Phosphorus limitation does not drive loss of bony lateral plates in freshwater stickleback (*Gasterosteus aculeatus*)

Running Title: Phosphorus limitation in stickleback

Sophie L. Archambeault[1,2,3], Daniel J. Durston[4], Alex Wan[5], Rana W. El-Sabaawi[4], Blake Matthews[6] and Catherine L. Peichel[1,2,3,*]

[1]Institute of Ecology and Evolution, University of Bern, Bern, Switzerland

[2]Graduate Program in Molecular and Cellular Biology, University of Washington, Seattle, Washington, USA

[3]Divisions of Basic Sciences and Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA

[4]Department of Biology, University of Victoria, Victoria, BC, Canada

[5]Aquaculture Nutrition and Aquafeed Research Unit (ANARU), Carna Research Station, Ryan Institute, NUI Galway, Ireland

[6]Department of Aquatic Ecology, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Centre for Ecology, Evolution and Biogeochemistry, Kastanienbaum, Switzerland

[*]Author for correspondence: Catherine L. Peichel, Institute of Ecology and Evolution, University of Bern, Baltzerstrasse 6, Bern, 3012 Switzerland; email: catherine.peichel@iee.unibe.ch

Authorship Contributions

SLA and CLP conceived and designed the study, with critical input from RWE and BM. AW formulated and produced the diets. SLA and DJD collected data and SLA analyzed the data. SLA and CLP wrote the manuscript with contributions from RWE, BM, DJD and AW.

Acknowledgments

We are grateful to: Hilary Poore and Melanie Hiltbrunner for help with fish husbandry, genotyping and phenotyping; Shaugnessy McCann, Jake Whitehurse, and Verena Saladin for their excellent fish care; Soraya Villalba for genotyping; all the Peichel lab members who carefully fed pellet food to the fish for a year; Dolph Schluter for advice on experimental design; Madi Gamble, Dave Beauchamp, Taylor Frierson and Will Dezan for help catching fish; and Daniel Steiner for performing the phosphorus analyses. This project was supported by the NIH Cell and Molecular Biology Training Grant T32 GM07270 (SLA), the Fred Hutchinson Cancer Research Center (CLP), an NSERC Discovery grant (DJD, RWE), and the University of Bern (CLP).

Data Accessibility Statement

The datasets used for analyses have been deposited on Dryad: https://doi.org/10.5061/dryad.stqjq2c1d.

Abstract

Connecting the selective forces that drive the evolution of phenotypes to their underlying genotypes is key to understanding adaptation, but such connections are rarely tested experimentally. Threespine stickleback (*Gasterosteus aculeatus*) are a powerful model for such tests because genotypes that underlie putatively adaptive traits have been identified. For example, a regulatory mutation in the *Ectodysplasin* (*Eda*) gene causes a reduction in the number of bony armor plates, which occurs rapidly and repeatedly when marine sticklebacks invade freshwater. However, the source of selection on plate loss in freshwater is unknown. Here, we tested whether dietary reduction of phosphorus can account for selection on plate loss due to a growth advantage of low-plated fish in freshwater. We crossed marine fish heterozygous for the 16 kilobase freshwater *Eda* haplotype and compared the growth of offspring with different genotypes under contrasting levels of dietary phosphorus in both saltwater and freshwater. *Eda* genotype was not associated with growth differences in any treatment, or with mechanisms that could mitigate the impacts of

phosphorus limitation, like differential phosphorus deposition, phosphorus excretion, or intestine length. This study highlights the importance of experimentally testing the putative selective forces acting on phenotypes and their underlying genotypes in the wild.

Keywords

Threespine stickleback, salinity, phosphorus, growth trade-off hypothesis, *Ectodysplasin* (*Eda*), ecological stoichiometry

Introduction

Understanding and predicting the process of evolution by natural selection requires connecting phenotypes, genotypes and fitness (Barrett and Hoekstra 2011). Experimental evolution suggests that evolution can be predictable (Lässig et al. 2017). However, predicting phenotypic changes in nature has been difficult due, in part, to the unpredictability of the ecological factors driving selection (Grant and Grant 2002; Ozgul et al. 2009; Nosil et al. 2018). This difficulty highlights the importance of understanding the ecological mechanisms driving adaptive change. While conceptually straightforward, direct tests of selective agents on the response of genotypes and phenotypes requires both experimental manipulations of selective agents and knowledge of the genetic basis of phenotypes to disentangle the effects of selection on the focal phenotype from selection on linked phenotypes (Wade and Kalisz 1990; MacColl 2011).

Threespine stickleback (*Gasterosteus aculeatus*) are an informative experimental and genetic model for the study of adaptive evolution due to their extensive phenotypic diversity, a rich history of ecological studies in the wild, ease of producing large crosses in the laboratory, and the availability of genomic and genetic tools (Wootton 1976; Bell and Foster 1994; Cresko et al. 2007; Kingsley and Peichel 2007; Peichel and Marques 2017). Marine stickleback have repeatedly colonized and adapted to freshwater environments across the Northern hemisphere in the last 10,000 - 20,000 years, leading to repeated and rapid shifts in both phenotypes and in allele frequencies at the genes under selection (Hohenlohe et al. 2010; Jones et al. 2012a; Jones et al. 2012b). Many of these adaptive alleles are present in the marine population at low frequency and are repeatedly selected in freshwater (Colosimo et al. 2005; Bassham et al. 2018). One of the most consistent allele frequency shifts in response to freshwater environments is at the gene *Ectodysplasin* (*Eda*), which is found in a 16 kb haplotype that is highly divergent between marine and freshwater populations (Hohenlohe et al. 2010; Jones et al. 2012b). Two additional protein-coding genes lie within this divergent haplotype, *Tnfsf13b* and *Garp*, however variation in *Eda* is known to affect the number of bony armor plates, patterning and number of neuromasts in the posterior lateral line (a mechanosensory system), and schooling ability (Colosimo et al. 2005; Mills et al. 2014; Greenwood et al. 2016; Wucherpfennig et al. 2019; Archambeault et al. 2020). Lateral plate number is the most

intensively studied of these phenotypes, and is one of many bony armor traits that are reduced or lost in freshwater (Bell and Foster 1994). Marine sticklebacks are completely-plated, with 31 - 36 plates per side and are homozygous for the marine *Eda* haplotype, while freshwater sticklebacks are usually low-plated with between 0 - 10 plates per side and are homozygous for the freshwater *Eda* haplotype (Colosimo et al. 2005). A reduction in plate number and an increase in *Eda* freshwater allele frequency occur over a few generations in natural and experimental populations, indicative of strong and predictable selection on both the phenotype and the genotype (Bell et al. 2004; Barrett et al. 2008; Gelmond et al. 2009; Le Rouzic et al. 2011; Bell and Aguirre 2013; Rennison et al. 2015).

Despite evidence that there is repeatable and strong selection for lateral plate loss in freshwater environments, the selective agent(s) remain unclear. Previous work has shown that armor plating increases the probability of escape and survival after predatory attacks by birds and other fish species, particularly in the clear, open water environments of marine habitats (Reimchen 1992, 2000; Leinonen et al. 2011). Indeed, sticklebacks found in clear and deep freshwater lakes with an abundance of vertebrate predators are often completely-plated like marine sticklebacks (Kitano et al. 2008; Reimchen et al. 2013). By contrast, escaping from vertebrate predators before capture might be more important in freshwater environments with abundant shelter, and low-plated sticklebacks do have a better fast-start performance (Reimchen 2000; Bergstrom 2002). It has also been hypothesized that increased predation by insects in freshwater might select for a reduction in lateral plates and other bony armor (Reimchen 1980), but two experiments have failed to find evidence of direct selection on lateral plates by insect predators (Marchinko 2009; Zeller et al. 2012). In addition to predation regime, several abiotic factors have been proposed as agents of selection for the reduction in lateral plates in freshwater, including salinity tolerance (Heuts 1947), swimming regime (Baumgartner and Bell 1984), increase in buoyancy by reducing body density (Myhre and Klepaker 2009), temperature (Heuts 1947; Smith et al. 2020), and a growth trade-off with building bone in environments with low ion concentrations (Giles 1983; Bell et al. 1993; Bourgeois et al. 1994).

A few direct tests of specific selective agents on armor plate loss have been performed, but none have provided strong evidence that a single selective agent is responsible for reduction of bony plates in freshwater (e.g. Heuts 1947; Marchinko and Schluter 2007; Barrett et al. 2008, 2009; Marchinko 2009; Leinonen et al. 2011; Spence et al. 2012; Zeller et al. 2012). In part, this is because some studies have ruled out a particular factor (e.g. insect predation: Marchinko 2009; Zeller et al. 2012), have confounded plate morph and population origin (e.g. Heuts 1947; Spence et al. 2012), or have not tested completely-plated versus low-plated fish (e.g. Leinonen et al. 2011). Furthermore, none of these studies have isolated the effects of the underlying *Eda* allele in a marine genomic background, which is needed to determine whether selection is acting directly on phenotypes controlled by *Eda* or on linked phenotypes. A few experiments have manipulated the *Eda* genotype in a marine genomic background using crosses from populations that are polymorphic for lateral

plate phenotypes and *Eda* genotype. However, these previous studies did not examine the extent of linkage disequilibrium of the *Eda* freshwater allele with other loci and therefore could not determine whether selection was acting directly on lateral plates, on other phenotypes affected by *Eda*, or on phenotypes affected by linked loci. Despite these caveats, both laboratory and pond experiments showed that low-plated and/or fish with *Eda* freshwater alleles have a growth advantage over completely-plated fish in freshwater (Marchinko and Schluter 2007; Barrett et al. 2008, 2009; Rennison et al. 2015). Rapid juvenile growth can be beneficial for predator avoidance (Foster et al. 1988; Marchinko 2009) and overwinter survival (Carlson et al. 2010), and adult size is positively correlated with reproductive output (reviewed by Baker et al. 2015). Taken together, these results suggest that growth rate mediated by genotype at *Eda* could be a target of selection in freshwater sticklebacks.

