzocaine, 5 parts water), after which eggs were stripped from the female and placed in a marked petri dish with embryo medium consisting of 15 ppt saltwater (see below) (done to

prevent spreading and mortality from fungi (Bell & Foster 1994)). The male testes were removed surgically, cut into pieces and mixed with a few drops of embryo medium before being added to the eggs for fertilization. After two minutes all excess liquid was removed by pipette, the fertilized eggs were submerged in embryo medium, and petri dishes were sealed. Approximately one day after fertilization, the eggs of each family were separated from each other using sterilized plastic probes. This is a simulation of what the threespine stickleback male does in the wild to increase access to oxygen rich water around the eggs. These methods are adapted from (Schluter 2012, https://www.zoology.ubc.ca/~schluter/wordpress/stickleback/raise-stickleback/).

The eggs, embryos and juveniles were kept at room temperature (22˚C) throughout the experiment. Dead embryos were removed daily and 80% of the embryo medium in each petri dish was changed twice a day until all eggs were hatched (Fig. 1). The surviving larvae of each family were split in two and transferred to their treatments in glass tanks filled with either fresh water (0 ppt salt) or salt water (25-30 ppt salt). The initial volume of the new tanks was 1 L of which 50% was changed twice a day. After one week this volume was increased to 2 L and 50% of the water was changed once a day. Fresh water came from the Oslo water reservoir, Maridalsvannet. Salt water was made by adding Instant Ocean Aquarium Sea Salt Mixture to fresh water. The ratio was 0.015 g/L for 15 ppt embryo medium and 0.030 g/L for the 25-30 ppt salt water treatment.

After the yolk sacs of the stickleback larvae were consumed they were fed ad libitum twice a day with nauplii of the brine shrimp *Artemia*. The *Artemia* were reared and hatched in the lab over a 24 hour cycle; two batches of *Artemia* were hatched every day with a 5 hour interval from each other. 32 days after all eggs had hatched the feeding regime was changed into a mix of brine shrimp and blood worms (*Chironomidae* larvae) that had been chopped into smaller pieces while frozen, then thawed and rinsed. The proportion of bloodworms in the food mix was gradually increased as more of the sticklebacks grew to a size where they were able to consume this kind of food until finally all fish were large enough to eat bloodworms. At this point, brine shrimp was removed from the diet. Over the last four weeks of the experiment, feeding was increased to 3 times a day in order to maximize growth rates.

When all fish within one family were larger than 1cm in length, they were transferred to new 40 L aquaria with automated water flow-through and filtration systems. This setup was

comprised of two individual racks of 18 40 L tanks (36 tanks total), each rack with its own water circulation system. One rack was filled with fresh water, and the other with 25-30 ppt salt water. Due to evaporation the water had to be refilled regularly, hence salinity varied somewhat in the saltwater tanks, but it was kept within the stated values. Each rack was equipped with a 150 L open sink for water storage. Before entering the sink, water passed through a UV-radiation filter (Aqua Medic Helix Max UV 55 W) to minimize the spread of potentially harmful microorganisms between the 40 L aquaria. The sinks also contained bio balls (Tetratec BioBaller 600/700/1200 800 ml, plastic balls with perforated surfaces), nitrogen filters that function as surfaces for nitrifying bacteria. These made sure that nitrogen levels did not accumulate to critical levels. In addition the sinks were outfitted with two air pumps each (Tetra Whisper Air Pumps) to make sure the water was properly oxygenated.

Lighting for the entire experiment was controlled by a timer with a 19L-5D light cycle mimicking summertime light conditions in Norway. The light used was kept within 500 and 600 nm of the EM-spectrum to reduce photosynthetic effectiveness and thereby reduce the level of algae growth in the tanks. Despite this, tanks had to be rinsed weekly using a sponge and a fine mesh net. The sticklebacks were kept and fed until all fish had reached at least 30 mm in length. The experiment ended on December 17th 2013 (Figure 1). The fish were euthanized using the benzocaine solution described previously. Fish were removed from their respective tanks and each half-clutch (all fish in one tank) were euthanized simultaneously. This experiment was approved by the Norwegian animal experimentation and care committee (University of Oslo experimental permit number 5514) and all efforts were made to minimize suffering.

