Salinity transitions, population differentiation and phenotypic plasticity in the threespine stickleback

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My own scientific life was a decent from higher to lower dimensions,
led by the desire to understand life. I went from animals to cells,
from cells to bacteria, from bacteria to molecules, from molecules to electrons.
The story had its irony, for molecules and electrons have no life at all.
On my way, life ran out between my fingers.

- *Albert Szent-Györgyi (1893-1986)*

"Fish are also individuals"

- *L. Asbjørn Vøllestad*

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Similarly to the time that has accumulated since I started this Phd, an equally increased number of people should be thanked- not only has my inner circle more than doubled, but moving work-place and settling down in a new town has also brought new colleagues and friendships. I want to start with a collective Thank You – I am very lucky to have support from such a variety of friends and family.

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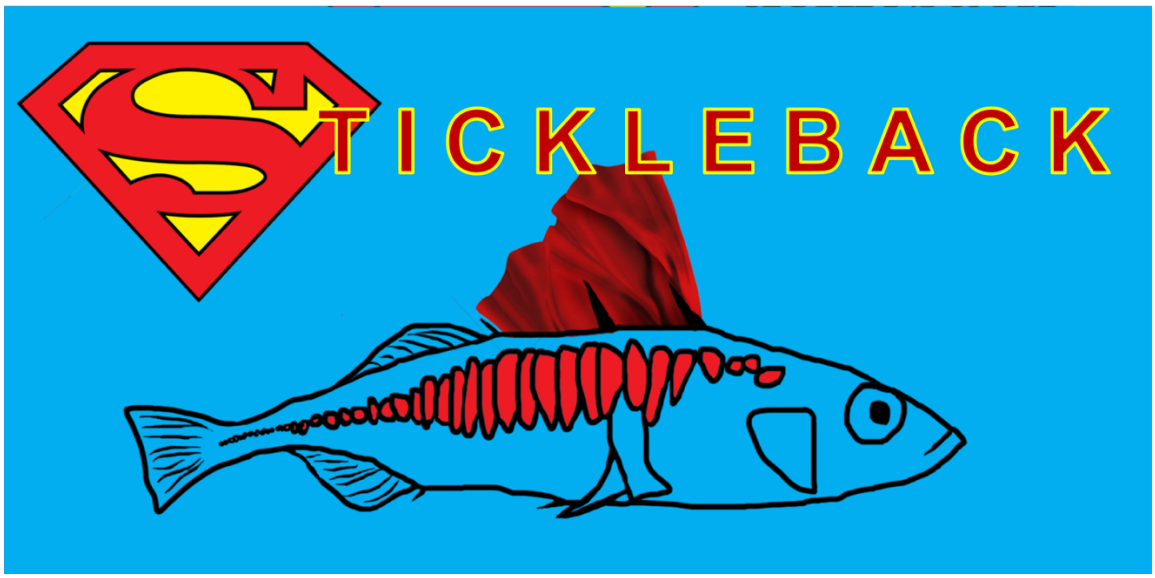
Torbjørn, Vemund and Håvard
- I love you to the moon and back! (and, *yes*, I do now know how far that is…)



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Paper I. A. Taugbøl, C. Junge, T.P. Quinn, A. Herland & L.A. Vøllestad. 2014. Genetic and morphometric divergence in threespine stickleback in the Chignik catchment, Alaska. Ecology and Evolution, 4 (2): 144-156

Paper II. A. Taugbøl, T.P. Quinn, K. Østbye & L.A. Vøllestad. 2020. Allometric relationships in morphological traits associated with foraging, swimming ability, and predator defense reveal adaptations toward brackish and freshwater environments in the threespine stickleback. Ecology and Evolution, 10: 13412-13426.

Paper III. K. Grøtan, K. Østbye, A. Taugbøl & L.A Vøllestad. 2012. No short-term effect of salinity on oxygen consumption in threespine stickleback (*Gasterosteus aculeatus*) from fresh, brackish, and salt water. Canadian Journal of Zoology, 90 (12): 1386-1393

Paper IV. A.Taugbøl, T. Arntsen, K. Østbye & L.A.Vøllestad. 2014. Small changes in gene expression of targeted osmoregulatory genes when exposing marine and freshwater threespine stickleback (*Gasterosteus aculeatus*) to abrupt salinity transfers. PLOS ONE, 9 (9): 144-156

Paper V. A. Taugbøl, M. Solbakken, K.S. Jacobsen & L.A. Vøllestad. 2022. Salinity-induced transcriptome profiles in marine and freshwater threespine stickleback. Ecology and Evolution, 12 (10)

Paper VI. A. Taugbøl, A.B. Mazzarella, E.R.A Cramer & T. Laskemoen. 2017. Salinity-induced phenotypic plasticity in threespine stickleback sperm activation. Biology Letters, 13 (10): 1-4

The following papers were published during the course of my PhD and are related to, but not part of my dissertation:

K. Østbye, A. Taugbøl, M. Ravinet, C. Harrod, R.A. Pettersen, L. Bernatchez. & L.A. Vøllestad. 2018. Ongoing niche differentiation under high gene flow in a polymorphic brackish water threespine stickleback (*Gasterosteus aculeatus*) population. BMC Evolutionary Biology, 18 (14): 1-18

A.B. Mazzarella, K. Voie, T. Hansson, A. Taugbøl & B. Fisher. 2015. Strong and parallel salinity-induced phenotypic plasticity in one generation of threespine stickleback. Journal of Evolutionary Biology. 28 (3): 667-677

A.C. Knag & A. Taugbøl. 2013. Offshore produced water has an effect on stress-responses in threespine stickleback (*Gasterosteus aculeatus*). Comparative Biochemistry and Physiology, Part C. 158 (3): 173-180

Summary

The transition from saltwater to freshwater has happened many times during the evolutionary history of time, a shift that has initiated adaptive radiation and speciation of many fish taxa. Whether in freshwater or seawater, teleost fish maintain the same salt concentration in their cells, where the surrounding salinity difference represents considerable diverse selective forces; water is leaking out from a saltwater fish, whereas a freshwater fish can "blow up" from all the water the cells receive in this environmental salinity. Hence, because saltwater and freshwater differ so strongly in solute concentration, the transition into freshwater must therefore involve adaption and evolution of ion and water balance regulations. Yet, we have limited understanding of the physiological modifications that facilitate this transition and how the fish revert back to steady states after experiencing new environmental cues.

This dissertation is (mostly) about how freshwater sticklebacks have adapted from their marine ancestral state, with the aim to gain insights into genetics and morphometrics of local adaptation in the two salinities. The threespine stickleback is a small fish (< 10 cm) that has intrigued researchers from many biological fields for decades due to its repeated adaptive morphological radiation in freshwater, functioning as a biological key with the potential to unlock many evolutionary questions. Following freshwater habitation, the stickleback typically evolves fewer lateral plates and more varied body shapes. In Paper I and Paper II, I was able to show that stickleback sampled from an open system in Alaska grouped by salinity, evident from both their genetic and morphometric differences. Paper III is a study on oxygen consumption in three Norwegian stickleback populations collected from saltwater, brackish-water and freshwater, being exposed to 0, 15 or 30 ‰ water in the lab. No consumption differences were observed for any of the experimental groups, which indicates that the stickleback has a very low osmoregulatory cost when it moves between salinities. I therefore moved into the regulation of genes expressed in the gills of fish that had been exposed to contrasting salinities by a targeting gene approach in Paper IV, and by a transcriptomic sequencing approach in Paper V. The experimental setup included groups of saltwater and freshwater fish being exposed to both their native (controls) and non-native salinities (experimental groups) for periods ranging from five minutes to three weeks. The targeted genes in Paper IV were selected based on results from published studies, with the aim to follow the gene expression patterns through the early adaptive- and the subsequent regulatory period of adaptation to the novel salinity, by exposing stickleback to native and contrasting salinities for nine time-periods. By sequencing the gill transcriptome from the fish in the six-hour group in Paper V, we revealed that freshwater fish being moved from freshwater to saltwater had a much higher difference in gene regulation than saltwater fish moved to freshwater. Paper VI explored mobility and phenotypic plasticity in the activation of sperm cells of saltwater and freshwater male sticklebacks. By activating one of the testes in saltwater and the other in freshwater, we found that the sperm cells of freshwater stickleback did not activate in saltwater, but sperm cells of freshwater stickleback that had been exposed to saltwater for only two days was activated and swam as good in saltwater as in freshwater.

Overall, my dissertation demonstrates that the stickleback is an extremely plastic fish, having the ability to osmoregulate and reproduce in a wide variety of salinities, seemingly without any large costs. The question of how this is possible, both within such short time interval and also following direct exposure to a highly different environments is still a mystery, ***but what would we scientists be, without a puzzle to explore?***

Sammendrag (in Norwegian)

Tilpasning til et liv i ferskvann fra et i saltvann har skjedd flere ganger i evolusjonens historie. Saltvannsfisk og ferskvannsfisk har den samme konsentrasjonen av salter i cellene, og for å opprettholde et stabilt indre miljø mot ulike saliniteter i miljøet rundt, må fiskene regulere salt- og ioneinnholdet. En saltvannsfisk har lavere saltkonsentrasjon mot det saltere havet, og vil derfor lekke vann til omgivelsene, noe som vil føre til at den tørker ut om den ikke drikker mye vann og aktivt skiller ut de ekstra saltene. For en ferskvannsfisk er det motsatt, her går vann passivt inn i fisken som er saltere enn omgivelsene sine, slik at det ekstra vannet må skilles ut via vannholdig urin og salter må aktivt tas opp for å unngå at fisken sveller opp. Det er fortsatt kunnskapsmangel rundt hvilke genetiske og fysiologiske prosesser som endres når ulike fiskearter endrer salinitet.