What could cause the growth differential between *Eda* genotypes in freshwater (Marchinko and Schluter 2007; Barrett et al. 2008, 2009)? As described above, genotype at *Eda* controls most of the variation in number of bony lateral plates, which are primarily composed of hydroxyapatite, a calcium- and phosphorus-rich crystal. Bone accounts for the majority of the calcium and phosphorus found in fishes (Hendrixson et al. 2007). Fish extract calcium from both the water and their diet, and can adjust their ability to extract calcium from either source (reviewed in Hossain and Yoshimatsu 2014). So while calcium levels are substantially lower in freshwater compared to marine environments (Krumgalz 1982; Atkinson and Bingman 1997; Jeziorski et al. 2008; Weyhenmeyer et al. 2019), stickleback can likely meet their calcium demands by adjusting calcium uptake (Rudman et al. 2019). In contrast, fish must obtain a substantial portion of phosphorus from their diets (Vielma and Lall 1998; Avila et al. 2000; Lall 2002; Sullivan et al. 2007). In stickleback, bones account for the majority of whole body phosphorus (Leal et al. 2017), the number of lateral plates is positively correlated with whole-body phosphorus (Durston and El-Sabaawi 2017), and ion concentrations (calcium, magnesium, silicon, pH and reactive phosphorus) are the top predictor of number of lateral plates in freshwater stickleback populations from the Cook Inlet of Alaska (Bourgeois et al. 1994). The total phosphorus content of stickleback varies from 2-6.5% (El-Sabaawi et al. 2016; Durston and El-Sabaawi 2017; Leal et al. 2017), whereas the mean phosphorus content of typical stickleback prey in marine and freshwater environments, such as zooplankton and macroinvertebrates (Schluter and McPhail 1992; Schluter 1993; Lankov et al. 2010; Jakubavičiūtė et al. 2017a; Jakubavičiūtė et al. 2017b), ranges from 0.61-1.24% (Supplemental Table 1). Therefore, stickleback might be confronted with a mismatch between their phosphorus requirements and the supply of dietary phosphorus in their environment. Such potential mismatches have led to the hypothesis that dietary phosphorus may limit the growth rate of fish in freshwater environments (Elser et al. 2000; Hood et al. 2005; Boersma et al. 2008; McIntyre and Flecker 2010; Vrede et al. 2011). Consistent with this hypothesis, reduction of dietary phosphorus has been shown to cause growth and bone mineralization defects in haddock and trout (Roy et al. 2002; Fontagné et al. 2009; Boros et al. 2015; Witten et al. 2016; Morales et al. 2018).

We hypothesized that dietary phosphorus is a limiting element for completely-plated marine stickleback in freshwater environments. We predicted that phosphorus limitation imposes a cost on these fish that could manifest in a variety of ways, including as a reduction in survival, somatic growth, bone mineral density, phosphorus excretion rate, or an increase in gastrointestinal tract lengths to facilitate phosphorus uptake. Furthermore, we hypothesized that reduction in lateral plate count would alleviate phosphorus demands on marine fish in freshwater, and therefore marine fish homozygous for the freshwater allele at *Eda* would display less severe responses to phosphorus limitation. To test our hypotheses, we caught marine stickleback that were heterozygous carriers of the freshwater *Eda* haplotype. We made F2 crosses between heterozygous F1 parents and split families by randomly assigning individuals to factorial combinations of salinity (saltwater and near freshwater) and diet (high and low phosphorus). We compared the survival and growth rates of fish that carried homozygous marine, heterozygous, and homozygous freshwater alleles at *Eda*, both before and during bony plate growth, as well as bone phosphorus content, phosphorus excretion, and intestine length of a subset of fish after bony plate growth.

Materials and Methods

Ethics statement

Marine stickleback were collected under the Washington Department of Fish and Wildlife scientific collection permits 15-033, 15-213, and 16-066. Animal care and handling procedures were approved by the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee (protocol 1575) and the Veterinary Service of the Department of Agriculture and Nature of the Canton of Bern (VTHa# BE4/16 and BE82/17).

Puget Sound fish collection and crosses

Wild marine stickleback were collected in Puget Sound, WA, USA in the summers of 2015 and 2016 as previously described (Archambeault et al. 2020). In 2015, fish were collected at the surface during a multi-day trawling survey conducted in water that was at least 30 meters deep in the Whidbey Basin and Bellingham Bay areas. In 2016, fish were collected with the help of the Washington Department of Fish and Wildlife in a beach seine in Clam Bay, near Manchester. Wild-caught fish were transferred to the Fred Hutchinson Cancer Research Center stickleback facility and initially genotyped at a single marker within the *Ectodysplasin* (*Eda*) haplotype (Stn382, see "DNA extractions and genotyping" below). Fish carrying one or two freshwater alleles at this marker were uniquely marked using spine clips and visible implant elastomer tags (Northwest Marine Technologies, Anacortes, WA, USA). Subsequent genotyping of these carriers revealed that some fish carried a partial freshwater haplotype, while other fish were heterozygous at all markers within the

haplotype. Thirty crosses between 37 wild-caught heterozygous fish carrying unique freshwater haplotypes were made and subsequently shipped to the University of Bern stickleback facility in September 2016. The F1 fish were grown to reproductive maturity and genotyped to identify heterozygous carriers of the full 16 kb haplotype (i.e. fish that were heterozygous at all markers within the haplotype, Figure 1). Of the 37 unique freshwater haplotypes transmitted to the F1 generation, 24 of these were full 16 kb *Eda* haplotypes, which were transmitted to the F2 experimental generation by intercrossing F1 fish heterozygous for these haplotypes.

F2 experimental cross setup

Experimental F2 crosses were made between F1 *Eda* heterozygotes, so that the resulting offspring would have a 1:2:1 ratio of homozygous marine : heterozygous : homozygous freshwater genotypes at *Eda*. The marine allele is referred to as C, and CC homozygotes have a completely-plated phenotype of 31 - 36 plates per side of the fish. L alleles are usually found in freshwater populations and LL fish are low-plated with between 0 - 10 plates per side (Figure 1). Crosses yielding at least 102 eggs were fertilized, separated into groups of ~10 embryos for better nutrient exchange and kept in Petri dishes containing clean freshwater for 2 days. Water was exchanged daily, and after 2 - 3 days, unfertilized or dead embryos were discarded, and the remaining developing embryos were split between four clean tanks with the following treatments: freshwater + low phosphorus (LowP) diet, freshwater + high phosphorus (HighP) diet, saltwater + LowP diet, and saltwater + HighP diet. Embryos were kept in plastic cups with mesh replacing the bottom of the cup to allow for air and water exchange while confining the embryos and hatchlings to the cup. Embryos generally hatched 9 - 10 days post fertilization (dpf), so between 10 - 12 dpf, hatchlings were counted within each tank, and equal numbers of larvae were released into each of the four tanks for that family. Released hatchlings per tank ranged from 20 - 25 per tank for Timepoint 1, and 20 - 38 per tank for Timepoints 2 and 3 (see "*Sampling and phenotyping of F2 experimental fish"* below).

Fish housing and care

Wild-caught and F1 fish were housed in freshwater conditions (3.5 parts per thousand (ppt) saltwater), as previously described (Archambeault et al. 2020). F2 crosses were made as described above (see "F2 Experimental cross setup") and housed in either full (35 ppt) saltwater or reduced (3.5 ppt) saltwater conditions (referred to as saltwater and freshwater, respectively, from here on). Fish were kept in 100-liter tanks on saltwater or freshwater recirculating systems. Conductivity was automatically monitored and maintained at 41.5 and 5.3 millisiemens cm^{-1} for the saltwater and freshwater systems, respectively, using a saturated solution of Instant Ocean sea salt (Instant Ocean, Aquarium Systems, Sarrebourg, France). Water temperature (15˚C) and pH (7.8 - 8.0) were monitored and maintained automatically for both the saltwater and freshwater systems. Lighting was programmed with 11 hours full sunlight (3450 lumens), 1 hour sunrise, 1 hour sunset, and 11 hours with a moon light (600 lumens) for nighttime for both recirculating systems.

Adult wild-caught and F1 fish were fed a mixture of freshly hatched *Artemia* nauplii every morning and frozen *Mysis* shrimp in the afternoons, three times per week. Newly hatched larvae and juvenile F1 fish were fed *Artemia* nauplii twice per day. Heterozygous F1s were fed *Mysis* shrimp every afternoon to induce reproduction prior to the start of the experiment. Experimental F2 fish were fed as described below (see "*Experimental Diets*").

DNA extractions and genotyping assays

DNA was extracted from fin tissue using a modified HotSHOT DNA extraction method (modified from Meeker et al. 2007) as previously described (Archambeault et al. 2020). Wild-caught fish were genotyped at markers listed in Supplemental Table 2. SNP4, SNP11110 and the NAKA SNP are segregating polymorphisms in the wild population. Although not useful for determining whether a fish carries a marine or a freshwater *Eda* haplotype, these SNPs were informative for distinguishing among the haplotypes in the F1s. F1 fish were genotyped at Stn382, IDH or LRR (sex markers; see Peichel et al. 2004 and Supplemental Table 2, respectively), and any informative markers that would distinguish the transmitted haplotype (i.e. SNPs12/13 distinguish short haplotypes from full 16 kb haplotypes). Heterozygous carriers of full haplotypes were marked within each cross as potential parents for the F2 experimental crosses.

F2 experimental fish were genotyped at Stn382 and IDH (or LRR) to identify their genotype at the *Eda* haplotype and their sex, respectively.

Experimental diets

Fish obtain phosphorus through their diet (Vielma and Lall 1998; Avila et al. 2000; Lall 2002; Sullivan et al. 2007), therefore we designed the experimental diets to differ only in phosphorus content, while otherwise being *iso*-caloric, *iso*-nitrogenous, and *iso*-lipidic (Supplemental Table 3). We targeted environmentally relevant levels of phosphorus near the low end of measured natural foods; the mean percentage of phosphorus in possible prey items that stickleback would encounter in freshwater and marine habitats ranges from 0.61-1.24% (overall mean = 0.9%; overall range 0.1-2.59%; Supplemental Table 1). We thus targeted 0.5% and 1% phosphorus (P) in the diets (equivalent to 0.5 and 1.0 mg kg^{-1}, respectively). However, after making the pellet diets, the measured concentrations of P were 0.48 ± 0.09% and 0.61 ± 0.05%. Despite this narrower than expected difference, these diets with reduced phosphorus allow us to test the relevant question: does the reduction of dietary phosphorus impose a growth cost on stickleback genetically programmed to build a complete set of bony lateral plates?

Experimental F2 fish were fed live *Artemia* twice per day on 11 - 13 dpf (approximately 1 day after hatching). At 14 dpf, fish were started on their experimental diets. Larvae were given the experimental diet in the morning and received live *Artemia* in the afternoon at 14 - 16 dpf. At 17 dpf, fish were fed pellet food twice per day, and no longer received any *Artemia*. The amount of pellet food provided per day was estimated to be 4% of body weight per day based on the number of larvae in the tank and was not adjusted as fish died. Therefore, fish received food effectively ad libitum. Pellet diets were ground and sieved to provide appropriate sizes for different age classes of stickleback. Larval fish were fed ground pellets that were smaller than 200 µm until 87 dpf. At 88 dpf, fish were moved to pellets sized between 320 and 500 µm.

Sampling and phenotyping of F2 experimental fish

In our crosses, new plates start forming at approximately 13 mm standard length (SL), and continue to form until 25 mm SL. After this stage, plates continue to grow in size, but new plates do not form (Supplemental Figure 1). Fish from Timepoint 1 were sampled between 37 and 39 dpf, so that fish were under 13.1 mm and therefore sampled prior to plate formation. Fish from Timepoint 2 were sampled between 128 and 132 dpf, so that the majority of fish in the freshwater tanks were between 21 and 30 mm SL and therefore sampled during and after plate formation. Fish from Timepoint 3 were sampled between 155 and 175 dpf when freshwater fish were between 18 and 55 mm SL (during and after plate formation).