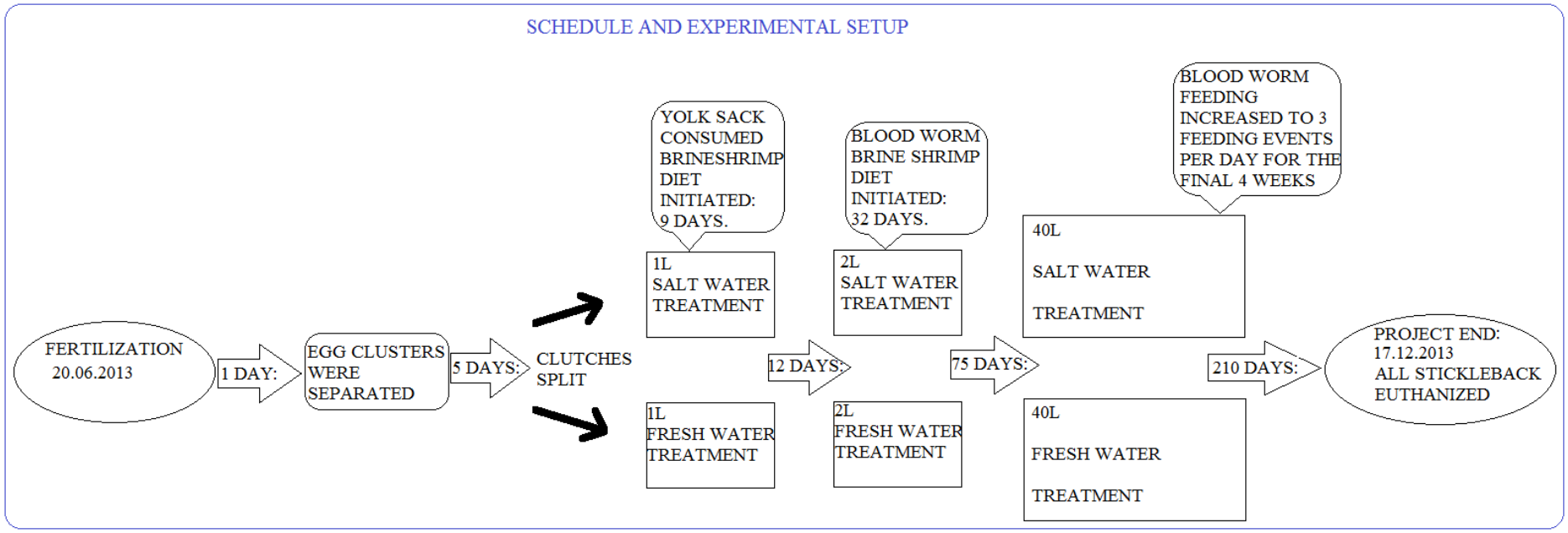

Figure 1.

Figure 1 demonstrates the procedure and timetable (start and end dates of the experiment, number of days between each step) for this experiment.

Data collection

Immediately after euthanization, sticklebacks were scanned using a Canon CanoScan 9000F flatbed scanner according to Herler *et al.* (2007). The images were used for length measurements of the fish. This was also done to collect data for morphological analysis in a sister paper (Mazzarella *et al.* 2014, submitted[1]). The scanner was modified for the imaging by removing the hood and constructing a rectangular basin (10 x 10 cm) with 2 cm high and 1cm thick walls on the scanning plate using white modeling clay. The basin was filled with water and fish with their labels were placed on the scanner along with a ruler as a scale. A black piece of cardboard was placed on top to block out light during scanning. The length of each fish was measured using the computer programs tpsUtil and tpsDig2 (Rohlf 2005). Length was measured by setting landmarks on the forward-most part of the upper lip and the end of the spinal cord at the beginning of the tail fin (figure 2) and subsequently exporting the coordinates and using the scale to transform them into length measurements.

Figure 2
Fish with landmarks (red dots) for length measurements and identification tag

After scanning, each half-clutch of fish was jointly strung on a fishing line, each individual together with its unique label to ensure later identification, and stored in 500 ml plastic jars on 96% ethanol. This was done to enable common bone staining of each half-clutch. In order to ease the process of counting lateral bony plates and to reduce the risk of miscounting or overlooking plates, the fish were stained in an Alizarin Red solution. This was done with slight modifications following the protocol from Dingerkus & Uhler (1977). The parents were also stained for plate counting. Plates were counted on both sides for every offspring and parent. Four half-families (96 sticklebacks in total) were chosen at random and recounted. This was done to estimate frequency of counting errors and to ensure that these were within acceptable levels.

Microsatellite analysis of one quantitative trait locus, Stn 382, was done on parental DNA samples extracted from fin clips in order to ensure that all fish were homozygotes for the low plated Eda allele. The marker Stn 382 is located within intron one of the Ectodysplacin (Eda) gene on linkage group IV (Colosimo *et al.* 2005). This marker has two alleles that are highly correlated with the three recognized stickleback morphs. DNA was extracted from each fish using Qiagen DNeasy kits (Qiagen, Hilden, Germany) and then the amplification reactions for this locus were performed as described in Colosimo *et al.* (2005). This marker has two alleles with fragment lengths of 151 ("a" allele) and 218 ("A" allele) base pairs, thus the individual genotypes were easily visualized on 2% agarose gels. Fragment size was verified with a size standard (Generuler, Fermentas) and internal gel controls for the two alleles. All stickleback parents were confirmed for being homozygotes of the low plated Eda allele "a".