Denne oppgaven handler (for det meste) om hvordan den opprinnelige marine stingsilden har tilpasset seg ulike ferskvannshabitater. Trepigget stingsild er en liten fisk (< 10 cm) som har fasinert forskere fra mange ulike biologiske disipliner i lang tid. En av grunnene til dette er at marine stingsild uavhengig har invadert tusenvis av ferskvann etter istiden, med det resultatet at de fleste av de nye ferskvannspopulasjonene har evolvert frem den samme fenotypen; et oppsett som forskere bruker som en nøkkel til å studere en rekke evolusjonære spørsmål. Marine stingsild har lite variasjon i kroppsform og laterale beinplater langs hele siden av kroppen, mens en stingsild i ferskvann har høyere variasjon i morfologiske trekk, og kun plater i den fremre delen av kroppen. I Artikkel I og Artikkel II viser jeg både med genetiske og morfologiske metoder at stingsild samlet inn fra et åpent system i Alaska kunne grupperes i forhold til salinitet og ikke morfologi. Artikkel III er en studie på oksygenforbruket til stingsild samlet inn fra ferskvann, brakkvann og saltvann, når de ble eksponert for 0, 15 og 30‰ saltholdighet i laboratoriet. Resultatene viste ingen forskjeller i oksygenforbruk mellom populasjoner eller morfer, en sterk indikasjon på at stingsild har veldig lave osmoregulatoriske kostnader ved salinitetsendringer i habitatet. Stingsild må allikevel osmoregulere ulik i saltvann og ferskvann, og Artikkel IV ser på potensielle endringer i genregulering for utvalgte gener når stingsild fra saltvann og ferskvann ble utsatt for direkte salinitetsbytte for ni tidsperioder, fra fem minutter opp til tre uker. Resultatene viste svært få regulerings-endringer mellom kontrollene og de eksperimentelle gruppene for de fire utvalgte genene. I Artikkel V ble hele transkriptomet til fiskene som hadde vært eksponert i seks timer sekvensert. Ved å sammenligne antallet produserte gener i saltvannsfisk som hadde vært eksponert i saltvann (kontroll) og ferskvann, samt ferskvannsfisk som hadde vært eksponert i ferskvann (kontroll) og saltvann, var det overaskende få forskjeller for saltvannsfisken, da kun 10 gener var signifikant opp- eller nedregulert, mens ~1500 gener hadde signifikant ulik genregulering mellom ferskvann og saltvann for ferskvannsfisken. Siden platemorfene i høy grad henger sammen med salinitet testet vi muligheten for redusert befruktning via svømmekapasiteten til sperm for stingsild samlet inn fra saltvann og ferskvann i Artikkel VI. Ved å aktivere sperm fra en av testiklene i saltvann og en i ferskvann for hver hann, viste de første resultatene at spermien til ferskvannsstingsilden ikke kunne svømme i saltvann, mens spermien til saltvanns-stingsilden kunne svømme i begge vannkvaliteter. Ved å teste ferskvanns-hanner som hadde blitt drettet opp i saltvann på labben fant vi at spermien her også svømte i begge vannkvalitetene, og en ytterligere test på vill-fangede hanner som var eksponert i saltvann for to dager hadde også svømmedyktig sperm i saltvann.

Avhandlingen konkluderer med at stingsild er en meget tilpasningsdyktig fisk som kan osmoregulere direkte i nye saliniteter tilsynelatende uten store kostnader.

Introduction

Background

Every living species and individual present today is a result of a long, unbroken line of sucessful breeding and survival, from the beginning of life. However, in the *tree of life*, most of the branches are actually not present any more, as about 99% of the species that have been existing at one time or another in evolutionary history have perished (Erwin, 2008; Valentine, 1970). What makes some species sucsessful through time, with an unbroken evolutionary history through speciation events, while others fail? Catastophic events and other coincidences to the side, one of the key ingredients for a sucsessfull line of ancestors is genetic variation. Even if all individuals within a species are 99,9% similar genetically, it is still enough variation in the genome for natural selection, and hence "survival of the fittest," to be an active force in the population (Jorde and Wooding, 2004). Natural selection can be summarized in three stages that works together; *i*) every individual is unique, *ii*) some of the qualities that makes individuals unique are transferred from the parental generation to their offspring, and *iii*) more individuals are born than can be sustained in the environment (Darwin, 1859). Natural selection is hence a process in which better adapted organisms have greater success and leave more offspring for future generations, that again are more fit to the current environment. Natural selection is therefore a principle of local adaptation to the environment, leading to evolutionary change and, over time, also makes speciation events possible through a range of mechanisms (Schluter, 2009; Via, 2009). Although the theory was put forward by Charles Darwin already in 1859 (Darwin, 1859), the model did not hold a centerpiece in evolutionary history until the modern synthesis in the 1930s (Gould, 2002), after Mendelian inheritance was combined with gradual evolution through population genetics (Futuyma and Kirkpatrick, 2017; Lewontin, 1974).

The theory of natural selection seems simple enough, but how can one individual be more fit to the environemnt than another individual, when they have almost all gene variants in common? Further, one individual only holds a small proportion of the population's gene pool during its life time, and, depending on the size of the population, the contributed genetic variation to future generations from one individual is usually small (Chen et al, 2019). *The solution is in the plenty*. It is in the population as a whole that alleles of genes interact in combinations, and where the continued interaction of genes in a gene pool provides a degree of integration that permits the population to act as a major unit of evolution. As such, understanding how much genetic variation that underlies ecologically important traits; how this trait variation is distributed across the genome; how variations of genetic diversity affects different populations and how genetic diversity is expressed across environmental gradients are all still central questions in evolutionary biology and genetics. In particular, as organisms are so fine tuned towards their environment, and as genomic variation is sparse, how are organisms able to adapt to new, and also changing environments, within a short time frame? In this thesis I have aimed to investigate how changes in salinity drives morphology, population genetic patters, energy use and plasticity in genetic expression and sperm viability with the use

of a model organism, the threespine stickleback (*Gasterosteus aculeatus*, hereafter "stickleback", Figure 1).

Figure 1. *Three threespine stickleback's caught in a trap; The upper is a spent female, the two below are males in their breeding colors. © Anna V. B. Mazzarella.*

The Threespine Stickleback

When first looking at a stickleback you might not find yourself very impressed; most of the year it is a small, rather drab little fish, that most people in Norway associate with a commonly used tackle ("stingsildsluken"). However, once you have laid eyes on a brightly colored male, building nests, courting females with his zigzag dance and caring for his fry, you are probably more fascinated (maybe even in love). The stickleback was first described literarily by Linneaus in 1758 (Linneaus, 1758), and it began to appear in written sources in the nineteenth century, while serious examination into the species began in the 1920s - a scientific relationship that has continued to the present day, with an impressive number of articles published every year (Figure 2). The increasing popularity spans from the stickleback's many favorable biological properties for science; it is hardy and directly adaptable to a large range of ecological environments; it displays large phenotypic variations within and among naturally occurring populations (Klepaker, 1995; Lucek et al, 2010; McKinnon and Rundle, 2002) and sexes (Aguirre and Akinpelu, 2010; Kitano et al, 2007) and are (at least according to ample literature) easy to keep in the laboratory. The stickleback is widely distributed in the northern hemisphere, and throughout its large range, the stickleback has colonized coastal freshwater environments from its ancestral marine environment, following the glacial retreat at the end of the Pleistocene (Bell, 1977), and is presently occurring in a wide range of environments, including the open ocean, coastal marine areas, estuaries, streams, sloughs, lakes and ponds (Bell and Foster, 1994; Wootton, 1976). The multiple independent colonization events to freshwater has been

associated with a variety of morphological, behavioural and physiological changes, including the well described evolutionary loss of lateral plates (Heuts, 1947), typically interpreted as the result of parallel genetic adaptation to a novel environment, i.e. fresh water (Colosimo et al, 2005; Foster and Baker, 2004; Jones et al, 2012b). As such, natural studies on stickleback have made important contributions to the fields of animal behavior, reproductive physiology, evolutionary biology, parasitology, ecology and genetics, and taken together, the stickleback has received more scientific attention than almost any other species of fish.

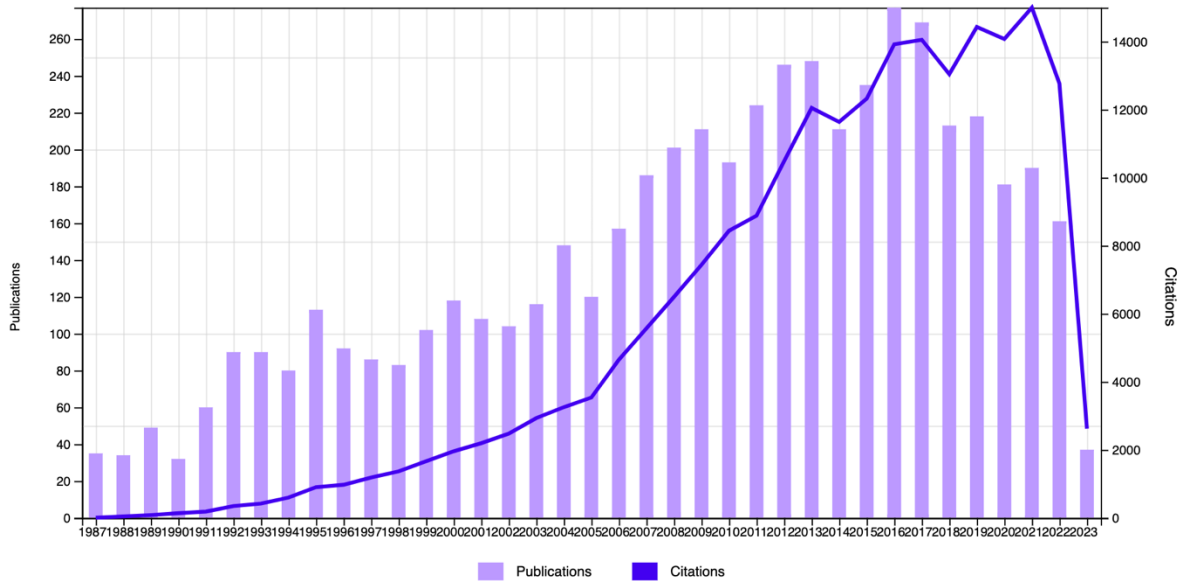

Figure 2. *Number of publications and citations using the search- word "stickleback" in ISI Web of Science, from 1987 to the beginning of April 2023.*

A Morphological Puzzle

The number of lateral bony plates is a common trait that varies within and among populations and salinity (**Figure 3**). Marine sticklebacks have a full row of lateral bony plates on each side (30 or more plates, referred to as the full plated morph), while freshwater individuals have evolved such that only some, or in extreme cases none, of this lateral armour remains (0-10 plates; referred to as the low plated morph) (Heuts, 1947; Klepaker, 1995). A partially plated morph with intermediate numbers of plates is typically found in brackish water (thought of as hybrid zones) (Hagen, 1967; Jones et al, 2008; Østbye et al, 2018), and occasionally also occur in freshwater or marine environments at different frequencies (Wootton, 1976). After completely plated morphs colonize freshwater, the evolutionary reduction of lateral plates has been observed to evolve incredibly rapidly, sometimes within just a few generations, interpreted as a strong selection pressure to have less plates in freshwater (Barrett and Schluter, 2008; Bell et al, 2004; Klepaker, 1993b). Indeed, there is direct experimental evidence that the low-plated morphs, or at least the genes coding for the low-plated morphology, have a selective advantage in freshwater lakes (Barrett and Schluter, 2008; Le Rouzic et al, 2011). The hypothesis that variation in plate numbers may be related to changes in predator regimes and predator intensity between habitats has received some support (Hagen and Gilbertson,

1973; Kitano et al, 2008; Moodie et al, 1973), but it is not conclusive.