Fish were euthanized with a lethal dose of 0.2% buffered MS-222 (tricaine methylsulfonate). Timepoint 1 fish were measured with manual calipers for SL to the nearest 0.05 mm and weighed to the nearest 0.01 mg. The caudal fin (and sometimes a portion of the posterior trunk) was sampled and placed in 95% ethanol for later DNA extraction. Timepoint 2 fish were measured to the nearest 0.05 mm with manual calipers for SL, weighed to the nearest mg, and photographed. A portion of the caudal fin was stored in 95% ethanol for DNA extraction. Timepoint 3 fish were placed into an excretion collection experiment (see "*Excretion collection and measurement*" below), then euthanized, measured to the nearest 0.05 mm, weighed to the nearest mg, and then frozen at -20°C. Later, Timepoint 3 fish were thawed in order to remove the pelvic girdle for phosphorus analysis. Additionally, the entire intestine was dissected, measured to the nearest 0.05 mm, and weighed to the nearest 0.01 mg, and the plates on the left side of the body were counted.

Excretion collection and measurement

Fish were fed 2 hours before the experiment began. They were left in the tank with the food for 1 hour, then caught and held in buckets until the start of the experiment. The experiment was carried

out in a climate-controlled room at 15°C using custom made chambers. These chambers were Ziploc® Twist/n Loc® 16 ounce containers (Johnson and Son, Inc., Racine, WI, USA) with a hole in the lid through which a hose for water exchange could pass.

System water (freshwater and saltwater) was filtered to 0.2 µm using a gravity filter (Platypus GravityWorks, Cascade Designs, Seattle, WA, USA). The hoses and chambers for excretion collection were rinsed with deionized water from the tap and then filled with 300 mL of filtered system water. Fish introduction to chambers was staggered by 1 minute (2 minutes if there were not as many fish to process) to allow for sample handling between fish. Up to 30 chambers were processed in a set (26 fish, 2 freshwater controls and 2 saltwater controls). Samples from each chamber (experimental and controls) were taken at 0 (just after fish introduction), 60, and 120 minutes.

Phosphorus does not homogenize as well as ammonium in the chamber, so unmixed samples can be very inaccurate. We spent approximately 40 seconds slowly withdrawing and then reinserting two full syringes (60 mL each) of water to mix the chambers before taking the 30 mL sample on the third pull. The 30 mL sample was then handled in the following way. First, a filter cartridge was attached to the syringe containing a GF-75 glass fiber filter (0.3 µm pore, AM.D. Manufacturing Inc., Mississauga, Ontario, Canada). Next, the filter was rinsed by pushing 5 mL of the sample through the filter cartridge. The collection tube was then rinsed twice with 5 mL of the sample, and then the final 15 mL of sample was filtered into the collection tube and sealed. Once all the samples were completed, the samples were frozen and stored at -20°C until analysis.

Bone and excretion phosphorus measurements

All water and bone samples for phosphorus analysis were digested and oxidized using a peroxydisulfate solution (10 grams of $K_2S_2O_8$ and 1.5 grams of NaOH in 1 liter of water), and analyzed on a Skalar San++ Continuous Flow P/N analyzer (as in Leal et al. 2017).

Survival analyses

Survival was calculated for each tank from the number of hatched larvae released into the tank and the number of live fish collected at the end of each timepoint. Differences in survival between conditions were analyzed using a linear mixed-effects model with family and tank as random effects, and water, diet and water x diet interaction as fixed effects.

For each timepoint, we also tested whether the CC, CL and LL genotypes had differential survival. The null hypothesis was that there is no difference in survival between genotypes. Assuming that each tank initially received a 1:2:1 ratio of genotypes, our expected ratios of genotypes at the end of each timepoint under the null hypothesis was 1:2:1. Due to low numbers of surviving fish (typically N < 20, except in FW tanks at Timepoint 1), the surviving fish were summed within genotype, but across families within each condition to produce a three genotype (CC, CL, LL) x four treatment table (FW-HighP, FW-LowP, SW-HighP, SW-LowP). Survival was then analyzed in two ways: 1) a chi-square analysis across the 3x4 table to see if the survival of genotypes varied by treatment; and 2) a chi-square analysis comparing the observed genotype ratios within each treatment to the 1:2:1 expected ratio.

Growth, intestine length, and bone phosphorus analyses

Growth was measured in two ways for fish from all three timepoints: standard length (SL measured in mm) and body condition. Intestine length and bone phosphorus content (%P) were measured in fish taken at Timepoint 3 only. Each of these measures were then analyzed for differences between treatments and genotypes using a linear mixed-effects model with family and tank as random effects, and water, diet, sex, genotype, and the interactions between water x genotype, water x diet, and diet x genotype as fixed effects. Analyses of fish from Timepoint 3 also included age as a fixed effect, since the fish ranged in age between 155 - 175 dpf. Body condition and intestine length were analyzed by running the model for the natural log of weight (or intestine length) and including the natural log of standard length as an additional fixed effect.

Excretion data analyses

Excretion rates were calculated for the 0 - 60 minute sampling interval as well as the 60 - 120 minute sampling interval using the equation:

$$Excretion\ rate = \frac{P_f * V_i - P_i * V_i}{t}$$ **(Error! No text of specified style in document.**.1)

where P_i and P_f are the μg L^{-1} phosphorus in the water at the start and end of the sampling interval, respectively, V_i is the volume of water (in liters) at the start of the sampling interval (before the end sample was removed), and t is the length of the sampling interval in minutes. The phosphorus excretion rate is therefore reported as μg min^{-1}.

During data exploration, we found that the excretion measurements varied from batch to batch on the Skalar San++ Continuous Flow P/N analyzer. We also found that excretion rates in the second time interval (60 - 120 minutes) were lower than in the first time interval (0 – 60 minutes) likely because the fish ceased excretion after 60 minutes (R.W.E., pers. observation). Therefore, the data

were trimmed to only include samples from the first time interval, and in which the 60 minute sample was analyzed in the same batch as the 0 minute sample for each individual fish. Therefore, effects of time interval and analysis batch were removed. Excretion rates for the 0 – 60 minute time interval were analyzed for differences between treatments and genotypes using a linear mixed-effects model. Family and collection day were set as random effects; water, diet, sex, weight, and genotype were fixed effects; and the interactions between water x genotype, water x diet, and diet x genotype were also fixed effects.

Results

Plate number is controlled by genotype at Eda

The *Eda* locus controls between 74 - 100% of the variation in number of plates, depending on the genetic modifiers present in the population or cross (Avise 1976; Colosimo et al. 2004). In the F2 fish at Timepoint 3, fish homozygous for the marine allele (CC) are completely-plated, fish homozygous for the freshwater allele (LL) are low-plated, and heterozygous fish (CL) are phenotypically indistinguishable from CC fish (Figure 1). Genotype at the *Eda* haplotype controls 93% of the variation in the number of lateral plates in this study.

Hatching rates equal between saltwater and freshwater conditions

We observed no differences in hatching rates (% eggs that hatched) between saltwater and freshwater conditions (Supplemental Figure 2). Hatching rates were analyzed separately for crosses analyzed at Timepoint 1, since these crosses were performed at a different time than the crosses analyzed at Timepoints 2 and 3, which were setup and hatched concurrently. Hatching rates of crosses analyzed at Timepoint 1 were 95% in both freshwater and saltwater ($F_{1, 56} = 0.14$, $p = 0.70$). Hatching rates of crosses analyzed at Timepoints 2 and 3 were 93% and 94% in freshwater and saltwater, respectively ($F_{1, 130} = 0.18$, $p = 0.68$).

Higher survival in freshwater than in saltwater

Water salinity, but not diet, affected survival rates at all three timepoints, and survival decreased over time (Figure 2). For fish analyzed at Timepoint 1 (37 dpf), survival was 84% and 55% in freshwater and saltwater, respectively ($F_{1, 54} = 64.9$, $p = 8.0e\text{-}11$), while diet had no effect ($F_{1, 54} = 0.39$, $p = 0.54$). For fish analyzed at Timepoint 2 (129-133 dpf), survival was 45% and 30% in freshwater and saltwater, respectively ($F_{1, 78} = 10.9$, $p = 0.001$), and again diet had no effect ($F_{1, 78} = 0.55$, $p = 0.46$). Survival of fish analyzed at Timepoint 3 (155-175 dpf) was 60% and 12% in freshwater and saltwater, respectively ($F_{1, 20} = 67.1$, $p = 8.1e\text{-}08$), with no significant effect of diet ($F_{1, 20} = 2.81$, $p = 0.11$).

No differential survival of Eda genotypes

We also analyzed the ratios of genotypes that were alive at the end of each timepoint, and found no difference in the ratios of genotypes (Timepoint 1: χ^2_6 = 3.6, p = 0.73; Timepoint 2: χ^2_6 = 5.5, p = 0.48; Timepoint 3: χ^2_6 = 3.4, p = 0.76). We also found no deviation from the expected 1:2:1 ratio of genotypes in any of the experimental conditions (Supplemental Figures 3-5).

No differential growth of Eda genotypes prior to plate development (Timepoint 1)

At Timepoint 1, fish were an average of 9.95 mm long (range 6.85 – 13.05 mm), thereby representing a timepoint before the lateral bony plates begin to develop (Supplemental Figure 1). The results of the mixed-effects linear model revealed significant effects of water on SL and body condition (the natural log of weight with the natural log of SL in the model) at this timepoint (Table 1; Figure 3; Supplemental Figure 6); fish were larger and had higher body condition when reared in saltwater. In addition, males were significantly larger than females across the entire experiment (Table 1). Overall, there was no effect of *Eda* genotype on any measure of growth at this timepoint before bony plates started to develop.

No differential growth of Eda genotypes during plate development (Timepoints 2 & 3)

Fish were an average of 27.85 mm long (range 13.05 - 44.00 mm) at Timepoint 2. Plate number is generally set by 25 mm SL (Supplemental Figure 1); importantly LL fish have fewer plates than CC and CL fish. Therefore, we considered this a reasonable time to further assess the differential effects of genotype on growth, because we expect considerable investment of phosphorus into plate development during this phase. However, the mixed-effects linear models for SL and body condition show no effect of genotype on size (Table 1; Figure 3; Supplemental Figure 7). Water salinity had a significant effect on SL (but not on body condition); however, the fish raised in freshwater were larger than fish grown in saltwater - a reversal from Timepoint 1.

At Timepoint 3, fish were an average of 34.59 mm long (range 15.35 - 50.40 mm). The results of the linear mixed-effects model suggest that water and sex have significant effects on size as measured by standard length (Table 1; Figure 3; Supplemental Figure 8). Similar to Timepoint 2, fish grown in freshwater were larger than their siblings grown in saltwater. Additionally, males were larger than females. At this stage, genotype has an effect on both standard length ($F_{2, 259}$ = 3.02, p = 0.05) and body condition ($F_{2, 259}$ = 4.14, p = 0.02). However, contrary to our hypothesis about dietary phosphorus limiting the growth of the completely-plated genotype, the CC fish were larger than their siblings (CL and LL fish were on average 1.98 and 1.77 mm smaller than CC fish in SL,

respectively) and had a higher body condition. This effect is even greater in saltwater, where the LL fish have an even lower body condition (water x genotype interaction) but these effects are slight, at best, and likely due to low numbers of surviving fish (Figure 3; Supplemental Figure 8).