Statistical analysis

All statistical analyzes described below were conducted in the statistical environment R version 3.0.1 (R Core Team 2013, Vienna, Austria, http://www.R-project.org/) using the package lme4/nlme (Pinheiro *et al.* 2013; Bates *et al.* 2014).

Difference in survival between the two treatments could potentially affect the output of the experiment. I therefore tested for differences in survival, defined as the proportion of fish in a tank that survived until experiment end, with a general linear mixed model (glmm) using salinity treatment as fixed effect and family as random variable.

Individual fish size could potentially have an effect on plate number. Size can also be strongly linked to density through density-dependent growth. I therefore evaluated the effects of density and treatment on size by using general linear mixed models using combinations of these variables, always including family as a random variable. The models were compared using the Akaike Information Criterion (AIC) score of the models (Akaike 1976).

I tested for effects of treatment, density and size on plate number using general linear mixed models. I included family as a random variable to account for nested family effects. Generalized linear models are usually fitted to count data using a Poisson link function. However, the total plate distribution appeared close to a normal distribution suggesting that a

general linear model might be suitable (figure 3). I therefore first evaluated whether a generalized linear mixed model with Poisson link function or a general linear mixed model without a link function, that instead assumes the data is normally distributed, would fit the data best, using the AIC. In this preliminary model only treatment was included as factor. Since the simpler one of the two model types, the general linear mixed model, fit the data better than the generalized linear mixed model, all further analyses were done using this simpler type of model.

After selection of the model type, I searched for the best model that fit the plate number data using treatment, density, and fish size as factor. I ran a standard model selection procedure using the AIC value.