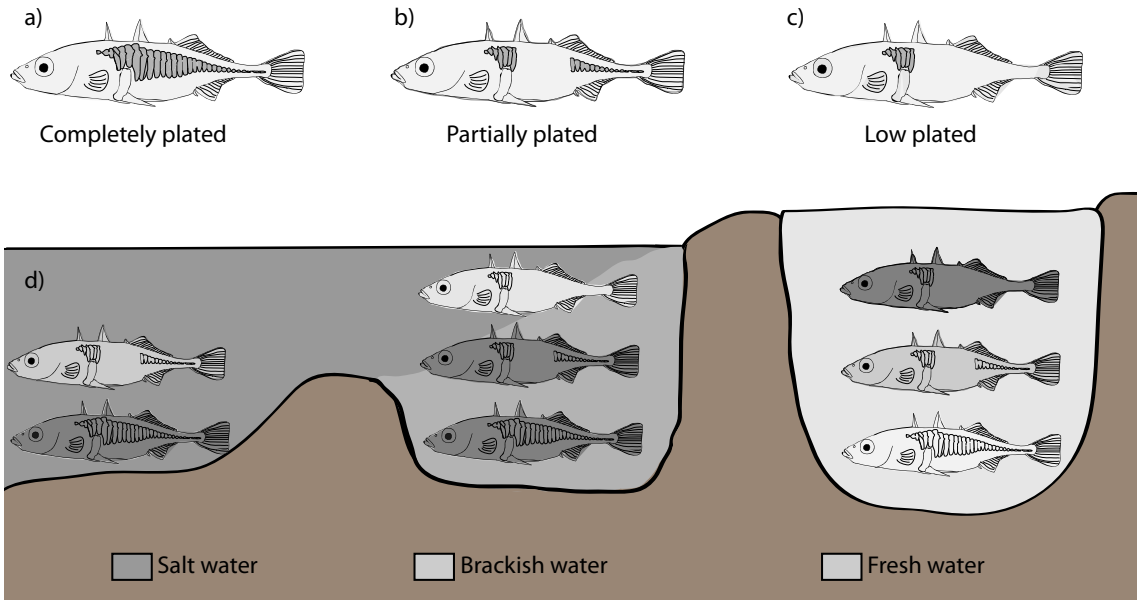

Figure 3. *Lateral plated morphs and the typical distribution in relation to salinity. a) the completely plated morph, with a continuous row of plates and a complete keel on the caudal peduncle, b) the partially plated morph, with one or several missing plates on one or both sides on the body and/or on the keel and c) the low plated morph with plates in the head region only, and no keel, d) the completely plated morph is mostly observed in marine and brackish water environments (illustrated with a darker fish-color), the partially plated morph in coastal marine and brackish water environments and also in fresh water, while the low plated morph is mainly found in fresh water and rarely in brackish water. All lateral plated morphs make vital offspring when crossed.*

It has also been hypothesized that the abundance of cover and shelter in littoral habitats might select for fewer plates, as acceleration and manoeuvrability may be adaptations to predator regimes where fast-start performance is important (e.g. a fast acceleration from rest) (Bergstrom, 2002; Walker et al, 2005). A hypothesis that has been less thoroughly investigated, but often mentioned is Giles's (1983) suggestion that the reduction in nonessential bony tissue in freshwater-resident sticklebacks is an adaptation to reduced calcium availability (Marchinko and Schluter, 2007), and buoyancy has been proposed as another selection factor, leaving more room for food in stomachs of low plated sticklebacks as less room is needed for a larger swimming bladder (Myhre and Klepaker, 2009). To further complicate the pinpointing of cause of the commonly found evolutionary loss of lateral plates in freshwater, the three lateral plates also coexist in many lakes, and has done so for a long time, across many generations (Narver, 1969). Hence, taken together, there is not one explanation that fits all the exciting freshwater radiations, and the mystery of the exact selection pressure(s) for plate morphology has yet to be sorted out.

Into the Genome Area

Having populations of stickleback that vary considerable in skeletal structures, but still make viable crosses in the lab, offers a great tool for genetic mapping experiments. Already in 2001, a genome-wide linkage map was developed for stickleback (Peichel et al, 2001). By availing monitoring of inheritance for different chromosomal regions in crosses between populations expressing different phenotypic traits, many of the classic armor and trophic traits could be narrowed down to particular chromosomal regions, and later, to specific genes (Chan et al, 2010; Colosimo et al, 2004). The selection pressure on the number of lateral plates is still undefined, but a gene responsible for about 70% of the lateral plate variation has been identified as *Ectosysplasin-A* (*EDA*) (Colosimo et al, 2004). An intron within the *EDA*-gene has two main alleles, with low-plated fish being homozygous for the low armour allele (aa), fully plated fish homozygous for the full armour allele (AA), and heterozygous fish usually being partially plated (Aa; Colosimo et al, 2004). The prevailing theory is that a low number of marine individuals are heterozygous for the *EDA* gene, where the standing genetic *EDA*-variation in the marine colonizers enables the rapid evolution of plate loss when inhabiting freshwater (Barrett and Schluter, 2008; Jones et al, 2012b; O'Brown et al, 2015). This theory is further supported by work where freshwater populations only has the "marine" alleles at *EDA* also have a full set of plates, but evolve to have smaller, thinner plates of lower bone density (Leinonen et al, 2012).

The Physiological Brainteaser

For aquatic organisms, the difference between saltwater and freshwater represents considerably different physiological selective forces on the cell's homeostasis, which is about 9‰. To maintain a more or less stable ion concentration, which is needed to stay alive, animal cells surrounded by an environment that are not equal to their internal concentration need a system for active ion-regulation against a concentration gradient, counteracting the effects of osmosis. In saltwater, a fish will have a lower concentration of inorganic ions and hence a lower osmotic pressure compared to the environment, and the fish will hence passively gain ions and loose water (**Figure 4**) (Evans et al, 2005). The situation for a freshwater fish is reversed, as the fish now has a higher concentration of ions when compared to the surroundings, and the fish passively gain water and loose inorganic ions (**Figure 4**). Consequently, to maintain homeostasis, saltwater fish drink saltwater, where excessive salts are actively secreted at the gills and water is absorbed in the intestine (Evans et al, 2005) and freshwater fish actively absorb ions at their gills, minimizes ion loss at their body surfaces, and actively reabsorb ions in their kidney to minimize urinary ion loss (Evans et al, 2005). If the fish do not counteract the effects of osmosis, they will either dry out or swell up (**Figure 4**).

Altogether, osmoregulation is costly, and is estimated to consume between 10-50% of the total energy budget (Boeuf and Payan 2001), where about 7% of the total energy budget can be spent in the gill tissue alone (Mommsen 1984). Therefore, taking the energy costs and the laws of natural selection in to account, it is surprising that the sticklebacks occupy such wide osmotic nice as they do, populating environments in pure fresh to hardy saltwater. Further, especially

for landlocked freshwater populations, that to a large extent have been separated from other populations (gene flow) and the likelihood of experiencing salinity changes for generations, natural selection should have reduced their ability to osmoregulate in non-native salinities due to local adaptation and genetic drift.

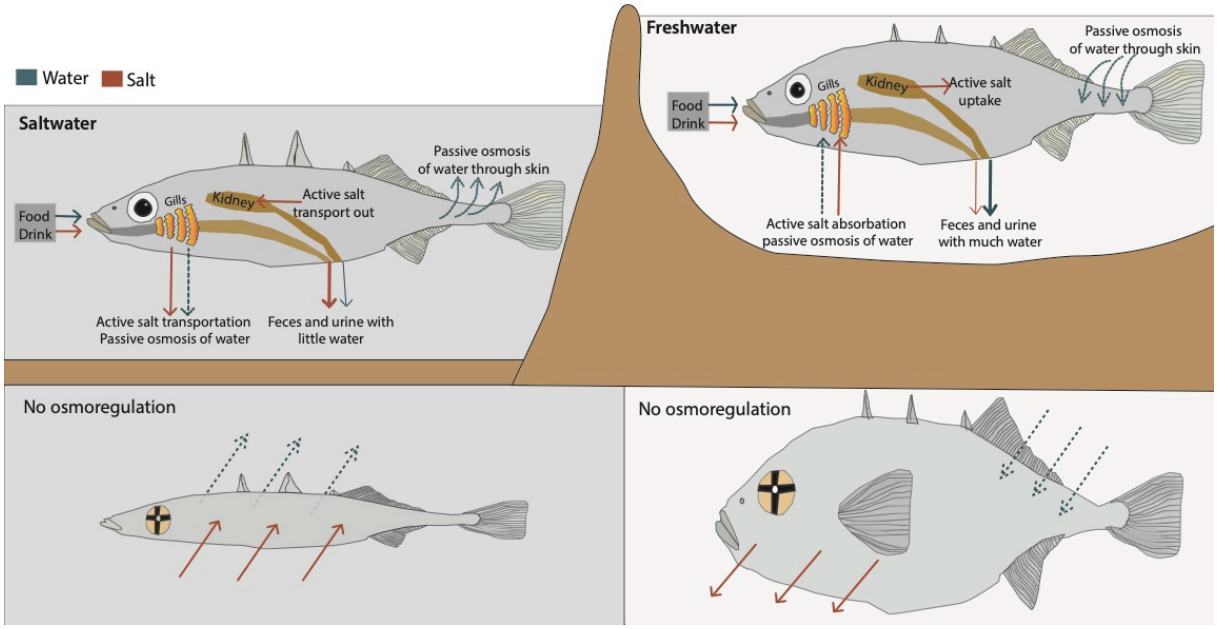

Figure 4. *osmoregulatory challenges in saltwater (dark grey) and freshwater (light grey). A fish in saltwater has a lower concentration of salt in the blood compared to the surrounding water and is therefore losing water by osmosis. The saltwater fish must therefore drink water and spend energy on active salt transportation out of the fish, which happens over the gills and through concentrated urine. If a fish fails to osmoregulate in saltwater, it will shrink and eventually die as the concentration of salt in the cells will reach deadly levels. In freshwater, the fish has opposite challenges, with water flowing into the fish due to its higher concentration of salts in the body, and at the same time it is loosing salt to the surrounding freshwater. To compensate, the freshwater fish must therefore actively absorb salts in the gills and kidney, and have a very diluted urine. If a freshwater fish fails to osmoregulate, the fish will swell up as excess water enters the body, drowning the cells.*

The stickleback genome was published in 2005 and further genome re-sequencing of 21 individuals from marine and freshwater habitats across their global distribution revealed, with very high resolution, a total of 81 loci underlying repeated parallel divergence in marine and freshwater ecotypes, including three chromosomal inversions (Jones et al, 2012b). This indicates that ecological selection to freshwater favors certain genes and/or gene regions, such as chromosomal rearrangements. Further studies on Single Nucleotide Polymorphism sites (SNPs) (Guo et al, 2015; Hohenlohe et al, 2010; Jones et al, 2012a) and microsatellites (DeFaveri et al, 2011; DeFaveri et al, 2013b) have also shown high levels of population differentiation towards salinity, and it has been revealed that freshwater-linked alleles can increase rapidly in newly colonized freshwater habitats (Lescak et al. 2015). These combined genetical differences between marine and saltwater clearly illustrate that the stickleback populations have adapted

to their native environment, but amazingly, they are for the most part still able to handle abrupt salinity changes, in both directions, and can survive without any seemingly large costs (which is almost like having a superpower).

How are organisms physiological state, so fine tuned in one environment able to "immediately" adapt to changing, or even reverse state, as with freshwater and saltwater? Phenotypic plasticity is the property of a given genotype to produce different phenotypes in response to distinct environmental conditions, which can be a continuous process throughout a lifetime for many traits. Having a wide possibility for plastic responses is hence often viewed as beneficial when adapting to new environments, as this gives a higher chance of expressing a "new phenotypic optima" directly, which then can be genetically assimilated in the new environment (Levis and Pfennig, 2016). Fixating environmentally induced plasticity through genetic assimilation should therefore reduce genetic- and plastic diversity in the derived population, where the rate of stabilizing selection depends on the number of loci that contribute to the additive genetic variance of the character(s) (Lande 1976). Set in a stickleback evolutionary perspective; having a wide range of phenotypic plasticity in the population has likely been one of the qualities that made the species so successful in invading and sustaining populations in new territories, on the other hand, genetic assimilation and natural selection on the derived, likely smaller population size, should reduce genetic variation, which again should reduce the reaction norm potential, and therefore counteract the possibilities of successfully occupying the original environment, here meaning saltwater. The expected reduction in fitness will also to some extent be linked to time, where populations that have been genetically molded into a derived environmental (freshwater) state, often for thousands of years, are more likely to experience a reduction in fitness if again finding themselves in the original habitat.