No difference in phosphorus *deposition or excretion, or intestine length* by Eda genotype (Timepoint 3)

In fish sampled at Timepoint 3, we examined whether fish compensated for reduced phosphorus in their diets by reducing phosphorus deposition in the bone, decreasing excretion of phosphorus, and/or increasing intestine length, which can be correlated with phosphorus uptake. The number of bony plates was affected by neither diet nor water ($F_{1, 261} = 0.33$, $p = 0.56$ and $F_{1, 263} = 1.04$, $p = 0.31$, respectively), suggesting that there was not a plastic response of plate development to the environmental treatments. However, to further investigate whether phosphorus deposition in bony structures changed in response to the treatments and/or due to *Eda* genotype, we also analyzed the phosphorus content of the bones constituting the pelvic girdle. The results of the mixed-effects linear model show that the percent phosphorus (%P) of the pelvic girdle is lower in fish reared in saltwater, in younger fish, and in males (Figure 4; Supplemental Figure 9; Table 2). The effect of water on phosphorus content of the pelvic girdle could be due to a difference in ontogeny: fish reared in saltwater are significantly smaller than those in freshwater and age also has a significant effect in the model (Table 2). Importantly, phosphorus content of the pelvic girdle bones does not differ between fish with different genotypes.

Fish may also compensate for dietary limitations by adjusting the excretion rates of limiting elements. To test this, we compared phosphorus excretion rates of fish across our experimental treatments. The mixed-effects linear model shows that water and diet have significant effects on phosphorus excretion rate. Fish reared in saltwater excrete less phosphorus, and, as expected, fish fed the HighP diet excrete more phosphorus (Figure 4; Supplemental Figure 10; Table 2). However, phosphorus excretion rates do not differ between fish with different genotypes at *Eda*.

Finally, fish might compensate for diet quality and increase phosphorus uptake through plastic changes in intestine length (Olsson et al. 2007; Wagner et al. 2009; German et al. 2010), so we examined whether intestine length varied in response to either lower phosphorus content in the diet or to the higher phosphorus demands of fish with more plates. We found that intestine length was strongly positively correlated with standard length, but there was no effect of *Eda* genotype or any other factor on intestine length (Figure 4; Table 2).

Discussion

Ancient haplotypes containing the gene *Ectodysplasin* (*Eda*) control bony plate number in threespine stickleback. Fish carrying the common marine allele, C, are completely-plated with 30 - 36 plates per side. Fish homozygous for the freshwater *Eda* allele, L, are low-plated with between 0 - 10 plates per side. The freshwater allele has been repeatedly fixed in freshwater environments, and selection on the allele can be quite strong (Colosimo et al. 2005; Jones et al. 2012b; Rennison et al. 2015), yet the agent of this natural selection on the allele is unknown. Previous work has found that LL fish grow faster than CC fish in freshwater but has not identified what causes this growth differential (Barrett et al. 2008, 2009). In some wild populations, CC fish have higher whole-body phosphorus content than LL adults, consistent with having more phosphorus-rich bony armor (El-Sabaawi et al. 2016; Durston and El-Sabaawi 2017). We therefore hypothesized that marine stickleback with more plates would experience greater dietary phosphorus limitation in freshwater habitats than those with fewer bony plates, as controlled by genotype at *Eda*. We used the offspring of laboratory crosses between marine fish heterozygous for the *Eda* haplotype to look for evidence of phosphorus limitation and alleviation in fish with a full set of bony plates (CC at *Eda*) and reduced set of bony plates (LL at *Eda*), respectively, across four combinations of two environmental treatments: saltwater and freshwater, and HighP and LowP diets.

Ecological stoichiometry predicts that when an element is limiting, consumers are forced to cope with that limitation or suffer fitness consequences (Sterner and Elser 2002). Mechanisms for coping with a limitation include differential survival or growth, as well as differential allocation to various tissues (e.g. bone), excretion, or element uptake (e.g. intestine length). In our laboratory crosses and conditions, we did not find the predicted differences in either survival or growth rate driven by diet or genotype either prior to or during bony plate development. Furthermore, we did not detect differences in either bone phosphorus content, phosphorus excretion rates or intestine lengths between sibling fish with different genotypes at *Eda*. Together, these data suggest that marine stickleback in freshwater environments may not suffer a cost due to low levels of dietary phosphorus and that phosphorus availability is therefore unlikely to be driving evolutionary changes in genotype at *Eda*, the major effect locus underlying plate phenotype.

We conclude that limited phosphorus availability is unlikely to be the selective agent favoring plate loss in freshwater habitats for several reasons. First, stickleback may not be phosphorus limited in the wild or in our experiment, although more work is needed to quantify habitat (e.g. freshwater, marine, littoral, pelagic) and population-specific variation in the availability of phosphorus in stickleback diets. Most fish thought to be living under phosphorus-limited growth conditions are herbivores (Hood et al. 2005; Frost et al. 2006), though phosphorus limitation is predicted to occur occasionally in carnivorous and insectivorous fishes as well (Schindler and Eby 1997; McIntyre and

Flecker 2010). Interestingly in our experiment, the fish fed a HighP diet (0.61% P) had a higher phosphorus excretion rate than fish fed the LowP diet (0.48% P) (Figure 4; Supplemental Figure 10; Table 2). Both of the experimental diets were relatively low in phosphorus compared to wild prey items (Supplemental Table 1). Thus, an increase in phosphorus excretion with an increase in %P diet in this range is consistent with either no phosphorus limitation at these dietary levels, or an inability to adjust the retention of phosphorus via the excretion rate of dietary phosphorus, as observed in some consumers (Hessen et al. 2013). If excretion rates are static, then we would expect to see growth differentials between fish with different *Eda* genotypes unless growth limitation is driven by something other than dietary phosphorus. This alternative hypothesis is consistent with the observation that phosphorus excretion rates are not inversely related to body phosphorus content in the wild, as is expected under limiting dietary phosphorus conditions (Durston and El-Sabaawi 2019; Rudman et al. 2019).

Second, it is possible that the previously identified growth advantage of *Eda* LL sticklebacks in freshwater may have been driven by a gene or genes linked to *Eda* and not by *Eda* itself. In our study, we found no evidence for increased growth of *Eda* LL fish in either freshwater or saltwater. We were able to identify most of the boundaries of the freshwater *Eda* haplotypes used in our crosses, whereas the previous studies did not determine whether additional freshwater alleles were in linkage disequilibrium with the *Eda* freshwater haplotype (Marchinko and Schluter 2007; Barrett et al. 2008, 2009). The authors of these studies raise the possibility that growth trade-offs could be facilitated by freshwater alleles at a neighboring, downstream gene, *Gjb1*. About half of the F0 fish used in our crosses carried the marine allele at this gene, meaning that the effects of genotypes at *Gjb1* and *Eda* were unlinked in our experiment (Archambeault et al. 2020). Consistent with a locus other than *Eda* conferring a growth advantage, selection on *Eda* in a freshwater pond experiment (Barrett et al. 2008) was attributed to both direct selection for plate loss and independent selection on as yet unidentified phenotypes mediated by pleiotropic effects of *Eda* and/or linked gene(s) (Rennison et al. 2015). This point highlights the importance of conducting studies using controlled genotypes to determine whether a particular agent of selection is acting upon the focal phenotypes rather than on linked phenotypes.

A third possibility is that the selective agent causing growth trade-offs in freshwater is not phosphorus, but something often correlated with phosphorus availability. Direct selection on plate loss in freshwater (Rennison et al. 2015) suggests that there may still be a trade-off between bone development and growth in freshwater. A reduction in environmental calcium has been proposed as a factor mediating this trade-off because the majority of calcium, like phosphorus, is found in the bone, and calcium homeostasis is critical for a range of cellular processes (Giles 1983). Although there is a positive correlation between extremely low levels of environmental calcium (< 3 mg L^{-1}) and a nearly complete reduction in bony armor in some freshwater populations, these studies have mostly focused on comparisons between un-plated and low-plated freshwater populations.

Furthermore, the relative importance of calcium limitation and predation regime in these studies is debated (Giles 1983; Bell et al. 1993; Spence et al. 2013; MacColl and Aucott 2014; Smith et al. 2014; Smith et al. 2020). It has been shown that completely-plated sticklebacks grow faster in high calcium concentrations, but this study somewhat confounded population and habitat origin with plate morph (Spence et al. 2012). Unfortunately, our experiment was not designed to test the role of environmentally relevant levels of calcium. While calcium levels were likely higher in the saltwater versus freshwater tanks, the level of calcium in the freshwater tanks was still quite high due to the source of our water (> 20 mg L^{-1}) and our use of 3.5 ppt saltwater for the freshwater tanks (see "*Fish housing and care*"). While 3.5 ppt saltwater is protective against freshwater parasites and benefits fish health, it does not fully recapitulate the environmental conditions faced by marine stickleback adapting to freshwater. Thus, our freshwater condition was unlikely to impose a calcium limitation as severe as that associated with the extreme reduction of bony armor observed in some freshwater populations. Given the potential interactions and co-limitations of ions such as calcium and phosphorus, the relatively high calcium and salinity levels in our experiment may also explain why we did not observe the previously reported growth advantage of fish with either reduced plates or the LL genotype in 0 ppt freshwater, although these studies did not report calcium levels (Marchinko and Schluter 2007; Barrett et al. 2009). Interestingly, a recent study of stickleback ionomes found that rare elements, particularly barium, differ consistently between marine and freshwater fish and their native habitats (Rudman et al. 2019). This might be a factor to test in future studies, given the lack of conclusive empirical support for either calcium or phosphorus limitation driving selection for the low-plated phenotype in freshwater.

It is also important to consider the potential effects of the limitations of this study on our results. The difference between the high (0.61 ± 0.05% P) and low (0.48 ± 0.09% P) diets was not as large as originally planned; nonetheless, no effect of genotype was seen in either diet treatment. It is possible that our targeted dietary phosphorus limitation was either too strict or not strict enough. Studies in trout and haddock observed growth trade-offs with dietary phosphorus contents of 0.5% and 0.42%, respectively, (and at least partially rescue it at 1.0% P) (Roy et al. 2002; Witten et al. 2016). However, the fish in our experiment grew and developed normally, at least in freshwater (for saltwater, see below). Based on work in trout, we would expect a severe phosphorus limitation to cause skeletal deformities (Fontagné et al. 2009; Deschamps et al. 2014; Deschamps et al. 2016; Witten et al. 2016), but a chi-square analysis of "abnormal" vs "normal" fish at Timepoint 2 detected no differences in the frequencies of skeletal deformities (assessed qualitatively) between the four conditions (8 of 269 in SW-HighP, 11 of 279 in SW-LowP, 8 of 506 in FW-HighP, 8 of 432 in FW-LowP; χ^2 = 5.30, df=3, n=1209, p=0.15), suggesting that the P limitation was not too severe. Furthermore, the level of phosphorus in both of these diets is consistent with the amount of phosphorus found in wild prey items available to stickleback in freshwater environments, which range from 0.1-2.6% P among prey taxa (Supplemental Table 1). The growth deficits observed in previous laboratory experiments with stickleback in freshwater resulted from diets of *Artemia*, *Daphnia* and/or chironomid larvae (Marchinko and Schluter 2007; Barrett et al. 2009), all of which have an estimated

% P content of 1.1-1.3% (Andersen and Hessen 1991; Hall et al. 2004; El-Sabaawi et al. 2016). However, the fish in these previous experiments were fed to satiation with extra food removed each day, while the fish in our experiment were fed *ad libitum* and therefore might have been able to obtain more phosphorus in their diets simply by consuming more food.