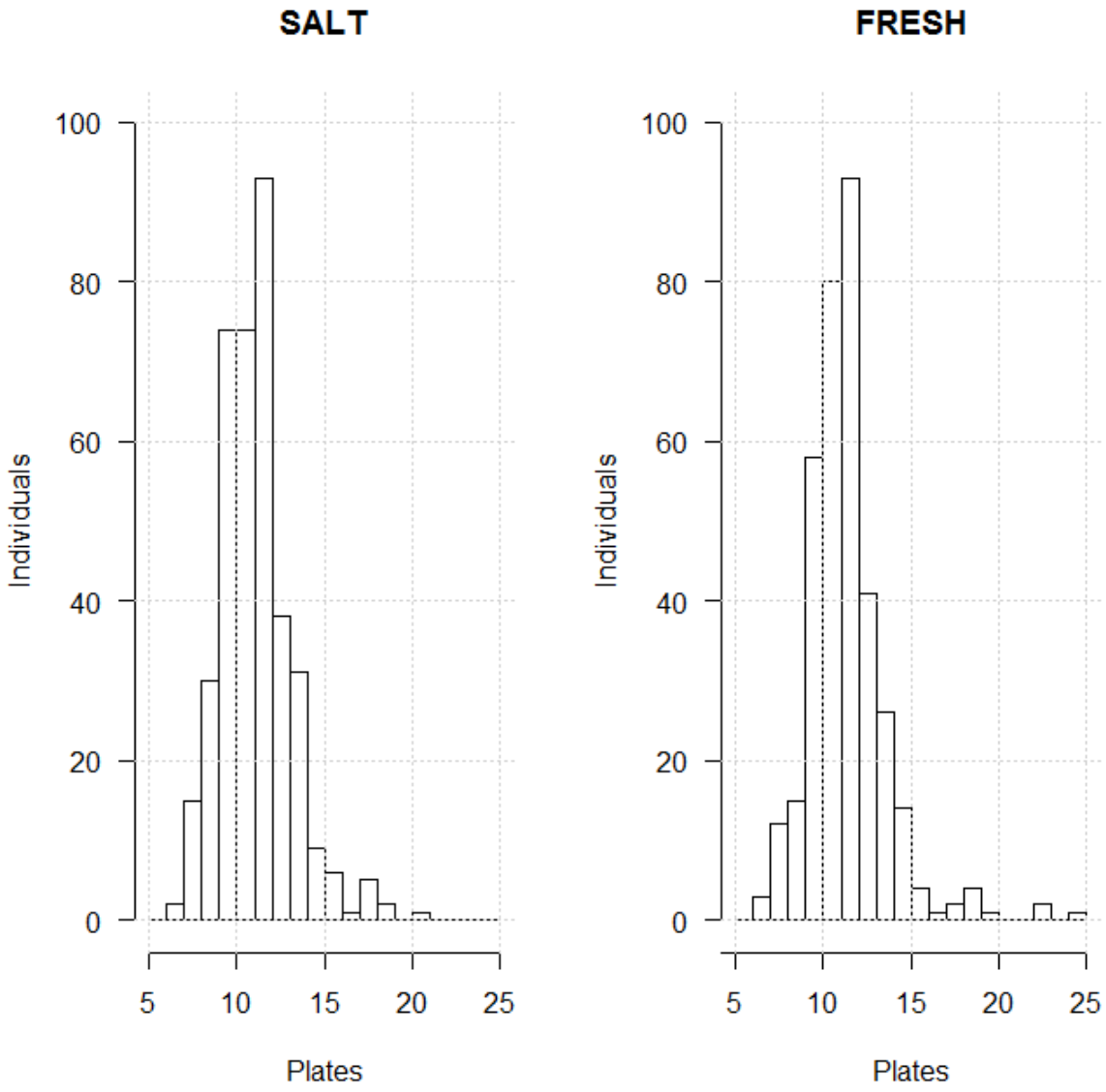

Figure 3

Histograms with frequency of individuals on the y-axis and total plate number (sum of plates on both sides of one fish) on the x-axis, for stickleback raised in salt water (left panel) and fresh water (right panel).

Results

Variation in lateral plate number

Stickleback that were raised in fresh water had on average in total 11.82 plates per fish, while stickleback raised in salt water had on average 11.55 plates per fish (table 1). Family identity was expected to affect plate number because of genetic differences between the parents. Family 15 had the highest average plate number in both treatments and family 16 had the lowest average plate number in salt water and second lowest in fresh water (table 1). Family 10 had the lowest average plate number of the fresh water treatment and the second lowest in salt water (table 1).

Table 1

Mean (± sd) number of total lateral plates for each stickleback family for the fresh water and salt water treatments, respectively.

Family Number	Freshwater	Saltwater
10	8.75 ± 1.13	10.26 ± 1.45
11	11.24 ± 1.45	11.0 ± 1.79
12	11.1 ± 1.77	9.96 ± 1.43
13	12.05 ± 1.17	12.25 ± 1.83
14	11.11 ± 1.45	12.04 ± 2.03
15	16.72 ± 4.08	14.45 ± 2.99
16	9.75 ± 1.67	9.00 ± 0.82
17	12.29 ± 1.51	11.66 ± 1.97
18	11.21 ± 0.98	10.95 ± 1.17
19	11.62 ± 1.07	10.29 ± 1.27
1	12.22 ± 1.15	11.42 ± 1.03
2	11.76 ± 1.7	11.78 ± 1.69
4	11.8 ± 1.74	11.96 ± 1.79
5	11.15 ± 1.43	10.32 ± 1.72
8	12.3 ± 2.54	12.0 ± 2.05
9	11.79 ± 1.12	12.54 ± 0.83
Average	11.82 ± 2.25	11.55 ± 2.04

To test whether salinity had an effect on plate number when taking density and size into account, I evaluated several general linear mixed models against each other (table 2). The model that explained the data best was the one with salinity as the sole explanatory variable (model 1A, table 2). Its AIC–value was at least two points lower than any other model I fitted (table 2). Plate number of fresh water sticklebacks was significantly different from that of salt water sticklebacks (table 2). This model (model 1A, table 2) indicates that sticklebacks raised in 25-30 ppt salt water have on average 0.31 fewer plates than those raised in 0 ppt salt water (fresh water). While sticklebacks in the fresh water treatment had an intercept of 11.72 plates in total per fish, those in the salt water treatment had an intercept 11.41 (table 3). The second best model had an additive effect of density (table 2, model 1B). In this model, plate number increased when density increased (table 4). However, the effect of salinity was still significant and similar to that for model 1A. Size had, independent of density, no effect on plate number. All the models used in the model selection are shown in table 2.

Table 2

Table showing model selection with model number (left), model type with variables (center right), AIC-values of each model (center left) and AIC difference from model 1A for each model (left). The explanatory variables (Treatment, Density and Size) are proceeded by the symbol "~". An "*" indicates an interaction effect. Significant effects of variables are indicated by bold and underline ($p < 0.05$). PlatesTotal = total plate number.

Model Number:	Model:	AIC -value	AIC model x − AIC model 1A
model 1A	lme: PlatesTotal ~ **Treatment**	3034.865	0
model 1B	lme: PlatesTotal ~ **Treatment** + **Density**	3036.921	2.056
model 1C	lme: PlatesTotal ~ **Treatment * Density**	3045.003	10.138
model 1D	lme: PlatesTotal ~ **Treatment** + Size	3042.038	7.173
model 1E	lme: PlatesTotal ~ Treatment * Size	3047.417	12.552
model 1F	lme: PlatesTotal ~ **Treatment** + **Density** + Size	3043.769	8.904
model 1G	lme: PlatesTotal ~ **Treatment** + **Density * Size**	3048.141	13.276
model 1H	lme: PlatesTotal ~ Treatment * **Density * Size**	3071.799	36.934
model 2A	lme: PlatesTotal ~ Size	3043.470	8.605
model 2B	lme: PlatesTotal ~ Density	3041.114	6.249
model 2C	lme: PlatesTotal ~ Density + Size	3048.136	13.271
model 2D	lme: PlatesTotal ~ **Density * Size**	3050.219	15.354

Table 3

Results of general linear mixed model for total plate number explained by treatment, with family number as a random variable. Shown are the relevant parameters describing the model fit: Value (treatment effect), standard error (SE), *t*-value (t), degrees of freedom for the t-test (DF), *p*-value (p). The intercept refers to the fresh water treatment. Plate number in salt water treatment differs from the intercept (plate number in fresh water treatment) by -0.3 plates.