Aims and goals

Freshwater covers 1% of the world surface; it includes a total of 0.01% of the total water resources and is still home to 40% of the world´s total number of fish species. Only a handful of fishes (roughly 100, about 0.5% of the total number of the known fish species) is distinctly anadromous, and a fraction of these handful of species can move freely between salt and freshwater at all life stages (Delgado and Ruzzante, 2020), as can the stickleback. The main aims of this thesis was to gain insight into how populations of stickleback vary in their genetics, both in their genome and in their transcriptome, linked to morphometrical differences, and the ability for populations inhabiting different salinities to also be able to reproduce across salinity gradients. Differences between lateral plated morphs are also quite central in this thesis and every paper includes at least two of the plate-morphs. Firstly, to explore the genetic variability of stickleback morphs across a salinity gradient, I used a mixture of microsatellites and geometric morphometric to entangle the population structure and morphological differences between sticklebacks inhabiting an open brackish-freshwater system in Chignik, Alaska, in Paper I, and allometric relationships of morphological traits for the same fish in Paper II. Findings in Paper I supported high levels of divergence between stickleback's assigned to brackish and freshwater populations, despite high potential for gene flow from the lagoon population into freshwater. Following the post-glacial freshwater invasion, the ancestral marine form has in most cases evolved from a completely plated population to a low plated population, and it therefore seems that there is a cost to having plates in freshwater. Obviously, one such cost could be osmoregulation, and Paper III investigates the differences in standard metabolic rate (oxygen consumption) of saltwater, brackish and freshwater sticklebacks in three different salinities for two hours. As no apparent differences in oxygen consumption was found between any of the experimental groups, I went on to include genetic expression of targeted osmoregulatory genes in marine and freshwater sticklebacks exposed to native and conflicting salinity for periods ranging from five minutes and up to three weeks in Paper IV. The original plan was to select genes based on findings from transcriptomic sequencing results in Paper V, where I explored gene regulations in gill tissue from fish exposed for six hours and where I would likely identify potential candidate genes important for osmoregulation in both salt- and freshwater. As the *original plan* ended up being delayed, the targeted genes in Paper IV were selected based on similar studies in the literature, resulting in testing genes that displayed few obvious changes across experimental groups or time points. Further, as I also found few regulatory differences in the transcriptome for the saltwater fish in Paper V, Paper VI covers a new twist to the osmoregulatory theory by examining the potential for gametic isolation caused by a lack of sperm activation in novel water qualities. This study was inspired by several findings in the literature on sticklebacks that indicated reduced breeding success across salinities, and the results from the first part of the project, where sperm from wild caught marine and freshwater males were filmed looked promising in that the sperm of freshwater sticklebacks did not activate in saltwater. However, the stickleback has a whole range of adaptabilities up it's plates; if freshwater fish were bred in saltwater, or only exposed to saltwater for two days, the sperm activated in saltwater and it was again concluded that the stickleback trumps with its plastic abilities.

Methodological Approaches

This thesis spans a diverse set of methods used to investigate the questions under consideration (Table 1). This section is therefore only a brief overview of the materials and methods used throughout the thesis, where detailed descriptions of the methods can be found within each specific paper.

Table 1. Summary of methods used **in Paper I-VI**.

Paper	DNA	mRNA	Geno-typing	qPCR	Illumina sequencing	Morphology	Common Garden
I	X		X			X	
II						X	
III	X		X				X
IV		X		X			X
V		X			X		X
VI							X

Sampling Sites and Sampling Efforts

The stickleback sampled for this thesis comes from two sites, the Chignik Lake system in Alaska, U.S.A. (Paper I, Paper II) and from the Oslofjord-area in Norway (Paper III- VI), see Figure 5. The sample sites within Chignik is geographically isolated, but there are no migrational obstacles within the stretch spanning the outlet to the lagoon and the upper lake. In the Oslofjord, the sample sites are also separated by geography, but waterfalls further separate the marine and brackish-water sites from the two freshwater sites. In particular, Glitredammen is estimated to be about 7800 years based on isostatic uplifting of the land since the last glacial period, and is currently located above several steep waterfalls, reducing geneflow to downward migration only.

The salinity in the sampling sites vary. In Chignik, the lagoon has a fluctuating salinity due to tidal influence, ranging between 0-30‰, whereas the rest of the sampling sites in freshwater is stable at 0‰. In the Oslofjord, Sandspollen is defined as a marine site with salinity ranging from 22-29‰, Engervann is a brackish water lagoon, partly influenced by freshwater floods and tidal effects, having a salinity that ranges between 0-17‰, and Sandvikselva being a freshwater river and Glitredammen is a freshwater pond, stable at 0‰. In Chignik, the fish were collected from four locations using beach seines, tow nets and fyke nets during the two last weeks of June 2009. Fish sampled in the Chignik (Paper I, Paper II) were euthanized and stored on 95% ethanol until processing. In the Oslofjord, Sandspollen was sampled using beach seine in the spring, Engervann, Sandvikselva and Glitredammen by baited minnow traps in May-August 2008 (Engervann and Sandvikselva) and 2010- 2012 (Glitredammen).

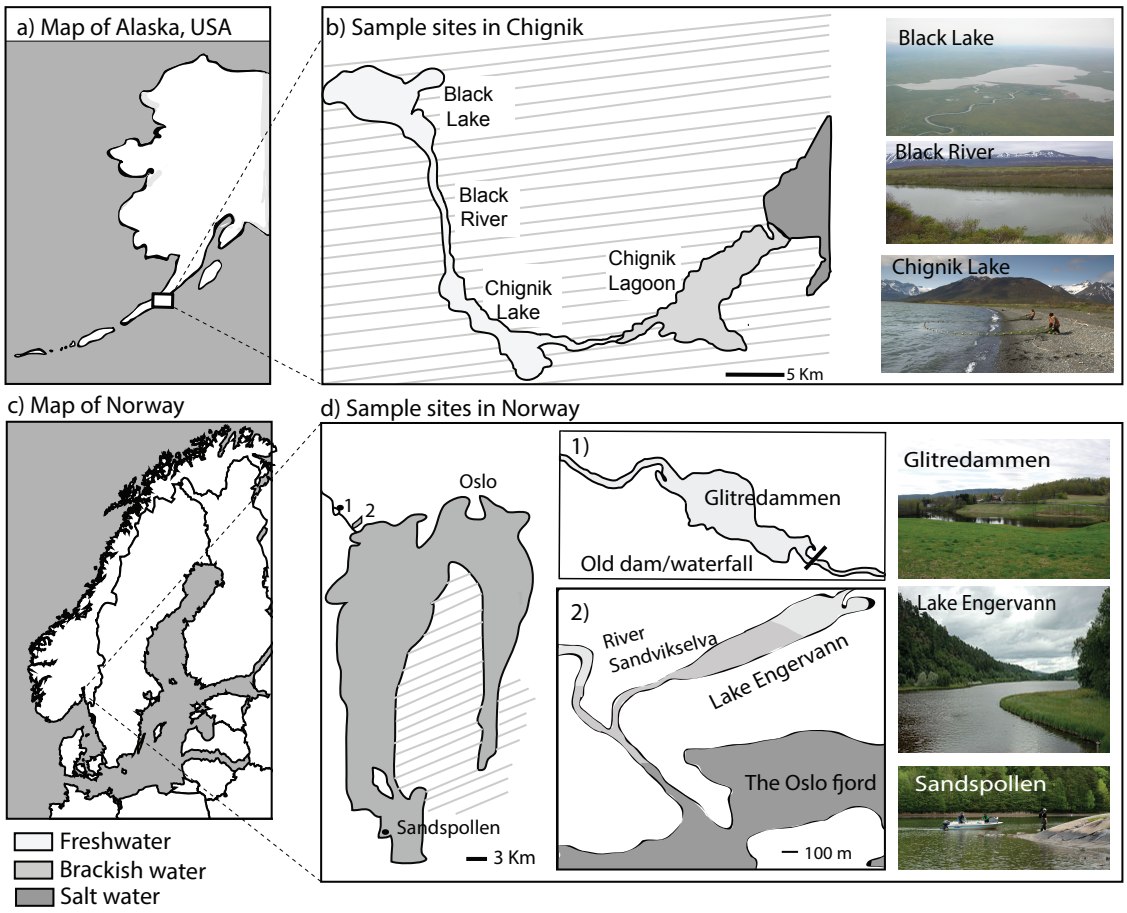

Figure 5. *Sampling sites. This Thesis comprises samples from both (a) Alaska, which includes samples from (b) several river and lakes in Chignik, the sampling sites illustrated by pictures (© Morgan Bond), and (c) Norway, focusing on locations in the Oslofjord (d). Glitredammen (1) is the upper freshwater lake, draining in River Sandvikselva and Lake Engervann (2), and Sandspollen is the marine site, close to Drøbak. The three sampling sites are illustrated with pictures (©Annette Taugbøl).*

Laboratory Conditions for Sampled Fish

The sampled adult fish from the Oslofjord area (**Paper III- VI**) were transported back to the aquarium facility at the University of Oslo and acclimated to laboratory conditions for up to three weeks prior to the experimental setups. In the lab, the fish were kept at room temperatures (~19-20°C), at a photoperiod of 18 day/6 night, and were fed red bloodworms once or twice a day. Initially, fish caught from Sandspollen (marine) were placed in artificially made saltwater (30‰) and the fish sampled from freshwater were kept in freshwater (**Paper III-VI**). The fish from the brackish water lake Engervann were placed in a freshwater holding tank prior to the experiments (**Paper III**). In **Paper VI**, one experimental group consisted of first-generation laboratory fish that had been raised in saltwater, but whose parent originated from freshwater (for more details on breeding and husbandry of the juvenile fish, see **Paper VI**). After each experimental setup, the sticklebacks were either euthanatized with benzocaine (**Paper III**) or killed with a swift blow to the head (**Paper IV-V**), before being processed for tissue collection and storage in 95% ethanol.

Common Garden Experiments

Several common garden experimental approaches were used in order to investigate phenotypic plasticity between populations sampled from different salinity regimes (**Paper III-VI**). For all the common garden experiments, except one group in **Paper VI**, the fish were wild caught. In **Paper III**, fish from saltwater (completely plated), brackishwater (completely-, partial- and lowplated) and freshwater (lowplated) were divided into five groups based on salinity and lateral plate morphs before being exposed to fresh-, brackish- and saltwater in a respirometer chamber, measuring their oxygen consumption. In **Paper IV** and **V**, groups of fish from saltwater and freshwater were exposed to both their natural (control) and the opposite salinity for a timespan ranging from five minutes to three weeks as to measure for potential gene regulatory changes in selected genes over time (**Paper IV**) and between the control groups and the exposure groups after six hours using illumina sequencing of the transcriptome from gills (**Paper V**). In **Paper VI**, two of the four experimental setups where common garden approaches, were first offspring of wild caught freshwater sticklebacks were raised to maturation in saltwater, and second, wild caught freshwater males where exposed to saltwater for two or seven days after one day of slow accumulation to 30‰ before sperm mobility was measured.