There is also the possibility that we did not accurately assess the compensatory mechanisms, such as excretion or incorporation of phosphorus into bone. For example, most of the fish used for the excretion analyses were smaller than have been used for other studies of wild-caught fish (El-Sabaawi et al. 2016). Consequently, we observed low values of excreted phosphorus, especially in the LowP diet (Figure 4). Nonetheless, our assay was still sensitive enough to detect that fish on the HighP diet excreted more phosphorus (Figure 4). For our measurement of phosphorus incorporation into bone, we measured the pelvic girdle, which was robust and accessible on all fish, whereas the plates on most fish were too small to sample reliably. It is therefore possible that a difference in phosphorus incorporation occurred in the plates themselves, which can account for between 5 and 22% of the entire phosphorus content of a stickleback (Durston and El-Sabaawi 2017; Leal et al. 2017). Finally, it is possible that additional compensatory mechanisms that were not measured here, such as regulation of phosphorus absorption rates within the intestine, might differ between fish with different *Eda* genotypes.

Although we did not observe the predicted effects of *Eda* genotype on any measured traits, we did observe effects of our experimental manipulations on stickleback survival and growth. We found that there was faster larval growth in saltwater compared to freshwater, but this was reversed at later timepoints with faster juvenile growth in freshwater (i.e. an interaction between salinity and development stage). This is surprising given that all of our fish are derived from marine sticklebacks, which normally hatch and develop in freshwater and then migrate as juveniles to saltwater. Our results are also in contrast to previous experiments have observed faster juvenile growth of marine sticklebacks in saltwater relative to freshwater (Barrett et al. 2009; Gibbons et al. 2017). We do not think this was due to the use of the pellet diets, as the growth rate of stickleback in our saltwater conditions (35 ppt) was higher than the growth rate of stickleback in the saltwater conditions used in previous studies (20 ppt in Gibbons et al. 2007 and 30 ppt in Barrett et al. 2009). However, the higher salinity in our experiment could explain the significantly lower survival in saltwater across all timepoints. Although this could be due to a higher than normal level of calcium or some other trace element in the saltwater tanks, we regularly raise marine sticklebacks in our saltwater facility on a diet of *Artemia* nauplii and *Mysis* shrimp with high survival and normal growth. Thus, it is possible that there was a nutritional deficit in the pellet diet that was manifest only under saltwater conditions; however, the diet was designed to be stable and nutritious in both saltwater and freshwater. Given that our main goal was to test the effect of the *Eda* freshwater haplotype under phosphorus limitation in freshwater, these results do not alter our main finding that *Eda* genotype

had no effect on survival or growth, or on phosphorus deposition, phosphorus excretion, or a proxy for phosphorus uptake (intestine length) in any of the environmental conditions tested.

Conclusions

Loss of armor plates in freshwater populations of threespine stickleback is a classic example of strong and repeatable adaptive evolution. Despite progress towards a complete genotype-phenotype-fitness map for this trait, the agent(s) of selection acting on plate loss have remained a mystery. Here we tested one plausible agent of selection, dietary phosphorus reduction, by raising marine sticklebacks that only vary in their genotype at the *Eda* 16kb haplotype and manipulating levels of phosphorus in their diets. We conclude that a biologically relevant level of 0.48% dietary phosphorus is not limiting to marine stickleback and that dietary phosphorus availability is unlikely to be the selective agent driving the increase in the *Eda* freshwater allele and bony plate loss in freshwater stickleback populations. These results emphasize the need to experimentally test plausible agents of natural selection, even when there is clear evidence that ecological factors drive changes in allele frequencies in the genes known to underlie specific phenotypes.

REFERENCES

Andersen, T. and D. O. Hessen. 1991. Carbon, nitrogen, and phosphorus content of freshwater zooplankton. Limnol. Oceanogr. 36:807-814.

Atkinson, M. J. and C. Bingman. 1997. Elemental composition of commercial seasalts. Journal of Aquariculture and Aquatic Sciences 8:39-43.

Archambeault, S. A., L. R. Bärtschi, A. D. Merminod, and C. L. Peichel. 2020. Adaptation via pleiotropy and linkage: association mapping reveals a complex genetic architecture within the stickleback *Eda* locus. Evol. Lett. In press. https://doi.org/10.1002/evl3.175

Avila, E. M., H. Tu, S. Basantes, and R. P. Ferraris. 2000. Dietary phosphorus regulates intestinal transport and plasma concentrations of phosphate in rainbow trout. J. Comp. Physiol. B 170:201-209.

Avise, J. C. 1976. Genetics of plate morphology in an unusual population of threespine sticklebacks (*Gasterosteus aculeatus*). Genet. Res. 27:33-46.

Baker, J. A., M. A. Wund, D. C. Heins, R. W. King, M. L. Reyes, and S. A. Foster. 2015. Life-history plasticity in female threespine stickleback. Heredity 115:322-334.

Barrett, R. D. H. and H. E. Hoekstra. 2011. Molecular spandrels: tests of adaptation at the genetic level. Nat. Rev. Genet. 12:767-780.

Barrett, R. D. H., S. M. Rogers, and D. Schluter. 2008. Natural selection on a major armor gene in threespine stickleback. Science 322:255-257.

Barrett, R. D. H., S. M. Rogers, and D. Schluter. 2009. Environment specific pleiotropy facilitates divergence at the *Ectodysplasin* locus in threespine stickleback. Evolution 63:2831-2837.

Bassham, S., J. Catchen, E. Lescak, F. A. von Hippel, and W. A. Cresko. 2018. Repeated selection of alternatively adapted haplotypes creates sweeping genomic remodeling in stickleback. Genetics 209:921-939.

Baumgartner, J. V. and M. A. Bell. 1984. Lateral plate morph variation in California populations of the threespine stickleback, *Gasterosteus aculeatus*. Evolution 38:665-674.

Bell, M. A. and W. E. Aguirre. 2013. Contemporary evolution, allelic recycling, and adaptive radiation of the threespine stickleback. Evol. Ecol. Res. 15:377-411.

Bell, M. A., W. E. Aguirre, and N. J. Buck. 2004. Twelve years of contemporary armor evolution in a threespine stickleback population. Evolution 58:814-824.

Bell, M. A. and S. A. Foster. 1994. The evolutionary biology of the threespine stickleback. Oxford University Press, New York, USA.

Bell, M. A., G. Orti, J. A. Walker, and J. P. Koenings. 1993. Evolution of pelvic reduction in threespine stickleback fish: a test of competing hypotheses. Evolution 47:906-914.

Bergstrom, C. A. 2002. Fast-start swimming performance and reduction in lateral plate number in threespine stickleback. Can. J. Zool. 80:207-213.

Boersma, M., N. Aberle, F. M. Hantzsche, K. L. Schoo, K. H. Wiltshire, and A. M. Malzahn. 2008. Nutritional limitation travels up the food chain. Internat. Rev. Hydrobiol. 93:479-488.

Boros, G., P. Sály, and M. J. Vanni. 2015. Ontogenetic variation in the body stoichiometry of two fish species. Oecologia 179:329-341.

Bourgeois, J. F., D. M. Blouw, J. P. Koenings, and M. A. Bell. 1994. Multivariate analysis of geographic covariance between phenotypes and environments in the threespine stickleback, *Gasterosteus aculeatus*, from the Cook Inlet area, Alaska. Can. J. Zool. 72:1497-1509.

Carlson, S. M., A. Kottas, and M. Mangel. 2010. Bayesian analysis of size-dependent overwinter mortality from size-frequency distributions. Ecology 91:1016-1024.

Colosimo, P. F., K. E. Hosemann, S. Balabhadra, G. Villarreal, Jr., M. Dickson, J. Grimwood, J. Schmutz, R. M. Myers, D. Schluter, and D. M. Kingsley. 2005. Widespread parallel evolution in sticklebacks by repeated fixation of *Ectodysplasin* alleles. Science 307:1928-1933.

Colosimo, P. F., C. L. Peichel, K. Nereng, B. K. Blackman, M. D. Shapiro, D. Schluter, and D. M. Kingsley. 2004. The genetic architecture of parallel armor plate reduction in threespine sticklebacks. PLoS Biol. 2:635-641.

Cresko, W. A., K. L. McGuigan, P. C. Phillips, and J. H. Postlethwait. 2007. Studies of threespine stickleback developmental evolution: progress and promise. Genetica 129:105-126.

Deschamps, M.-H., N. Poirier Stewart, A. Demanche, and G. W. Vandenberg. 2016. Preliminary study for description of bone tissue responsiveness to prolonged dietary phosphorus deficiency in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquac. Res. 47:900-911.

Deschamps, M. H., N. Poirier Stewart, A. Demanche, and G. W. Vandenberg. 2014. Preliminary study for phenotypic description of vertebral abnormalities in triploid trout subjected to prolonged deficiency in phosphorus. J. Appl. Ichthyol. 30:833-839.

Durston, D. J. and R. W. El-Sabaawi. 2017. Bony traits and genetics drive intraspecific variation in vertebrate elemental composition. Funct. Ecol. 31:2128-2137.

Durston, D. J. and R. W. El-Sabaawi. 2019. The utility of stoichiometric and metabolic theory for understanding the foraging habitat and excretion of threespine stickleback (*Gasterosteus aculeatus*). Evol. Ecol. Res. 20:193-211.

El-Sabaawi, R. W., M. L. Warbanski, S. M. Rudman, R. Hovel, and B. Matthews. 2016. Investment in boney defensive traits alters organismal stoichiometry and excretion in fish. Oecologia 181:1209-1220.

Elser, J. J., R. W. Sterner, E. Gorokhova, W. F. Fagan, T. A. Markow, J. B. Cotner, J. F. Harrison, S. E. Hobbie, G. M. Odell, and L. W. Weider. 2000. Biological stoichiometry from genes to ecosystems. Ecol. Lett. 3:540-550.

Fontagné, S., N. Silva, D. Bazin, A. Ramos, P. Aguirre, A. Surget, A. Abrantes, S. J. Kaushik, and D. M. Power. 2009. Effects of dietary phosphorus and calcium level on growth and skeletal development in rainbow trout (*Oncorhynchus mykiss*) fry. Aquaculture 297:141-150.