	Estimate	**SE**	**t**	**DF**	**p**
Intercept	11.7	0.34	34.1	721	<0.001
Treatment	-0.3	0.13	-2.3	721	0.0184

Table 4

Results of general linear mixed model for total plate number explained by treatment and Density (additive effect), with family number as a random variable. Shown are the relevant parameters describing the model fit: Value (treatment effect), standard error (SE), *t*-value (t), degrees of freedom for the t-test (DF), *p*-value (p). The intercept refers to the fresh water treatment. Plate number in salt water treatment differs from the intercept (plate number in fresh water treatment) by -0.4 plates while aquarium tank density differs from intercept by 0.06 plates.

	Estimate	SE	t	DF	p
Intercept	10.18	0.74	13,58	720	< 0.001
Treatment	-0.40	0.13	-2.88	720	0.004
Density	0.06	0.02	2.28	720	0.022

To ensure that my results were not biased by errors in the counting of plate number, I recounted the plate numbers of 97 randomly chosen stickleback. Out of these there were a total of 7 instances where repeated counts differed. The correlation coefficient between the counts and recounts was 0.97 for plates on the right side and 0.98 for plates on the left side, which indicates a very low error rate, thus the results of this experiment are unlikely to be affected by such errors.

Survival rates and effects of density and size

Large differences in survival, density and size could potentially affect my results. However, survival and size were very similar among treatments.

Although the number of individuals varied greatly between families, both at the start and at the end of the experiment (Table 5), survival did not differ between treatments. A total of 766 individuals from 16 families survived the experimental period (397 in saltwater, 369 in fresh water). The number of surviving individuals varied between 13 fish in family 16 to 97 fish in family 8. On average, 47.8 individuals survived per family and 23.9 individuals survived per tank. The average survival rate in fresh water was 0.85 (standard deviation (hereafter sd) = 0.14), while average survival rate in salt water was at 0.8 (sd = 0.08). Average overall survival rate was 0.82 (sd = 0.07). There was no difference in survival probability between the two treatments (glmm; $t_{15} = 1.39$, p = 0.18, table 6).

Table 5

Table showing number of stickleback in each treatment at start and end of experiment, as well as survival rates for each treatment.

	SALT			FRESH		
Family	No. of larvae transferred	Surviving Individuals	Survival	No. Of larvae transferred	Surviving Individuals	Survival
1	29	26	0.90	29	27	0.93
2	35	28	0.80	34	29	0.85
4	32	30	0.94	32	27	0.84
5	32	28	0.88	32	27	0.84
8	48	41	0.85	49	43	0.88
9	24	24	1.00	24	14	0.58
10	19	19	1.00	20	16	0.80
11	26	25	0.96	26	21	0.81
12	33	27	0.82	32	22	0.69
13	27	24	0.89	27	22	0.81
14	27	24	0.89	26	19	0.73
15	24	22	0.92	24	18	0.75
16	9	5	0.56	9	8	0.89
17	39	36	0.92	39	34	0.87
18	27	24	0.89	26	21	0.81
19	28	14	0.50	29	21	0.72

Table 6

Results of general linear mixed model survival of stickleback explained by treatment, with family number as a random variable. Shown are the relevant parameters describing the model fit: Value (treatment effect), standard error (SE), *t*-value (t), degrees of freedom for the t-test (DF), *p*-value (p). The intercept refers to the fresh water treatment.

	Estimate	**SE**	**t**	**DF**	**p**
Intercept	0.8	0.03	27.4	15	< 0.001
Treatment	0.05	0.04	1.39	15	0.1839

There was also some variation in mean individual size (standard length) among families. Size decreased as density increased in both salt and fresh water treatments (figure 4, table 7). However, the effect was stronger in the salt water treatment (correlation = -0.33) than in the

fresh water treatment (correlation = -0.15) (figure 4). The average size of sticklebacks did not differ between treatments. In fresh water it was 35.5 mm (sd = 2.9 mm) and in salt water 35.8 mm (sd = 2.9 mm). Overall, average size was 35.6 mm (sd = 2.9 mm) at the end of the experiment.

Table 7

Results of general linear mixed model for size explained by treatment in interaction with density (number of fish per aquarium tank), with family number as a random variable. Shown are the relevant parameters describing the model fit: Value (treatment effect), standard error (SE), *t*-value (t), degrees of freedom for the t-test (DF), *p*-value (p). The intercept refers to the fresh water treatment.