Population Genetic Markers

Microsatellites are stretches of DNA that consists of short tandem repeats, occurring all through the genome. The discovery of microsatellites and techniques to copy individual repeats by the thousands in polymerase chain reactions (PCR) revolutionized biology, as the method allowed for non-lethal sampling of very small quantities of source material. Further, as a co-dominant marker, the microsatellites also allow for studies of different evolutionary forces on population structuring. Microsatellites are often neutral (differences are not under selection) and have a higher mutation rate compared to other areas of the genome, giving them high levels of polymorphism within populations. In **Paper I**, DNA was extracted from a small piece of the pectoral fin for 389 individuals, each DNA sample was run in five separate PCRs with primer-pairs that could be separated by color and/or base-pair length on an agarose gel. A total of 14 neutral microsatellites were genotyped on micro gels on a 3730 DNA analyzer, and descriptive statistics of microsatellite diversity for was calculated using different programs.

Microsatellites within introns of certain genes can influence the individual phenotype, thereby functioning as a quantitative trait loci (QTL; *not* neutral). By targeting the microsatellite within the first intron of the EDA-gene (*stn382*), each fish was assigned a "plate-morph-genotype" through gel electrophoresis on a standardized 2% gel. The genotypes were separated by visual inspection on photographs of the *a*-allele (151 bp long; linked to less plates) and the A-allele (218 bp long; linked to more plates). As the populations under study in **Paper I** and **III** consisted of all the three lateral plate morphs, the *stn382* QTL were genotyped for all individuals. In **Paper I**, **IV** and **V**, an additional sex-linked QTL was also genotyped for all fish. The *IDH*-locus also has two alleles that was separated on a 2% agarose gel, where females are homozygous for the

longer allele (302 bp) and males are heterozygous, having two alleles at the same loci (302 and 271 bp long).

Population Genetic Statistics

A group of fish sampled at one site do not necessarily belong to the same population. To test for this, I investigated the genetic population structure through genetic clustering analysis, using Markov chain Monte Carlo simulations, to assign individuals to genetic clusters (*K*) on the basis of their multilocus genotypes in STRUCTURE (Pritchard et al, 2000). The program detects putative populations under the assumption of Hardy-Weinberg equilibrium (HWE) and linkage equilibrium within each cluster, using unlinked genotype data. Each individual is probabilistically assigned to one or more genetic clusters (*K*), each of which are characterized by a set of allele frequencies at each locus, and the program estimates a proportion of ancestry to each cluster (*Q*) for each individual. Values of *Q* can therefore be used to assign each individual to genetic clusters irrespective of sampling location. If the alleles and genotype frequencies in a population is constant from generation to generation, the population is in so called Hardy- Weinberg equilibrium, which is often the null hypothesis in population genetics. If a population is not in Hardy-Weinberg equilibrium, the population is influenced by either mate choice, mutation, selection, genetic drift, gene flow or meiotic drive, where one or several of these factors usually play a role in every population. Significant deviations from HWE were tested by computing the expected and observed heterozygositiy, tested for significance by Markov Chain parameters, and tested for population differentiation between the fish sampled at the different sites.

Transcriptomics and Genetic Expression

The gills play an important role in the maintenance of the blood's ion and acid–base balance in fish (Evans et al, 2005; Evans, 2008; Krogh, 1937), and hence the gill-tissue of the experimental fish in **Paper IV** and **V** were collected and stored on RNA-Later. In the lab, mRNA was extracted and transcribed to cDNA before the samples from all nine time-points were tested for four targeted genes (ATPA13, CFTR, KCNH4 and HSP70) in quantitative PCRs (qPCR) in **Paper IV**, and illumina sequencing of the transcriptome for the six-hour exposure in **Paper V**. The amount of tissue and genetic concentration were controlled by weighing the gill tissue before crushing it with beads, and the mRNA-concentration was measured and diluted to a fixed concentration before cDNA synthesis. In **Paper IV**, the expression was further normalized by the use of two reference genes (GADPH and EF1α), with the means that these genes should be equally expressed in gills despite changes in salinity. Each individual sample (fish) and primer pair were run on duplicated plates, along with three negative controls, a serial dilution as a positive control and plate reference. Variations in fold change of expression of the different targeted genes were tested using general linear models (GLM's), by applying the average C_q – values for the reference genes and the first timepoint (5 minutes) as a control group. In **Paper V**, the transcripts were aligned to the stickleback genome and the counts of each gene was analyzed between each experimental group with glmQLF tests in edgeR (Robinson et al, 2010) in R (R Development Core Team, 2021).

Geometric Morphometrics and Morphological Measures of Metric Traits

As stickleback populations are represented by a diversity of morphological variances, I also tested for geometric morphometric differences in the genetically identified populations in **Paper I** and allometric size differences in morphometric traits in **Paper II**. Geometric morphometrics is the statistical study of shape and their covariation with other variables. In order to compare shapes between groups of individuals, each fish was stained with alizarin red as to make the placements of digital landmarks more accurate for each photographed fish. Homologous landmarks were selected based on several criteria; firstly, the accuracy of repeatability for each fish was tested, secondly, the landmark should be biologically meaningful, and lastly, the landmarks should catch the overall shape of each fish. As all landmarks are dependent on each other, the raw coordinates of each specimen were transformed into shape variables by procrustes superimpositions in MorphoJ (Klingenberg, 2011). This operation separates size and shape, and also projects shape coordinates into a Euclidean space tangent to the Procrustes shape space. In short, the procrustes superimposition are least-squares methods to estimate superimpositions parameters (scale, rotation, translation) between configurations; aiming to minimize the squared distances between similar landmarks of configuration by allowing size, rotation and translation to be adjusted, where the sum is called procrustes distances. The projection into the tangent space is performed because standard statistical methods, such as regression and analysis of variance generally require data to be in a flat Euclidean space. In simple terms, this means that a distance between two observations is a straight line computed using the theorem of Pythagoras (or its multivariate extension). However, because the Procrustes shape space is curved, it has to be approximated by a tangent Euclidean space using a projection computed as a cartographer would do to draw the curved surface of the Earth onto a flat map.

Size and shape are only correlated to a certain extent, where the degree of correlation is being determined by the allometric relationships of the various body parts. To analyze for allometric scaling relationships on linear traits, I extracted line measurements for 15 morphology traits for the low- and partially plated fish and 24 traits for the completely plated fish by adding 16 homologous landmarks on the photographs from each completely plated fish in **Paper I** for the allometric testing in **Paper II**.

Phenotype Statistics

Potential differences in the extracted procrustes distances between the predefined groups in **Paper I** were visualized by the use of a canonical variates analysis (CVA). CVA is a method that first performs a principal component analysis (PCA) on the pooled within-group variation to construct a coordinate system in which the position of each group can be positioned. After rescaling the axis proportionate to the elongation of the average fish, the program solves for the direction in which the fish seems to be farthest apart in the rescaled space by performing a PCA on the group centroids, producing the canonical variates (CVs). The scores of individuals on the CVs are the projection of the individuals onto these new coordinate axes (Zelditch et al,

2004). The purpose of CVA is hence to find the linear combinations that reveal differences among groups, trying to maximize the means between the preselected groups. As all deviations from the centered data are expressed in the same metrics, it is possible to quantitatively visualize the shape change associated with a given principal component using warped outline drawings.

Since evolutionary allometry is the log–log regression of the mean trait size on mean body length across populations, standardized major axis regression (Warton et al, 2006) on all measured line-traits between the different groups were used as a first step to test for differences in allometric scaling relationships (slope and intercept) in **Paper II**. When the slope and/or the intercept of a trait differed significantly, I tested which groups that differed from each other by mean-centering the log body length of the fish around zero to make the intercept in the model equal to the trait mean within each treatment (mean of zero, sd = 1). This standardization enabled me to estimate the proportional trait change across morph type and genetic population as the ratio between the intercepts by using a general linear model (GLM): trait ~ bodylength × morph. Residuals were extracted with qq-plots and intergroup differences were applied on the covariance matrix by PCA's.

Measurements of Sperm Movements

Relatively little research has been done to investigate potential prezygotic mechanisms that might act to isolate populations at the level of the gamete for sticklebacks inhabiting marine and freshwater environments. As the stickleback reproduce externally, it is possible to both observe the sperm in its natural environment (in **Paper VI**, the native salinity), as well as manipulating the environment into something novel (**Paper VI**, salinity transfer). In **Paper VI**, the sperm mobility of 16 marine and 40 freshwater sticklebacks was measured using a microscope and a video camera at room temperature. For each male, the two testis were removed surgically and used in a paired comparison after they had been mixed in either fresh (0‰) or artificially made salt-water (30‰). The first testis was examined in freshwater for half the fish and in saltwater for the other half, in randomized order. As the diluted sperm was transferred and filmed on a chambered microscope slide, several locations on the slide could be filmed and compared for each individual, and four or six locations were filmed for each male. Using CEROS sperm tracker, the curvilinear velocity (VCL) was measured for 0.5 seconds at each of the recorded locations. As blood, small air bobbles and other non-sperm contaminants could induce inaccuracy on the analyzes, these and other poorly tracked cells were manually deleted. Further, since there were no statistically significant differences between the filmed locations, these were averaged for each male and used in a two-way T-test, testing for differences in VCL between the two salinity treatments. A total of four freshwater males (10%) were excluded from the data as they either had only one reading frame with moving cells (n=2) or they had no moving cells in any of the filming locations.

Results presented by individual papers

Paper I. Genetic and morphometric divergence in threespine stickleback in the Chignic catchment, Alaska

Here we test for population genetics in stickleback from four connecting sites, spanning from a brackish water lagoon to a freshwater lake ~ 40 km upstream (Figure 5). The Chigink system has been habituating stickleback in abundance at least since the late 1960's (Narver, 1969), where large migratory schools have been observed during spring/ early summer (Harvey et al, 1997), indicating a high potential for gene flow on the breeding grounds. The three lateral plated morphs had been described in the system, with the completely plated lagoon stickleback reportedly being morphologically differentiated from the completely plated stickleback mostly sampled from the freshwater habitats (Narver, 1969). The aim of the study was to test if and how the sticklebacks were genetically structured; if there were sufficient effective gene flow for the system to be one effective breeding population, or if the fish were structured on geography, plate morph or salinity. After genotyping 14 neutral microsatellites, the individuals where genetically assigned putative populations, and it was evident that the fish grouped in two genetic populations; one mainly sampled in the brackish lagoon and one in freshwater (Figure 6).