Foster, S. A., V. B. Garcia, and M. Y. Town. 1988. Cannibalism as the cause of an ontogenetic shift in habitat use by fry of the threespine stickleback. Oecologia 74:577-585.

Frost, P. C., J. P. Benstead, W. F. Cross, H. Hillebrand, J. H. Larson, M. A. Xenopoulos, and T. Yoshida. 2006. Threshold elemental ratios of carbon and phosphorus in aquatic consumers. Ecol. Lett. 9:774-779.

Gelmond, O., F. A. Von Hippel, and M. S. Christy. 2009. Rapid ecological speciation in three-spined stickleback *Gasterosteus aculeatus* from Middleton Island, Alaska: the roles of selection and geographic isolation. J. Fish Biol. 75:2037-2051.

German, D. P., B. C. Nagle, J. M. Villeda, A. M. Ruiz, A. W. Thomson, S. C. Balderas, and D. H. Evans. 2010. Evolution of herbivory in a carnivorous clade of minnows (Teleostei: Cyprinidae): effects on gut size and digestive physiology. Physiol. Biochem. Zool. 83:1-18.

Gibbons, T. C., S. M. Rudman, and P. M. Schulte. 2017. Low temperature and low salinity drive putatively adaptive growth differences in populations of threespine stickleback. Sci. Rep. 7:16766.

Giles, N. 1983. The possible role of environmental calcium levels during the evolution of phenotypic diversity in Outer Hebridean populations of the three-spined stickleback, *Gasterosteus aculeatus*. J. Zool. 199:535-544.

Grant, P. R. and B. R. Grant. 2002. Unpredictable evolution in a 30-year study of Darwin's finches. Science 296:707-711.

Greenwood, A. K., M. G. Mills, A. R. Wark, S. L. Archambeault, and C. L. Peichel. 2016. Evolution of schooling behavior in threespine sticklebacks is shaped by the *Eda* gene. Genetics 203:677-681.

Hall, S. R., M. A. Leibold, D. A. Lytle, and V. H. Smith. 2004. Stoichiometry and planktonic grazer composition over gradients of light, nutrients, and predation risk. Ecology 85:2291-2301.

Hendrixson, H. A., R. W. Sterner, and A. D. Kay. 2007. Elemental stoichiometry of freshwater fishes in relation to phylogeny, allometry and ecology. J. Fish Biol. 70:121-140.

Hessen, D. O., J. J. Elser, R. W. Sterner, and J. Urabe. 2013. Ecological stoichiometry: An elementary approach using basic principles. Limnol. Oceanogr. 58:2219-2236.

Heuts, M. J. 1947. Experimental studies on adaptive evolution in *Gasterosteus aculeatus* L. Evolution 1:89-102.

Hohenlohe, P. A., S. Bassham, P. D. Etter, N. Stiffler, E. A. Johnson, and W. A. Cresko. 2010. Population genomics of parallel adaptation in threespine stickleback using sequenced RAD tags. PLoS Genet. 6:e1000862.

Hood, J. M., M. J. Vanni, and A. S. Flecker. 2005. Nutrient recycling by two phosphorus-rich grazing catfish: the potential for phosphorus-limitation of fish growth. Oecologia 146:247-257.

Hossain, M. A. and T. Yoshimatsu. 2014. Dietary calcium requirement in fishes. Aquac. Nutr. 20:1-11.

Jakubavičiūtė, E., U. Bergström, J. S. Eklöf, Q. Haenel, and S. J. Bourlat. 2017a. DNA metabarcoding reveals diverse diet of the three-spined stickleback in a coastal ecosystem. PLoS ONE 12:e0186929.

Jakubavičiūtė, E., M. Casini, L. Ložys, and J. Olsson. 2017b. Seasonal dynamics in the diet of pelagic fish species in the southwest Baltic Proper. ICES J. Mar. Sci. 74:750-758.

Jeziorski, A., N. D. Yan, A. M. Paterson, A. M. DeSellas, M. A. Turner, D. S. Jeffries, B. Keller, R. C. Weeber, D. K. McNicol, M. E. Palmer, K. McIver, K. Arseneau, B. K. Ginn, B. F. Cumming, and J. P. Smol. 2008. The widespread threat of calcium decline in fresh waters. Science 322:1374-1377.

Jones, F. C., Y. F. Chan, J. Schmutz, J. Grimwood, S. D. Brady, A. M. Southwick, D. M. Absher, R. M. Myers, T. E. Reimchen, B. E. Deagle, D. Schluter, and D. M. Kingsley. 2012a. A genome-wide SNP genotyping array reveals patterns of global and repeated species-pair divergence in sticklebacks. Curr. Biol. 22:83-90.

Jones, F. C., M. G. Grabherr, Y. F. Chan, P. Russell, E. Mauceli, J. Johnson, R. Swofford, M. Pirun, M. C. Zody, S. White, E. Birney, S. Searle, J. Schmutz, J. Grimwood, M. C. Dickson, R. M. Myers, C. T. Miller, B. R. Summers, A. K. Knecht, S. D. Brady, H. Zhang, A. A. Pollen, T. Howes, C. Amemiya, E. S. Lander, F. Di Palma, K. Lindblad-Toh, and D. M. Kingsley. 2012b. The genomic basis of adaptive evolution in threespine sticklebacks. Nature 484:55-61.

Kingsley, D. M. and C. L. Peichel. 2007. The molecular genetics of evolutionary change in sticklebacks. Pp. 41-81 *in* S. Ostlund-Nilsson, I. Mayer, and F. A. Huntingford, eds. Biology of the Three-Spined Stickleback. CRC Press, Boca Raton, FL, USA.

Kitano, J., D. I. Bolnick, D. A. Beauchamp, M. M. Mazur, S. Mori, T. Nakano, and C. L. Peichel. 2008. Reverse evolution of armor plates in the threespine stickleback. Curr. Biol. 18:769-774.

Krumgalz, B. S. 1982. Calcium distribution in the world ocean waters. Oceanol. Acta 5:121-128.

Lall, S. P. 2002. The minerals. Pp. 259-308 *in* J. E. Halver, and R. W. Hardy, eds. Fish Nutrition. Academic Press Inc., San Diego, USA.

Lankov, A., H. Ojaveer, M. Simm, M. Põllupüü, and C. Möllmann. 2010. Feeding ecology of pelagic fish species in the Gulf of Riga (Baltic Sea): the importance of changes in the zooplankton community. J. Fish Biol. 77:2268-2284.

Lässig, M., V. Mustonen, and A. M. Walczak. 2017. Predicting evolution. Nat. Ecol. Evol. 1:0077.

Le Rouzic, A., K. Østbye, T. O. Klepaker, T. F. Hansen, L. Bernatchez, D. Schluter, and L. A. Vøllestad. 2011. Strong and consistent natural selection associated with armour reduction in sticklebacks. Mol. Ecol. 20:2483-2493.

Leal, M. C., R. J. Best, D. Durston, R. W. El-Sabaawi, and B. Matthews. 2017. Stoichiometric traits of stickleback: effects of genetic background, rearing environment, and ontogeny. Ecol. Evol. 7:2617-2625.

Leinonen, T., G. Herczeg, J. M. Cano, and J. Merilä. 2011. Predation-imposed selection on threespine stickleback (*Gasterosteus aculeatus*) morphology: a test of the refuge use hypothesis. Evolution 65:2916-2926.

MacColl, A. D. C. 2011. The ecological causes of evolution. Trends Ecol. Evol. 26:514-522.

MacColl, A. D. C. and B. Aucott. 2014. Inappropriate analysis does not reveal the ecological causes of evolution of stickleback armour: a critique of Spence et al. 2013. Ecol. Evol. 4:3509-3513.

Marchinko, K. B. 2009. Predation's role in repeated phenotypic and genetic divergence of armor in threespine stickleback. Evolution 63:127-138.

Marchinko, K. B. and D. Schluter. 2007. Parallel evolution by correlated response: lateral plate reduction in threespine stickleback. Evolution 61:1084-1090.

McIntyre, P. B. and A. S. Flecker. 2010. Ecological stoichiometry as an integrative framework in stream fish ecology. Am. Fish. Soc. Symp. 73:539-558.

Meeker, N. D., S. A. Hutchinson, L. Ho, and N. S. Trede. 2007. Method for isolation of PCR-ready genomic DNA from zebrafish tissues. BioTechniques 43:610-614.

Mills, M. G., A. K. Greenwood, and C. L. Peichel. 2014. Pleiotropic effects of a single gene on skeletal development and sensory system patterning in sticklebacks. EvoDevo 5:5.

Morales, G. A., R. L. Azcuy, M. E. Casaretto, L. Márquez, A. J. Hernández, F. Gómez, W. Koppe, and A. Mereu. 2018. Effect of different inorganic phosphorus sources on growth performance,

digestibility, retention efficiency and discharge of nutrients in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 495:568-574.

Myhre, F. and T. Klepaker. 2009. Body armour and lateral-plate reduction in freshwater three-spined stickleback *Gasterosteus aculeatus*: adaptations to a different buoyancy regime? J. Fish Biol. 75:2062-2074.

Nosil, P., R. Villoutreix, C. F. de Carvalho, T. E. Farkas, V. Soria-Carrasco, J. L. Feder, B. J. Crespi, and Z. Gompert. 2018. Natural selection and the predictability of evolution in *Timema* stick insects. Science 359:765-770.

Olsson, J., M. Quevedo, C. Colson, and R. Svanbäck. 2007. Gut length plasticity in perch: into the bowels of resource polymorphisms. Biol. J. Linn. Soc. 90:517-523.

Ozgul, A., S. Tuljapurkar, T. G. Benton, J. M. Pemberton, T. H. Clutton-Brock, and T. Coulson. 2009. The dynamics of phenotypic change and the shrinking sheep of St. Kilda. Science 325:464-467.

Peichel, C. L. and D. A. Marques. 2017. The genetic and molecular architecture of phenotypic diversity in sticklebacks. Phil. Trans. R. Soc. Lond. B 372:20150486.

Peichel, C. L., J. A. Ross, C. K. Matson, M. Dickson, J. Grimwood, J. Schmutz, R. M. Myers, S. Mori, D. Schluter, and D. M. Kingsley. 2004. The master sex-determination locus in threespine sticklebacks is on a nascent Y chromosome. Curr. Biol. 14:1416-1424.

Reimchen, T. E. 1980. Spine deficiency and polymorphism in a population of *Gasterosteus aculeatus*: an adaptation to predators? Can. J. Zool. 58:1232-1244.

Reimchen, T. E. 1992. Injuries on stickleback from attacks by a toothed predator (*Oncorhynchus*) and implications for the evolution of lateral plates. Evolution 46:1224-1230.

Reimchen, T. E. 2000. Predator handling failures of lateral plate morphs in *Gasterosteus aculeatus*: functional implications for the ancestral plate condition. Behaviour 137:1081-1096.

Reimchen, T. E., C. Bergstrom, and P. Nosil. 2013. Natural selection and the adaptive radiation of Haida Gwaii stickleback. Evol. Ecol. Res. 15:241-269.