	Estimate	**SE**	**t**	**DF**	**p**
Intercept	37.78	0.82	45.93	719	< 0.001
Treatment	2.76	0.74	3.74	719	< 0.001
Density	-0.09	0.03	-3.08	719	0.002
Treatment:Density	-0.09	0.03	-3.57	719	< 0.001

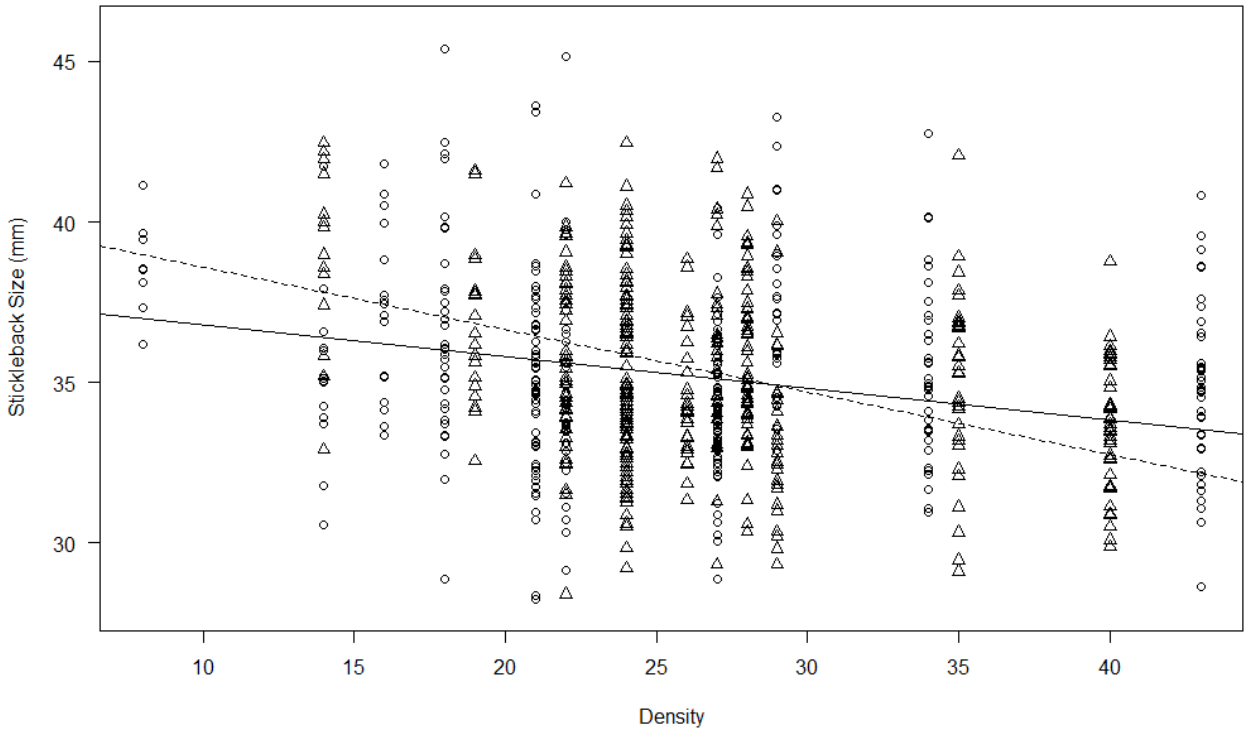

Figure 4

Figure displaying standard length of sticklebacks (size) on the y-axis and fish density of aquarium tanks on the x-axis. ○ = fresh water treatment fish, Δ = salt water treatment fish. The solid line represents fresh water treatment stickleback size across density gradient, dashed line represents salt water treatment stickleback size across density gradient. Both are regression lines fitted to the model output from the glmm; size explained by treatment in interaction with density (table 7).

Discussion

In this study I test for plastic response in lateral plate number to two different salinity treatments, 0 ppt fresh water and 30 ppt salt water. My results show a statistically significant difference in plate numbers between the two treatments, with fish developing fewer plates when raised in salt water than when raised in fresh water. The variation in stickleback lateral plate number and structure across populations has been the focus of many studies (Hagen & Gilbertson 1972; Myhre & Klepaker 2009; Song *et al.* 2010). Although plate morphs to a large extent can be explained by the *Eda* gene model (Colosimo *et al.* 2004, 2005), an explanation for the observed plate number variations within the different morphs is lacking. While many traits in stickleback have been shown to be plastic (Shama & Wegner 2014; McCairns & Bernatchez 2012; Baker and Foster 2002; Wund *et al.* 2008; Day *et al.* 1994; Baker & Foster 2002; Mazzarella *et al.* 2014 submitted[1]), this study is the first to address the question of whether salinity could affect plate number via phenotypic plasticity.

Salinity-induced plasticity in plate number

My study shows that stickleback from the Glitredammen population experiences phenotypic plasticity in reaction to salinity, leading to a reduction of plate number in a salt water habitat of, on average, 0.31 plates (model 1A, table 2 & table 3). These results are in contrast to the hypothesis that phenotypic plasticity would cause increased plate number in salt water compared to fresh water, mirroring plate number diversity in the wild.