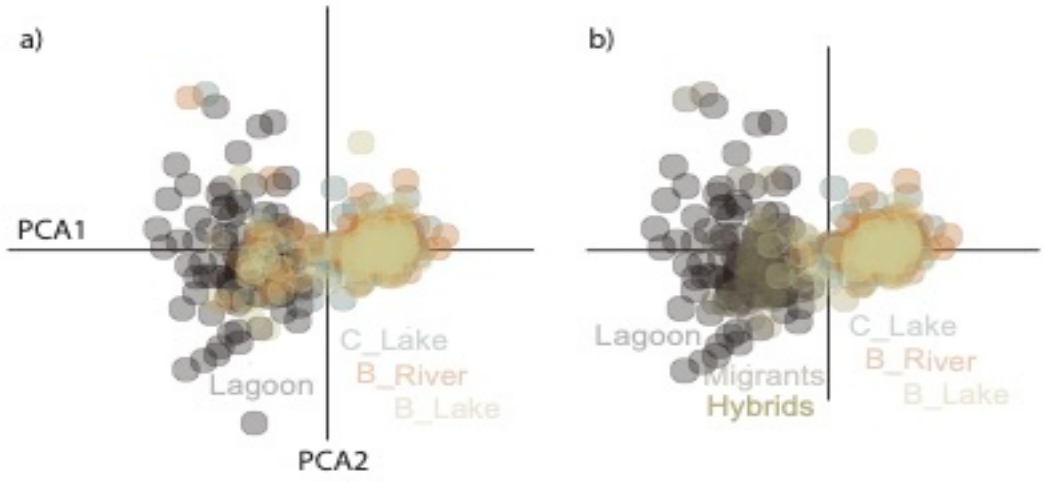

***Figure 6**. Discriminant Principle Component Analysis. By the use of genetic markers, the stickleback in this system could be assigned genetic populations as an entity instead of using individuals sampled at sampling sites as a group/population, thereby increasing the preciseness and interpretation of the results. (a) Illustrates individuals sorted by sampling sites (raw data), and (b) illustrates the individuals sorted by assigned genetic populations, where the freshwater group is divided in original sampling sites (C_lake= Chignik Lake, B_River= Black River, B_Lake= Black Lake).*

The lagoon sticklebacks were all of the completely plated morph, whereas the freshwater fish were sympatric for all three morphs. The plate morphs were typically, but not completely, linked to the *EDA*- alleles. Further, it was also obvious from both genetics, size-measurements and geometric morphometrics that lagoon-sticklebacks migrated into freshwater, as far up as the Black Lake (the upper lake), in fact, migrants from the lagoon were detected at all freshwater sites with increasing frequencies as the distance to the lagoon increased. Regardless of a high potential for gene flow, few hybrids were (genetically) identified, and the F_{ST} values

between the populations also signified low gene flow between the two putative populations. When testing the genetic populations (lagoon, migrants, hybrids and freshwater) for phenotypic differences in body shape, the two genetically assigned populations differed in geometric shape, with the lagoon fish having more streamlined bodies, with thinner heads and smaller eyes. Overall, there were few males in the dataset (17%), and only the lagoon and freshwater populations were included when analyzing for morphometric differences between males and females. The sexes separated on the second axis, with females having thinner bodies when compared to males

Paper II. Allometric relationships in morphological traits associated with foraging, swimming ability, and predator defense reveal adaptations toward brackish and freshwater environments in the threespine stickleback

Freshwater colonization by threespine stickleback has led to divergence in morphology between ancestral marine and derived freshwater populations, making them ideal for studying natural selection on phenotypes. Following the findings of two genetic populations separated by salinity and also differing in geometric shape in Paper I, the aim of this study was to search out morphological trait(s) that separated the populations in geometric shape, as shape and size are not perfectly correlated. To this end, a total of 23 phenotypes likely to be important for different life-history events were extracted and divided into four groups: (a) traits reflecting head shape and thus important for feeding; (b) spine traits important as defense against predators; (c) traits important for swimming/movement, and (d) lateral plate traits important as defense against predators. Overall, the three lateral plate morphs in freshwater displayed few significant changes in trait sizes, but the low plated expressed feeding traits more associated with benthic habitats. When comparing the completely plated genetically assigned populations, many of the linear traits had different slopes and intercepts in trait-size regressions, precluding our ability to directly compare all traits simultaneously, which most likely results from low variation in body length for the lagoon and migrant population. Overall, the lagoon population seemed more specialized toward the littoral zone, displaying benthic traits such as large, deep bodies with smaller eyes compared to the freshwater completely plated morph. Further, the lagoon and migrant fish had an overall higher body coverage of lateral plates compared to freshwater fish, and the dorsal and pelvic spines were longer.

Paper III. No short-term effect of salinity on oxygen consumption in threespine stickleback (*Gasterosteus aculeatus*) from fresh, brackish and salt water

Evidently, one of the major differences between saltwater and freshwater is the salinity, where one obvious test in the plate-morph puzzle is to find out if the three plate morphs spend less energy in the salinity in which it typically occurs. Paper III sums up results from an experiment to test for differences in standard metabolic rate (SMR- measured as oxygen consumption rate at rest) between populations inhabiting salt-, brackish- and freshwater when they are exposed to salinities of 0‰, 15‰ and 30‰. Also, as the marine stickleback typically evolve less plates following freshwater colonization, we also tested for a correlation between SMR and lateral

plate number, and between SMR and the associated *EDA*-alleles, as these also varied within the populations. Contrary to our expectations, the results indicated that the metabolic costs were equal for populations originating in all three water qualities, and across the three lateral plated morphs. Further, there was a slight trend for the freshwater fish to consume more oxygen at 0‰ compared to 30‰, but this was not significant (**Figure 7**). There were no further differences in any of the other experimental groups

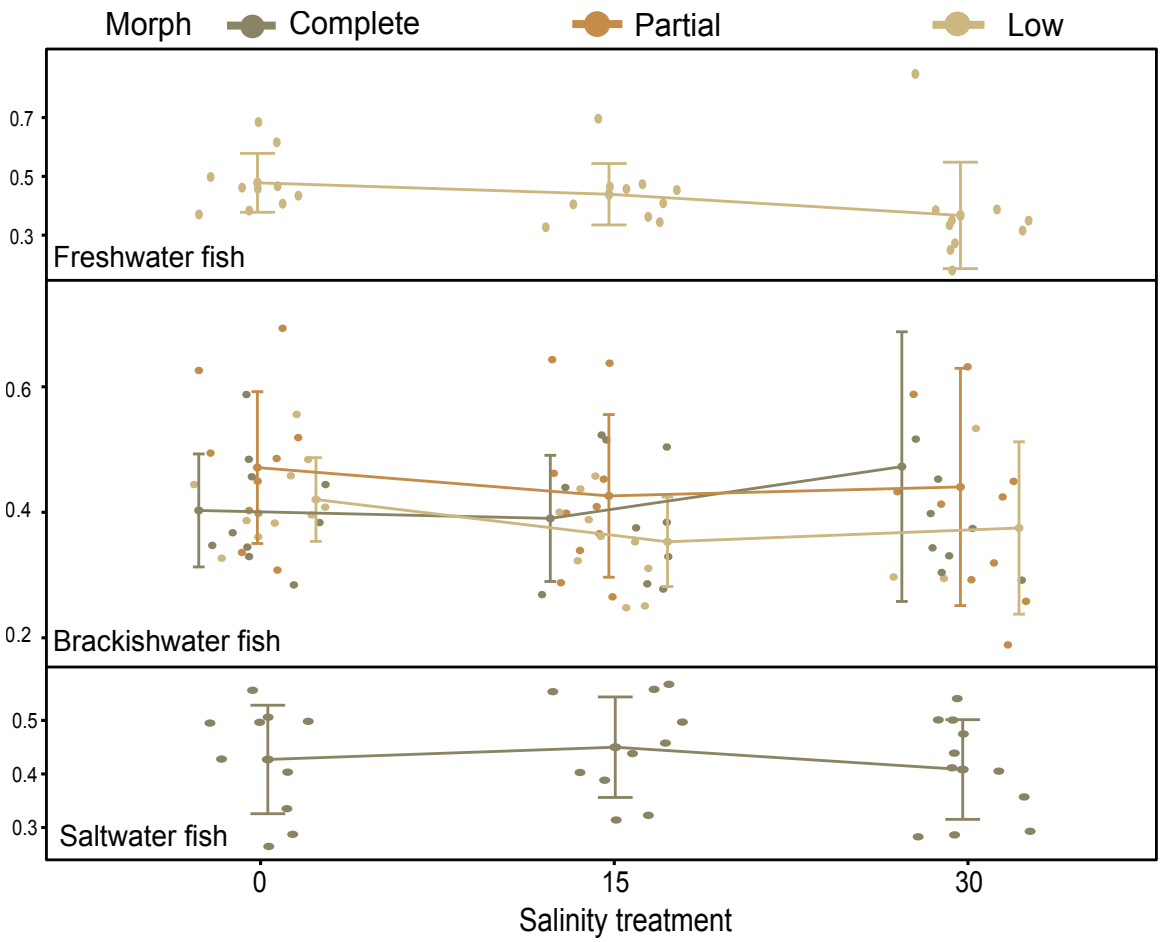

***Figure 7**. Dotplot with mean and standard errors showing the variation in mass specific oxygen consumption rate for the three morphs originating from freshwater, brackish water and saltwater.*

Paper IV. Small changes in gene expression of targeted osmoregulatory genes when exposing marine and freshwater threespine stickleback (*Gasterosteus aculeatus*) to abrupt salinity transfers

The aim of this paper was to explore gene expression patterns for stickleback originating within the same geographic areas as for the fish in **Paper III**, and test for differences in gene regulation across salinity. Fish from allopatric saltwater and freshwater were exposed to their own salinity (control groups) and their non-native saltines for time periods ranging from 5 minutes to three weeks. When 21 stickleback genomes were published in 2012, it also made it possible to

identify and design primers for genomic regions that were seemingly under salinity selection, and two of the osmoregulatory primers were partly based on the publication of Jones et al. (2012b); ATP1A3 and KCNH4. The ATP1A3-gene is a plasma membrane protein that maintains the electrochemical gradients of sodium- and potassium-ions across the plasma membrane, powering salt secretion in saltwater fish and absorption in freshwater fish. During the three-week exposure, the overall expression of ATP1A3-gene increased throughout the experiment, where the saltwater control and the freshwater exposed to saltwater-fish increased the expression toward the later periods of the setup, indicating that ATP1A3 has a higher regulation in saltwater (Figure 8).