Rennison, D. J., K. Heilbron, R. D. H. Barrett, and D. Schluter. 2015. Discriminating selection on lateral plate phenotype and its underlying gene, *Ectodysplasin*, in threespine stickleback. Am. Nat. 185:150-156.

Roy, P. K., P. E. Witten, B. K. Hall, and S. P. Lall. 2002. Effects of dietary phosphorus on bone growth and mineralisation of vertebrae in haddock (*Melanogrammus aeglefinus* L.). Fish Physiol. Biochem. 27:35-48.

Rudman, S. M., J. M. Goos, J. B. Burant, K. V. Brix, T. C. Gibbons, C. J. Brauner, and P. D. Jeyasingh. 2019. Ionome and elemental transport kinetics shaped by parallel evolution in threespine stickleback. Ecol. Lett. 22:645-653.

Schindler, D. E. and L. A. Eby. 1997. Stoichiometry of fishes and their prey: implications for nutrient recycling. Ecology 78:1816-1831.

Schluter, D. 1993. Adaptive radiation in sticklebacks: size, shape, and habitat use efficiency. Ecology 74:699-709.

Schluter, D. and J. D. McPhail. 1992. Ecological character displacement and speciation in sticklebacks. Am. Nat. 140:85-108.

Smith, C., R. Spence, I. Barber, M. Przybylski, and R. J. Wootton. 2014. The role of calcium and predation on plate morph evolution in the three-spined stickleback (*Gasterosteus aculeatus*). Ecol. Evol. 4:3550-3554.

Smith, C., G. Zięba, R. Spence, T. Klepaker, and M. Przybylski. 2020. Three-spined stickleback armour predicted by body size, minimum winter temperature and pH. J. Zool. 311:13-22.

Spence, R., R. J. Wootton, I. Barber, M. Przybylski, and C. Smith. 2013. Ecological causes of morphological evolution in the three-spined stickleback. Ecol. Evol. 3:1717-1726.

Spence, R., R. J. Wootton, M. Przybylski, G. Zięba, K. Macdonald, and C. Smith. 2012. Calcium and salinity as selective factors in plate morph evolution of the three-spined stickleback (*Gasterosteus aculeatus*). J. Evol. Biol. 25:1965-1974.

Sterner, R. W. and J. J. Elser. 2002. Ecological stoichiometry: the biology of elements from molecules to the biosphere. Princeton University Press, Princeton, NJ.

Sullivan, M., S. W. J. Reid, H. Ternent, N. J. Manchester, R. J. Roberts, D. A. J. Stone, and R. W. Hardy. 2007. The aetiology of spinal deformity in Atlantic salmon, *Salmo salar* L.: influence of different commercial diets on the incidence and severity of the preclinical condition in salmon parr under two contrasting husbandry regimes. J. Fish Dis. 30:759-767.

Vielma, J. and S. P. Lall. 1998. Control of phosphorus homeostasis of Atlantic salmon (*Salmo salar*) in fresh water. Fish Physiol. Biochem. 19:83-93.

Vrede, T., S. Drakare, P. Eklöv, A. Hein, A. Liess, J. Olsson, J. Persson, M. Quevedo, H. R. Stabo, and R. Svanbäck. 2011. Ecological stoichiometry of Eurasian perch – intraspecific variation due to size, habitat and diet. Oikos 120:886-896.

Wade, M. J. and S. Kalisz. 1990. The causes of natural selection. Evolution 44:1947-1955.

Wagner, C. E., P. B. McIntyre, K. S. Buels, D. M. Gilbert, and E. Michel. 2009. Diet predicts intestine length in Lake Tanganyika's cichlid fishes. Funct. Ecol. 23:1122-1131.

Weyhenmeyer, G. A., J. Hartmann, D. O. Hessen, J. Kopáček, J. Hejzlar, S. Jacquet, S. K. Hamilton, P. Verburg, T. H. Leach, M. Schmid, G. Flaim, T. Nõges, P. Nõges, V. C. Wentzky, M. Rogora, J. A. Rusak, S. Kosten, A. M. Paterson, K. Teubner, S. N. Higgins, G. Lawrence, K. Kangur, I. Kokorite, L. Cerasino, C. Funk, R. Harvey, F. Moatar, H. A. de Wit, and T. Zechmeister. 2019. Widespread diminishing anthropogenic effects on calcium in freshwaters. Sci. Rep. 9:10450.

Witten, P. E., M. A. G. Owen, R. Fontanillas, M. Soenens, C. McGurk, and A. Obach. 2016. A primary phosphorus-deficient skeletal phenotype in juvenile Atlantic salmon *Salmo salar*: the uncoupling of bone formation and mineralization. J. Fish Biol. 88:690-708.

Wootton, R. J. 1976. The biology of the sticklebacks. Academic Press, London, UK.

Wucherpfennig, J. I., C. T. Miller, and D. M. Kingsley. 2019. Efficient CRISPR-Cas 9 editing of major evolutionary loci in sticklebacks. Evol. Ecol. Res. 20:107-132.

Zeller, M., K. Lucek, M. P. Haesler, O. Seehausen, and A. Sivasundar. 2012. Little evidence for a selective advantage of armour-reduced threespined stickleback individuals in an invertebrate predation experiment. Evol. Ecol. 26:1293-1309.

Figure Legends

Figure 1. Crosses and experimental design. (A) Crosses were established between laboratory reared F1 fish heterozygous at *Eda* for the marine (C) and freshwater (L) allele, which produced the expected 1:2:1 ratios of genotypes at *Eda*. (B) The numbers of lateral bony plates were counted on F2 offspring older than 140 days and > 25 mm standard length (SL). Boxplots show the mean (middle line), upper and lower quartiles (box boundaries). The whiskers extend the range of the data up to 1.5 times the interquartile range. The single CL fish with 3 plates on the left side had 31 plates on the right side. (C) For each timepoint, eggs were transferred into experimental tanks at 2 days post fertilization (dpf). Fish were collected, measured and genotyped for Timepoint 1 between 37-39 dpf (prior to bony plate formation) and Timepoint 2 between 129-133 dpf (during and after bony plate formation). For Timepoint 3, fish were collected for excretion and bone samples, measured and genotyped between 155-175 dpf (during and after bony plate formation).

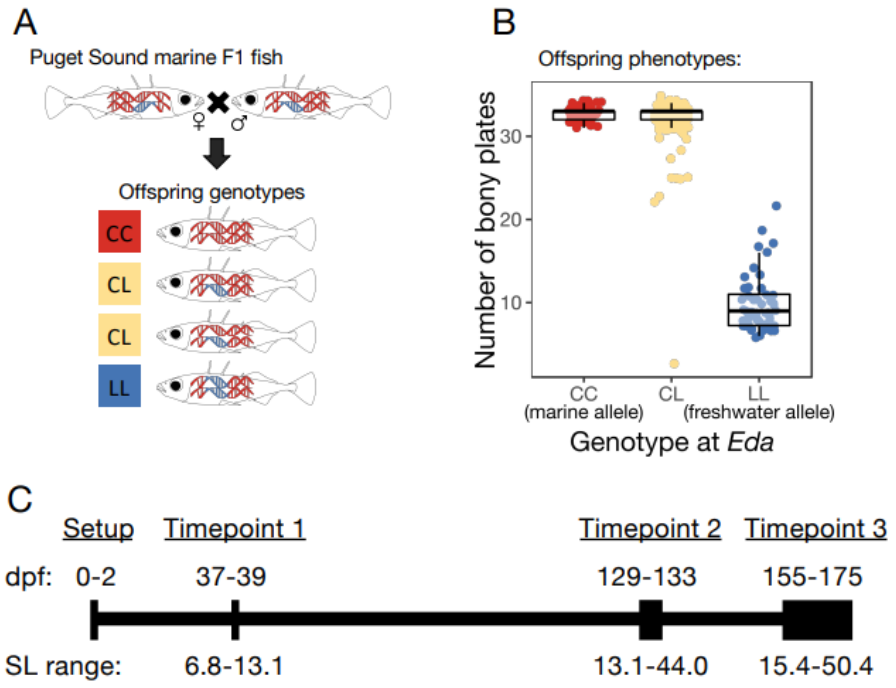

Figure 2. Survival varied between freshwater and saltwater tanks, but not between high and low phosphorus diets. Survival is plotted for each family and condition. Boxplots show the mean (middle line), upper and lower quartiles (box boundaries). The whiskers extend the range of the data up to 1.5 times the interquartile range. Survival was significantly lower in saltwater (SW) than in freshwater (FW) at all timepoints. Survival was highest at Timepoint 1 at 37 days post fertilization (dpf)), lower at 129-133 dpf in Timepoint 2, and lowest Timepoint 3, especially in SW conditions. There was no effect of diet or water x diet interaction on survival at any of the timepoints.

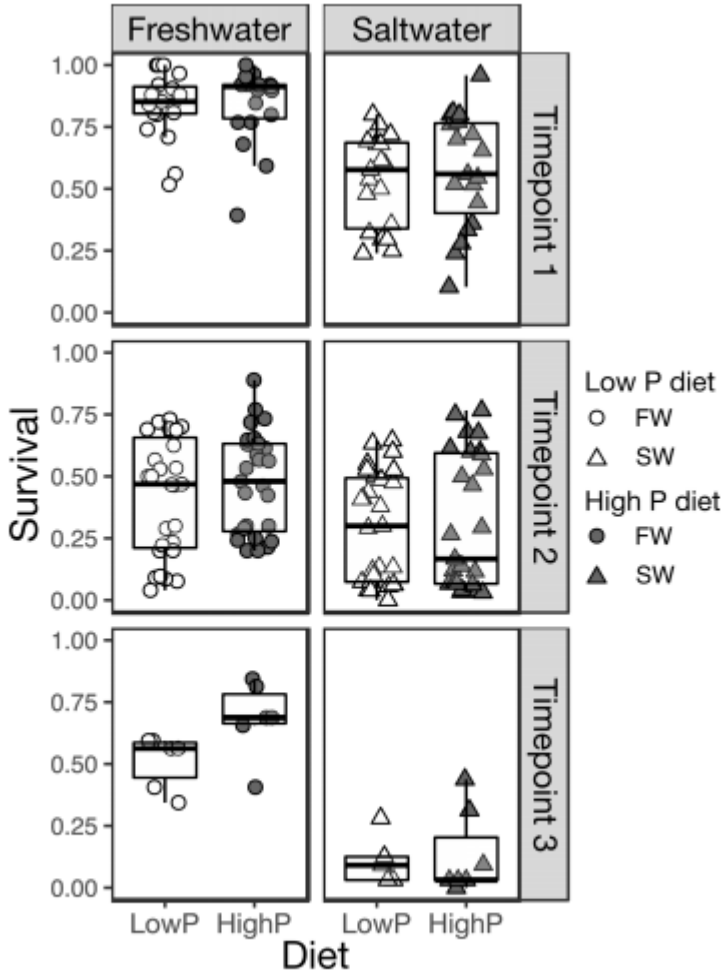

Figure 3. Growth differs most strongly between stickleback raised in saltwater and freshwater. Estimated marginal means and 95% confidence intervals for standard length and size-corrected weight are plotted by sex, water, and genotype at Timepoints 1, 2, and 3 in LowP diet conditions (diet had no effect on these traits, so only LowP samples were plotted). Size-corrected weight, or body condition, was assessed by including standard length in the mixed-effects linear model. These results visualize the ANOVA results in Table 1 and summarize the results plotted by family in Supplemental Figures 6-8.