Many morphological traits have previously been shown to be plastic in stickleback. For example, Shama & Wegner (2014) found that family size and egg size respond plastically to temperature in a trans-generational manner over three generations. And McCairns & Bernatchez (2012) found some morphological differences between sticklebacks from interconnected fresh water and salt water populations that were caused by environmental effects. Previously mentioned work by Baker & Foster (2002) found plastic responses in parental size at breeding, family size and egg size to life in a stream type environment versus life in a pond type environment, and Wund *et al.* (2008) found that marine ancestral populations through plastic responses mirror phenotypes of local fresh water populations when reared in similar environments. Day *et al.* (1994) additionally found that reversal of

diets between sister populations of stickleback leads to trait switching, i.e. they developed traits associated with the new diet instead of the diet they ate in the wild. Thus, we see that the threespine stickleback is a species that exhibits a high degree of phenotypic plasticity in its traits.

The large body of work showing plastic effects in response to other environmental cues and the strong interest in stickleback morphological variation makes it somewhat surprising that no one has investigated the question of whether salinity could affect plate number variation through phenotypic plasticity. A major environmental difference between habitats of threespine stickleback populations is salinity. Indeed the largest morphological divide between stickleback populations seems to be between those inhabiting salt water and those inhabiting fresh water. Even smaller differences in water salinity, such as populations found in brackish water, seem to be morphologically distinct, and intermediate to the fresh water and salt water groups, underscoring the importance of this study.

The only body of work which also addresses this question is a pilot experiment by Matthew Wund (personal communications). He created several freshwater stickleback families from a freshwater population in Alaska and raised them with a split-clutch design in two salinity treatments: 0 ppt and 6 ppt salt water. Wund found that the stickleback raised in the salt water treatment had smaller plates than the fish raised in the fresh water treatment. What makes this study interesting in relation to mine is that the plastic response to different salinities also resulted in an overall reduction of plate coverage in water with an increased salinity. The study by Wund found no salinity-induced response in plate number. However, his study comprised of a small number of families with fewer individuals per family, and a very low salinity was used, so it is quite possible that the plate number effect could have been revealed with more samples, a stronger salinity treatment, or both. Moreover, in agreement with my results, the plastic response in plate height that Wund found was in the same direction as the plastic response that I detected in plate number. These two studies together suggest a plastic reduction of plate coverage in fresh water sticklebacks induced by increased salinity. This might imply that the plastic plate-coverage reduction happens in stickleback populations in general, and not just in the local Norwegian population used in my study.

Interpretations and implications

Although the hypothesis that salinity induced plasticity may cause variation in plate number was confirmed, the direction of the detected response was surprising. The detected response, that sticklebacks raised in fresh water had on average 0.31 plates more than those raised in salt water, was a reversal of what one might expect considering that salt water populations generally are dominated by fully plated morphs and fresh water populations by low plated morphs (Hagen & Gilbertson 1972; Myhre & Klepaker 2009; Song *et al.* 2010).

Furthermore model 1B (table 2 & table 4), which scored only two AIC units higher than model 1A, tells us that the additive effect of density on treatment caused an increase in plate number as density increased. Interestingly the treatment effect was also higher in this model, which shows that density probably adds to the larger effect of treatment. The implications of this could be that because stickleback grew more slowly in tanks with higher density (table 7, figure 4), they spent more time within the stages where plate development takes place, buying time for more plates to be grown.

The fact that the plastic response seen is in the opposite direction of what one might expect, and that the plate number difference is quite small, are strong indications that this is a case of non-adaptive plasticity, i.e. that the observed plasticity is a reaction to a novel environment that yields no fitness advantage. If the reason salt water fish generally have more plates than fresh water fish is that there is a selection pressure for more plates in salt water - and most observations of wild stickleback populations seem to suggest this - then the fact that I found fewer plates in the fish reared in salt water is most likely not a result of adaptation, nor a trait which yields any direct advantage.

Another question arising when interpreting these results is whether or not a difference in plate number of 0.31 plates could be of biological importance. What this number means is that around 3 out of 10 stickleback in the fresh water treatment had about one plate more than stickleback in the salt water treatment. Whether or not such a low difference could be of biological importance is debatable, however, Hagen & Gilbertson (1973) found that 7 plated stickleback had a selective advantage over those with 6 or 8 plates on each side in lakes with high degree of predation. Thus it is not completely implausible that a plate difference of one

plate could be of biological importance. However, since our results explain a difference of only 0.31 plates in a system that can vary from 2 to 12 plates (on each side) (figure 3), it is relatively unlikely that this effect is of very much biological importance.