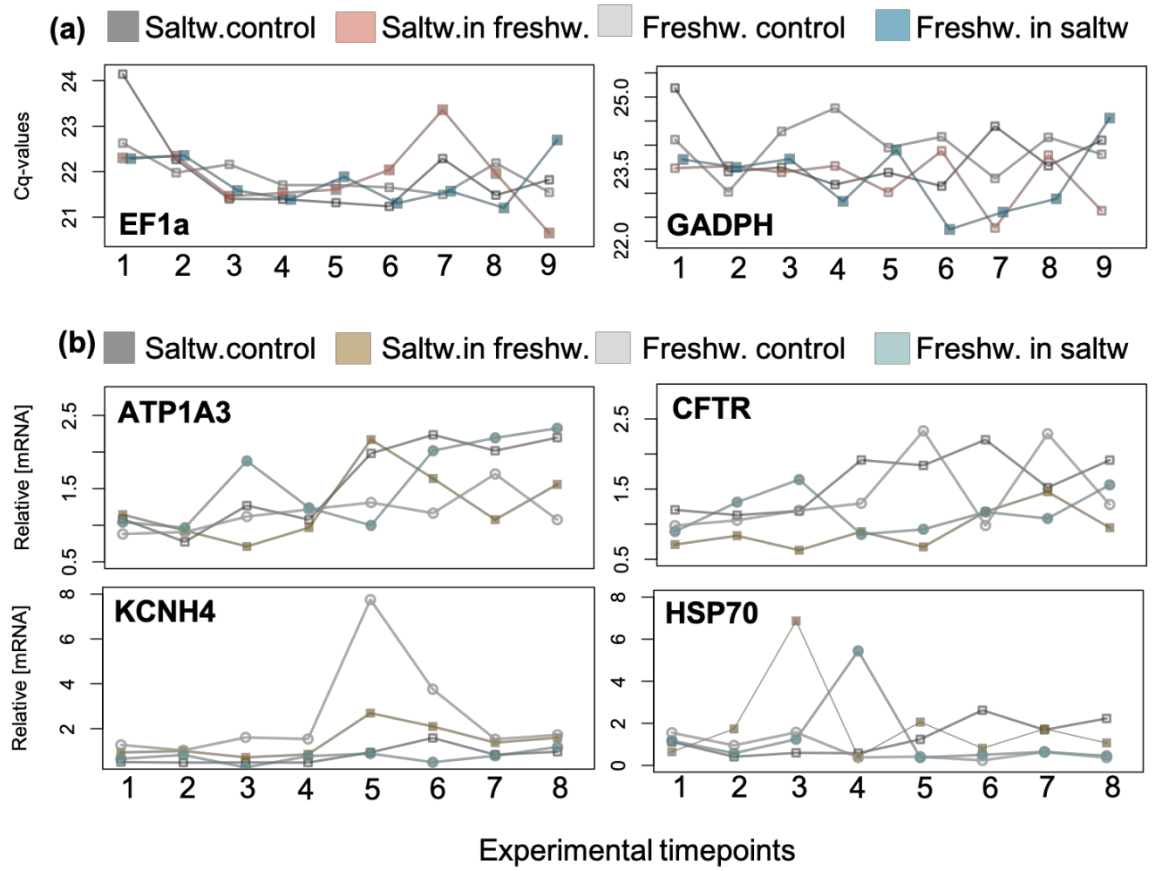

Figure 8. *Relative mRNA-expression for (a) the control genes (AF1a and GADPH) and (b) the four targeted genes for the four experimental groups (saltwater control, saltwater exposed to freshwater, freshwater control, freshwater exposed to saltwater). The plots express normalized qPCR-values across the eight timepoints, timepoint 1= 5 minutes being used as an additional control (1= 30 minutes, 2= 90 minutes, 3= 6 hours,4= 24 hours, 5= 48 hours, 6= seven days,7= 14 days and 8= 21 days).*

Jones et al. (2012b) also revealed an interesting inversion between the sequenced saltwater and freshwater fish, and as the endpoints of both inversions resulted in almost identical base-pairs, indicating parallel ecological selection on the breaking sites of the inversion. Chromosomal rearrangements have been linked to speciation (Fuller et al, 2019) and they are known to alter gene activity, either by causing non-functionalization of the gene, generating alternative splice sites or by altering gene regulatory networks (Gould et al, 2018). Hence, primers were designed to investigate if the KCNH4-gene were differentially expressed. The gene encodes for a voltage-gated ion channel protein that are sensitive to voltage changes in

the cell membrane and helps regulating cell volumes and maintaining resting currents. The freshwater controls had elevated expression profiles at 24 and one week (with high variation), as had the saltwater fish exposed to freshwater, stabilized again at two weeks, then at expression levels slightly higher than the groups in saltwater, indicating a potential, weak, regulatory effect of KCNH4 on osmoregulation (Figure 8). The CFTR-gene were selected based on interesting findings in a similar exposure study (McCairns and Bernatchez, 2010) and links to salinity by being involved in chloride transportation, as well as controlling the function of other channels that are transporting sodium ions across cell membranes. Overall, the expression profile for CFTR increased with time, and both control treatments expressed higher levels of the gene-product compared to the two experimental groups, especially after 24 to 48 hours' exposure (Figure 8). To test for expected stress-related physiological responses, a Heat shock protein-gene, HSP70, were also included, where the expression increased for both experimental groups; the saltwater fish exposed to freshwater showed significantly increased expression after six hours, and the freshwater fish exposed to saltwater after 24 hours (Figure 8). Overall, the levels of expression were higher for the fish originating in saltwater compared to native freshwater fish after the first 24 hours.

Paper V. Salinity-induced transcriptome profiles in marine and freshwater threespine stickleback after an abrupt 6-hour exposure

Most published studies on osmoregulatory expression in stickleback are either focusing on targeted genes (as e.g. in Paper III and in McCairns and Bernatchez, 2010), or are in general focusing on the regular period of adaptation, as the exposure times have been 30 days or longer (Gibbons et al, 2017; Wang et al, 2014). The experimental setup in Paper IV is the same as in Paper III, where the six-hour time point was selected for transcriptomic sequencing. As there were no evident trends for the selected genes in Paper III, the aim with Paper IV was to discover which candidate genes that could have a significant role in the early physiological changes when stickleback are exposed to non-native salinities (from saltwater to freshwater or freshwater to saltwater); how the whole genomic expression profiles differed between the controls (by comparing freshwater fish in freshwater to saltwater fish in saltwater), and ecotype (genes that were similarly expressed within freshwater fish and saltwater fish regardless of salinity, but significantly different between freshwater and saltwater fish), Figure 9. A total of 2712 (16.7%) unique transcript were identified, in which expression was differentiated between any of the groups that were compared by the use of GLM (FDR < 0.05). Contrarily to our expectations that many genes would be differentially regulated between the exposed fish and their controls, only 10 transcripts were significantly different when saltwater stickleback exposed to freshwater were compared to their controls, whereas 1536 transcripts were significant between freshwater sticklebacks exposed to saltwater and their controls in freshwater. The few significantly regulated transcripts identified in the saltwater fish in different salinities made for few tests for interactive regulatory patterns in this dataset. A total of 1314 transcripts were significant between the two control groups, including 502 that were expressed differently between saltwater- and freshwater ecotypes, signifying genetic assimilation patterns to their native salinity.

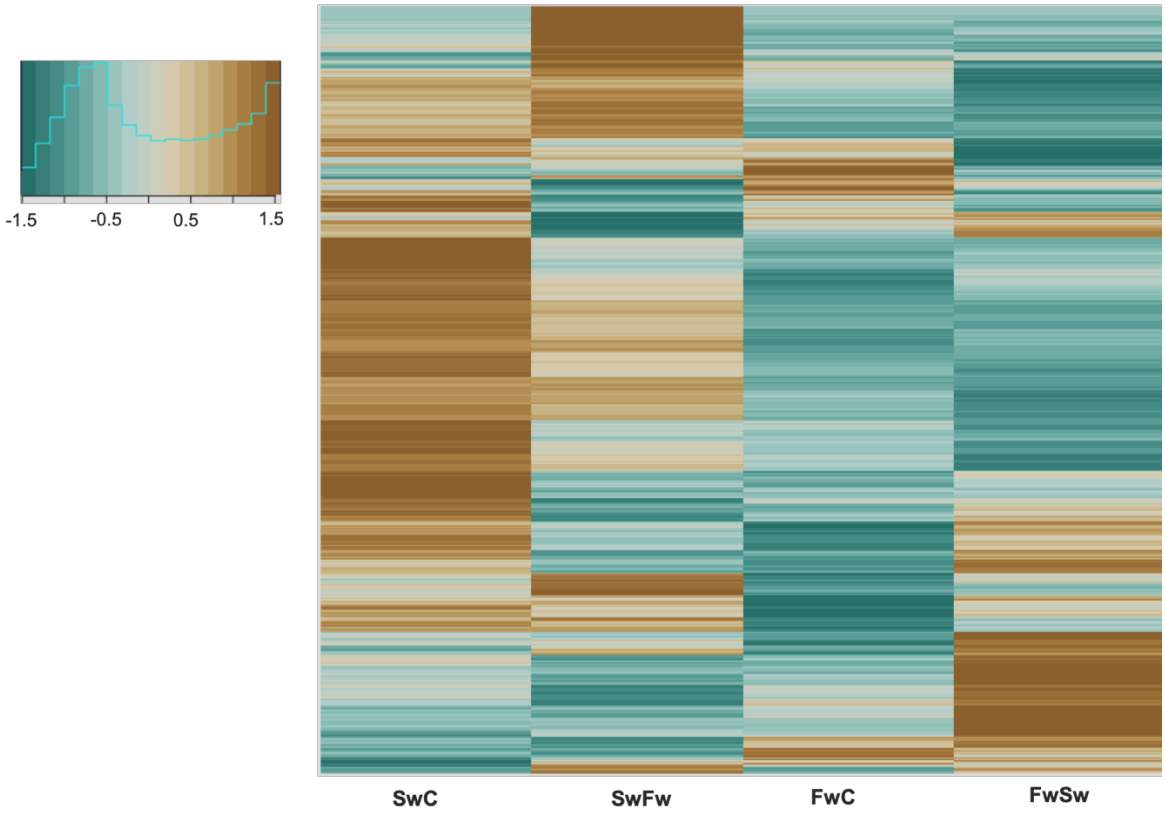

Figure 9. *illustration of the mean raw data per experimental group for the 16.211 transcripts that were kept after filtering (the data are centered and scaled). Each row is a transcript, and as the number of transcripts are too many to be illustrated separately, the figure is meant as an illustration of overall variation before normalization, glm-test's and group comparisons in EdgeR as is presented in the published version of* ***Paper V****. SWC = saltwater fish exposed to saltwater (control), SwFw =saltwater fish exposed to freshwater, FwC = freshwater fish exposed to freshwater (control), and FwSw) freshwater firsh exposed to saltwater; brown color indicates a larger transcriptomic expression level, and blue color a lower expression level than the mean of all samples.*

Paper VI. Salinity-induced phenotypic plasticity in threespine stickleback sperm activation

Population genetic studies consistently reveal high levels of divergence between marine and freshwater populations (Paper I), indicating low levels of gene flow between the two habitats despite the ability for stickleback from both saltwater and freshwater to osmoregulate in both water qualities (Paper II-IV). It is therefore unlikely that osmoregulation in itself is the key selective agent behind the lateral plate distribution. However, the stickleback reproduces externally (oviparous species), and have gametes that might experience a dramatically different environment after being released into the water surrounding the nest (Wootton, 1976). The sperm cells of teleosts have been found to be inactive in the seminal plasma and to be activated by osmotic shock for most fish species, through release in either hypertonic (for marine fish) or hypotonic (for freshwater fish) water. If this is the case, the regulatory system of sperm mobility should be reversed in saltwater and freshwater fish, and there should be selection for an optimal ion concentration in the seminal tract according to spawning salinity. Several studies on stickleback suggest that sperm and/or eggs are most successful in the parental native salinity, indicating that there could be a gametic barrier between saltwater and freshwater populations of stickleback (Elofsson et al, 2003a; Marchinko and Schluter, 2007). The

hypothesis of reduced fertility across salinity were tested by first activating sperm from saltwater and freshwater populations in their native (control) and opposite salinity. The sperm from saltwater stickleback could be activated in either salinity, which matches the freshwater colonization history of the species, whereas the sperm from the freshwater population acted as predicted to the osmotic shock theory and was activated in the control treatment only. As the freshwater population used here was calculated to be about 8000 thousand years old, the next step was to test whether the genetic regulation(s) responsible for sperm activation could have been genetically assimilated (canalized), or if the trait(s) were plastic, and the sperm could still be activated in saltwater after physical exposure to saltwater. To test this, freshwater sticklebacks were raised to maturation in saltwater, where the males now had obtained active sperm in both salt and freshwater. Further, we also found the sperm of wild-caught freshwater stickleback to be active in saltwater after exposing mature males to saltwater for only two days.