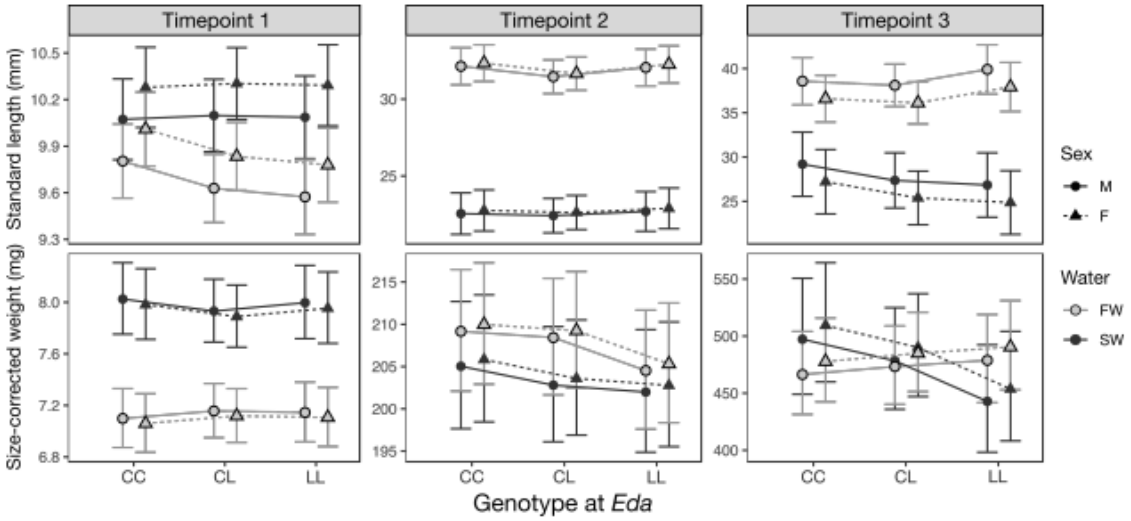

Figure 4. Stickleback on a high phosphorus diet have a higher phosphorus excretion rate. Estimated marginal means and 95 % confidence intervals for bone phosphorus content, phosphorus excretion rate (in µg per minute), and size-corrected intestine length are plotted by sex, water, and genotype for fish collected at Timepoint 3. Size-corrected intestine length was assessed by including standard length in the mixed-effects linear model. These results visualize the ANOVA results in Table 2 and summarize the results plotted by family in Supplemental Figures 9-10.

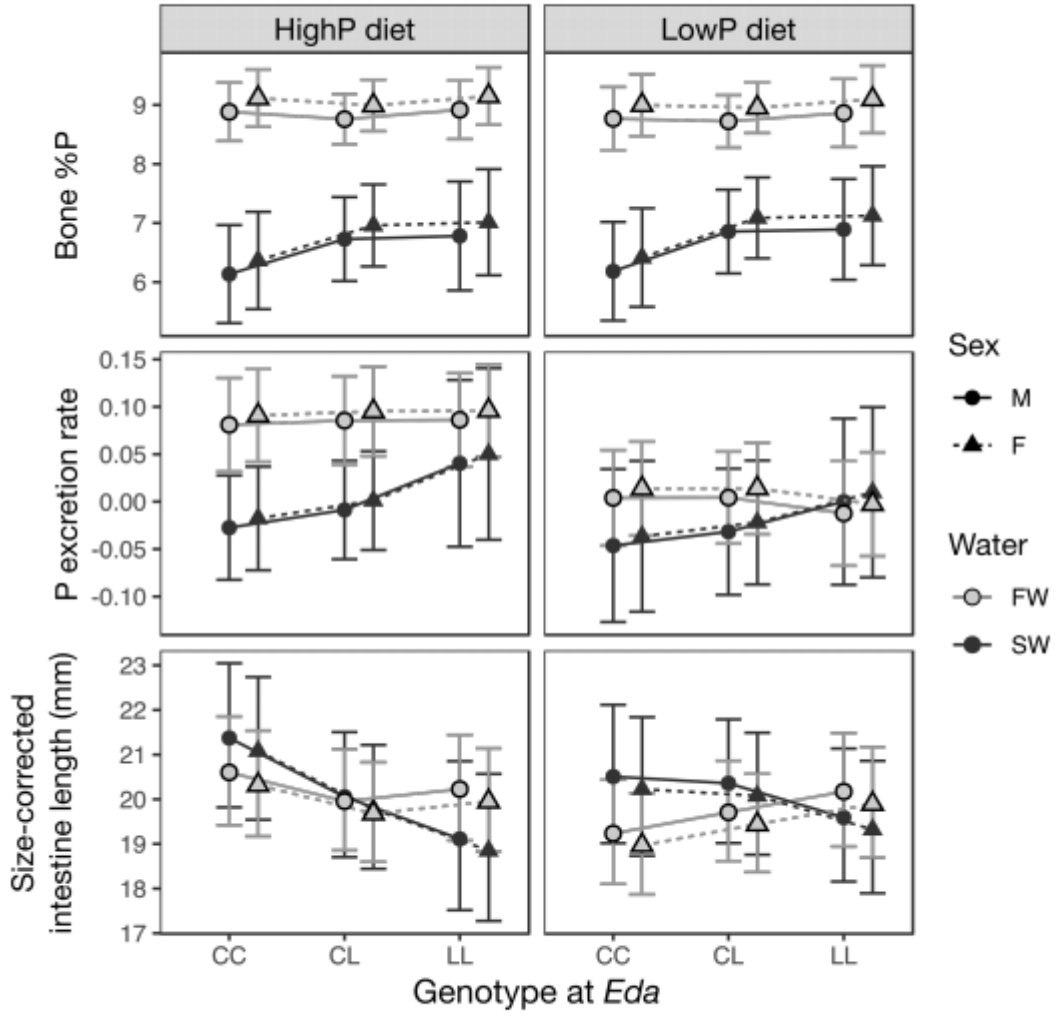

Table 1. Genotype has no effect on growth prior to or during plate development. Results from the type III ANOVA using Satterthwaite's method of the mixed effects linear models on standard length and body condition are shown for Timepoints 1, 2, and 3. Standard length was used as a measure of growth, while body condition was assessed as natural log of weight as the response variable with natural log of standard length as a predictor variable. For a visual representation of these data, see Figure 3 and Supplemental Figures 6-8. N.d.f. = numerator degrees of freedom; D.d.f = denominator degrees of freedom; *P < 0.05; ***P < 0.001

		Timepoint 1		n = 1342	Timepoint 2		n = 1209	Timepoint 3		n = 277
Response	Predictor	N.d.f., D.d.f.	F-value	p-value	N.d.f., D.d.f.	F-value	p-value	N.d.f., D.d.f.	F-value	p-value
Standard length	Water	1, 56	22.49	1.5E-05***	1, 66	577.70	<2e-16***	1, 17	135.51	2.19E-09***
	Diet	1, 55	0.10	0.75	1, 52	0.23	0.63	1, 15	1.83	0.20
	Sex	1, 1288	20.06	8.2E-06***	1, 1152	0.63	0.43	1, 259	14.00	0.00***
	Genotype	2, 1291	0.24	0.78	2, 1150	0.57	0.57	2, 259	3.02	0.05
	Age	-	-	-	-	-	-	1, 4	6.31	0.06
	Water x Genotype	2, 1291	2.06	0.13	2, 1149	0.39	0.67	2, 259	1.61	0.20
	Water x Diet	1, 52	0.01	0.92	1, 47	0.03	0.85	1, 13	0.19	0.67
	Diet x Genotype	2, 1290	2.55	0.08	2, 1151	1.10	0.33	2, 258	1.88	0.15
Body condition	ln(Standard length)	1, 1160	10414.50	<2e-16***	1, 1142	50637.90	<2e-16***	1, 258	5291.11	<2e-16***
	Water	1, 52	194.40	<2e-16***	1, 62	1.00	0.32	1, 18	0.62	0.44
	Diet	1, 45	1.84	0.18	1, 44	0.79	0.38	1, 10	0.45	0.52
	Sex	1, 1263	0.68	0.41	1, 1109	0.59	0.44	1, 251	3.63	0.06
	Genotype	2, 1262	0.77	0.46	2, 1106	2.20	0.11	2, 259	4.14	0.02*
	Age	-	-	-	-	-	-	1, 4	0.62	0.47
	Water x Genotype	2, 1263	0.78	0.46	2, 1107	0.75	0.47	2, 260	4.16	0.02*

Water x Diet	1, 39	0.00	0.95	1, 43	0.22	0.64	1, 9	0.76	0.41
Diet x Genotype	2, 1261	0.98	0.38	2, 1104	1.13	0.32	2, 250	1.25	0.29

Table 2. Phosphorus content of bone, phosphorus excretion rates, and intestine lengths do not vary between genotypes. Results from type III ANOVA using Satterthwaite's method of the mixed effects linear models for bone phosphorus content, phosphorus excretion rate, and intestine length at Timepoint 3. The natural log of intestine length was calculated prior to use in this analysis as a response variable. For a visual representation of these data, see Figure 4 and Supplemental Figures 9-10. N.d.f. = numerator degrees of freedom; D.d.f = denominator degrees of freedom; *P < 0.05; **P < 0.01; ***P < 0.001

Response	Predictor	N.d.f., D.d.f.	F-value	p-value
Bone %P	Water	1, 17	82.60	5.878E-08***
	Diet	1, 15	0.00	0.96
	Sex	1, 257	3.33	0.07
	Genotype	2, 262	1.36	0.26
	Age	1, 4	10.00	0.03*
	Water x Genotype	2, 263	1.62	0.20
	Water x Diet	1, 14	0.12	0.73
	Diet x Genotype	2, 258	0.03	0.97
P excretion rate	Water	1, 189	8.58	0.00**
	Diet	1, 74	5.71	0.02*
	Weight	1, 188	0.06	0.81
	Sex	1, 179	1.68	0.20
	Genotype	2, 187	0.63	0.54
	Water x Genotype	2, 186	0.97	0.38
	Water x Diet	1, 185	3.57	0.06
	Diet x Genotype	2, 183	0.41	0.66

Intestine length	ln(Standard length)	1, 260	916.15	<2e-16***
	Water	1, 70	0.17	0.68
	Diet	1, 28	0.88	0.36
	Sex	1, 255	1.65	0.20
	Genotype	2, 257	1.31	0.27
	Age	1, 4	3.02	0.16
	Water x Genotype	2, 256	2.64	0.07
	Water x Diet	1, 24	0.90	0.35
	Diet x Genotype	2, 258	2.84	0.06