The detected effect could alternatively be a reaction to being raised in a novel and therefore potentially stressful environment. The stickleback in this experiment all originate from an isolated fresh water population, meaning that their physiology most likely has become adapted to that environment. While they retain their ability to osmoregulate and survive in both fresh and salt water, osmoregulation in salt water is a trait that probably has not been beneficial to these sticklebacks for thousands years, and it would be expected for these fish to be somewhat less effective at osmoregulation in salt than in fresh water. Adaptations to a local environment have been documented in many stickleback populations, both in fresh water and in salt water (Lavin & Mcphail 1985).

One additional habitat difference that inevitably follows in a salt water environment is higher calcium concentration. The sea salt used in this experiment contains a calcium concentration roughly equal to sea water (400 mg/L calcium ion). Many papers have suggested that a reduction in plate number in fresh water sticklebacks could be due to reduced calcium availability (Spence *et al.* 2012). This study, together with Wund's pilot work, are in line with more recent findings that a reduction of plates in fresh water is not caused by calcium limitations (Voje *et al.* 2013; MacColl & Aucott 2014).

Osmoregulation itself has also been linked to plate reduction in some studies (Heuts 1947; Marchinko & Schluter 2007). If the plate number reduction detected in this study is indeed a consequence of changing osmoregulatory challenges, it would also be interesting to investigate whether this is an adaptation enabling offspring to better survive in a new and different environment, or merely a result of higher energy requirements from growing up in a suboptimal environment. It may be that because this population has adapted itself to fresh water, living in a salt water habitat simply is more energy-consuming, which in turn reduces energy available for plate development. In that case, however, the plastic effect in plate number could be due to indirect adaptive plasticity, where in order to better survive the higher energy requirements of unfavorable salinity, sticklebacks sacrifice some of their plate coverage in order to conserve their resources. And indeed, although it is more likely that this is non-adaptive plasticity, stickleback populations have, throughout much of their

evolutionary history, migrated between fresh water and salt water habitats (Bell 1976; Johnson & Taylor 2004). An adaptation to ease such transitions between differing salinities could be advantageous. A follow up experiment using stickleback from a salt water population would likely shed more light on these questions and would therefore be a logical next step. Such an experiment might also be helpful in further understanding several other plastic traits that have been shown to be affected by salinity, as for example stickleback body shape (Mazzarella *et al.* 2014, submitted[1]).

Another potentially interesting experiment could be to look for grandparental effects of salinity treatment in both fresh water and salt water populations. As previously mentioned, Shama and Wegner (2014) found grandparental effects in several traits, demonstrating that trans-generational plasticity does occur in stickleback. It is possible that the effect I found could be enhanced in the next generation. This is something that should also be further investigated in future studies.

Experimental limitations

Whenever an experiment like this is conducted one should consider the possibility that the results have been influenced by the experiment methods. One potential weakness in this study was the use of 15 ppt embryo medium at the beginning of the experiment. In order to reduce mortality from pathogens and particularly to prevent fungal infections (Bell & Foster 1994), fertilization and hatching of eggs was carried out in 15 ppt saltwater. Although it may seem unlikely, there is a chance that this could have already triggered plastic changes in stickleback larvae in response to saltwater, thereby causing fish that were later transferred to freshwater treatments to develop phenotypes which otherwise only would develop in saltwater stickleback. My results show a fairly small difference between stickleback reared in salt water versus stickleback reared in fresh water. It is possible that this difference would have been greater had the sticklebacks been separated into treatments from the day of fertilization. Unfortunately this was necessary in order to ensure enough offspring survived for the study to yield useful results. It should be noted however, that transfer to treatments was done as soon as all fish were hatched, and long before lateral bony plates start to develop (Hagen & Gilbertson 1972). As such, the risk of this weakness biasing the results is very small.

Another potential weakness of the experimental methods was that segregation of offspring into treatments 0 and 30 ppt salt water was not carried out until after all eggs were hatched, which means that all larvae experienced the same salinity treatment in very early development. If adult plate number is determined in this short developmental phase, the joint early exposure to saltwater and the fact that some of the larvae had slightly more time to develop before being transferred to a treatment group could have affected the results. Again however, the larvae were transferred to treatments within two days, which is long before lateral-bony-plates development starts (Hagen & Gilbertson 1972).

Conclusion

I found that sticklebacks from a Norwegian fresh water population reared in fresh water had significantly higher total plate numbers than those from the same population reared in salt water. Thus, a plastic response to salinity has occurred. This is, to date, the first large-scale study conducted to find out if salinity-induced phenotypic plasticity can explain some of the variation found in stickleback lateral plate numbers. Although the relatively low difference in plate numbers between treatments along with the fact that the response seen was a reversal of what was hypothesized based on observations in stickleback populations worldwide, point strongly towards non-adaptive plasticity, adaptive plasticity cannot be entirely excluded as an explanation. Further studies are needed in order to give a more definitive answer to this question. As such, the obvious next step should be to conduct a similar experiment with stickleback originating from a saltwater population and possibly extend it to include a second (F2) generation of offspring.

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