Discussion

Genetic variability is crucial for a population to adapt to novel or changing environmental conditions, as the smallest genetic change can give the small advantage needed in the struggle of life and reproductive success. In this Thesis, I have explored different types of genetic links between stickleback originating from different salinity habitats and lateral plate morphs, where the main questions, results and implications for each paper is summarized in Table 2.

Instead of morph or geographic distance, the stickleback sampled in **Paper I** clearly separated genetically on salinity of origin, with high levels of anadromous migrants swimming into freshwater in the breeding period, basically mirroring the evolutionary history of sticklebacks; gene flow in the marine to freshwater direction (Bell and Foster, 1994; Bell, 1977). The finding of genetic structuring to salinity were in contrast to other anadromous stickleback systems, where typically little genetic differentiations between migratory and stationary populations had been reported (Pedersen et al, 2017; Raeymaekers et al, 2005), but the results are in agreement to findings in other open river systems (McCairns and Bernatchez, 2008), and in the Baltic sea, where sticklebacks also have diverged in accordance with local salinity (DeFaveri et al, 2013a; Guo et al, 2015). Further, in **Paper I** and **II**, there were no signs of genetic or morphometric differentiation between the three morphs assigned to the freshwater population for the neutral markers (low, un-significant F_{ST}-values between morphs), genetic results that compare to other sympatric lateral plate morph systems (Mazzarella et al, 2016; Østbye et al, 2018). As expected due to historic founder effects (Nei et al, 1975), the assigned freshwater population in **Paper I** also had somewhat lower heterozygosity-values than the lagoon fish, a result commonly found also in other freshwater stickleback comparisons (Mäkinen et al, 2006; Raeymaekers et al, 2005; Taylor and McPhail, 2000). However, the overall heterozygosity was high for both genetic populations, which is likely a result of a productive ecosystem inhabiting vast amounts of sticklebacks (Harvey et al, 1997), and even if the evidence for hybridization in this system was low, there were some gene flow between the migrating lagoon individuals to the freshwater population. Taken together, the apparent large population sizes and diminutive evidence for gene flow makes it more likely that the observed genetic differences between the populations originating in the two salinities are a result of natural selection on adaptive traits, rather than random effects of drift and gene flow.

Table 2. Summary of the main research questions, results and implications from **Paper I-V.**

Main Question	Main Result	Implications
I. How many genetic populations are inhabiting the Chignik system and if there are more than one, do they also differ in morphology	Two main genetic populations were detected, with migrants and a few putative hybrids. The populations were also morphologically different	Salinity drives population differentiation despite a large potential for effective gene flow through upstream migrants. Less differentiation within freshwater than would have been expected.
II. Which are the most important allometric morphological traits that separates the two populations found in Paper I	The three lateral plated morphs have similar allometric relationships, where lowplates express more benthic traits. The lagoon fish are more heavily armored	Evidence for adaptive differentiation to salinity between the completely plated morphs as plate coverage and spine lengths was reduced in freshwater
III. Are populations originating in salt, brackish and freshwater consuming oxygen at higher rate in non-native salinities, and is there a link to lateral plates	No effects of oxygen consumption rate were found for either populations directly or for the three lateral plate morphs	The stickleback seems able to move between salinities without large short-term costs in osmoregulation
IV. How are targeted osmoregulatory genes regulated in response to abrupt salinity transfers for saltwater and freshwater populations?	There were few major trends in the overall genetic expression profiles for the targeted genes	The stickleback must have alternative mechanisms for keeping the ionic balance in the cells constant and/or other genes are of higher importance
V. How are transcriptomes regulated plastically in response to abrupt salinity transfers for saltwater and freshwater populations?	There were few transcripts that were significantly regulated for saltwater fish and many for freshwater fish	The freshwater stickleback has a higher regulatory cost in their non-native salinity than has the saltwater fish and use more genes to counterbalance changes in osmoregulation.
VI. Is salinity operating as a mating barrier through reduced or failed sperm activation in non-native salinities	The sperm cells could be activated by both salt and fresh water, but the freshwater populations had first to be acclimated to saltwater conditions	That stickleback sperm can activate in both salt and freshwater directly is rather unique, but is not explaining the lateral plate morph distribution

When an allele is beneficial in a new environment, and contributes positively to a phenotypic trait by increasing individual fitness, the allele is expected to undergo a selective sweep in the population; increasing in frequency generation by generation until it (usually) is fixated (Barrett and Hoekstra, 2011; Stephan, 2019). For the stickleback lateral plated morphs, there is clearly an advantage of being low-plated in freshwater, as this by far is the most commonly found morph in this water quality, across the northern hemisphere (Bell and Foster, 1994). It was therefore slightly surprising that such few percent of the freshwater sample were low-plated in the Chignik system in Paper I and II. The contemporary small numbers of the low plated morphs are especially intriguing set in context of the rapid evolution of plate reduction observed in other stickleback populations (Barrett and Schluter, 2008; Bell et al, 2004; Klepaker, 1993b), since the morphotype has been present in the Chignik system for many stickleback generations, and their frequency of ~17% of the freshwater population sample had also not changed much since the 1960's (Narver, 1966; Narver, 1969). There are likely many selective pressures acting on the plate numbers across salinities, seemingly also with a separate selection pressure on attaining the anterior plates (Mazzarella et al, 2016). The anterior plates are thought to function as direct protection against puncturing injuries from predators, and they also buttress the dorsal and pelvic spines, that, when erect, increase the overall size of the stickleback (Hoogland et al, 1957; Reimchen, 1983). The spines probably also function as a warning to gape-limited piscivores (Hoogland et al, 1957), or at least increase the stickleback's chances of escape if the spines hook into the predator. In areas where predators are common, the stickleback spines are often significantly longer than in areas where predators are absent or sparse (Hagen and Gilbertson, 1972; Zeller et al, 2012). So, as the theory goes, robust plates and spines makes for better defence against predation. The lagoon fish in Paper I and II were all completely plated, and they had a higher percentage body-coverage of lateral plates when compared to the completely plated fish in freshwater, and also longer spines. The observed size reduction of the lateral plates in the freshwater population can be comparable to the observed reduction of plate sizes found for some populations in Finland, that lack the lowplated *EDA*-allele variant to evolve a lower number of plates (Leinonen et al, 2012). One advantage of having less plates could be a reduced chance of getting attacked by predators in the first place, as suggested by strong negative relationships found between armour robustness and startle performance (Bergstrom, 2002), and the link between "faster starts" and the ability to avoid predation (Walker et al, 2005). A mix of selection pressures are likely the cause of sustaining the three morphs in freshwater, and the overall differences in morphological traits were small.

The low-plated morphs had somewhat larger mouths and longer heads. This could indicate a larger maximum gape, which again could signify selection on feeding performance on larger prey for the low plated morph. Low plated morphs from a brackish water lake in Norway fed more efficiently on larger benthic prey than the two other morphs (Bjærke et al, 2010), implying that the low-plated morph in Chignik likely has a more benthic lifestyle, and therefore are more adapted to freshwater habitats, or habitats that have productive benthic zones. Adaptation to benthic and limnetic food resources has been found to cause specially adapted morphotypes in fish (Bernatchez, 2004). Benthic and limnetic morphotypes are also commonly found in threespine stickleback populations, and although the differentiation along this benthic-limnetic axis is generally continuous, a few stickleback populations have diverged into sympatric populations (species pairs) that feed exclusively on one prey type or the other (McPhail, 1984). Fish from the Chignik system also seemed to follow the benthic-limnetic axis in the overall bodshape, where the lagoon fish had more streamlined bodies and had smaller heads when

compared to the bulkier freshwater fish (**Paper I**; lagoon fish vs all freshwater morphs). The finding that the larger lagoon fish had smaller heads can be due to selection for streamlined bodies or due to scaling relationships, as larger stickleback have relatively smaller heads (McGuigan et al, 2010; Walker, 1997). The most conserved trait across genotype and morph were caudal area, which were linked to swimming performance in **Paper II**. Selection on a streamlined body is likely under selection for a small fish that are to swim over 25 Km to breed, and could also be linked to fast turns and predator avoidance, as discussed above. Taken together, the observed phenotypic differences observed between the salinity-linked populations in **Paper I** and **II** could be resulting from genetic factors building up without gene flow (Hendry et al, 2002; Jones et al, 2012b; Leinonen et al, 2011; McPhail, 1977); by phenotypic plasticity as they inhabit diverse habitats (McCairns and Bernatchez, 2012; Pfennig et al, 2010), or a combination of both factors. Phenotypic plasticity within developmental phases is however likely not the main cause for the observed differences in geometric shape, as "pure-bred" offspring's of the lagoon migrants would have lagoon genetics and freshwater morphometrics- given that the lagoon migrants are successful at the breeding site; a combination that was not found in the dataset for **Paper I**. Further, evolutionary constraints due to allometric scaling relationships could also explain the observed, overall restricted, differences in morphology, as most traits in **Paper II** were found to have diversified in common allometric trajectories, as was also found in a split-clutch breeding design of Norwegian sticklebacks from Glitredammen (Mazzarella et al, 2015) and from 74 wild caught Norwegian stickleback populations (Voje et al, 2013).

Being larger is also defence against predation due to gape-limited hunters, and the lagoon stickleback were uniformly longer than the freshwater population, as they were ~17% longer than the completely plated morph in freshwater. The lagoon stickleback have also been found to mature a year younger than the shorter freshwater fish (Narver, 1969). The size-differences may therefore be explained by increased growth potential in the brackish/marine environment relative to the freshwater environments in the Chignik system (Bond, 2013). Body size also appears to be an important trait for mate selection in sticklebacks (Albert, 2005; Conte and Schluter, 2012; McKinnon et al, 2004; Nagel and Schluter, 1998). The low hybridization rate observed **Paper I**, despite the large potential for gene flow indicates the presence of pre- or post-zygotic barriers to gene flow (De Cara et al, 2008). Positive assortative mating between conspecific members in areas where the anadromous and freshwater forms coexist have also been reported from other study sites (Hay and McPhail, 1975; McKinnon et al, 2004), and experimental studies have further indicated that body size alone could function as a mating signal between the highly morphologically different benthic and limnetic species pairs found in British Colombia (Conte and Schluter, 2012). The lagoon migrants were mostly females, having an average length of 8.0 cm to the ~ 6.0 cm average freshwater males- hence, one simple, speculative theory on a physical reproductive barrier could be the size of the freshwater male's nest not being big enough for the lagoon females, but this needs to be checked out further.

The genotyped *EDA*-intron alleles in both **Paper I** and **III** had a tight linkage to the lateral plated morphs, but there were also mismatches in the associations, as a few completely plated individuals in **Paper I** carried an *a* on both chromosomes and a few low-plated individuals carried *AA*. This is typically also found in other populations, and Colisimo et al. (2004) estimated that the *EDA*-gene explains about 80% of the recurrent plate loss in freshwater fish, implying that the complete reduction in plate morphs are also caused by other genetic loci with smaller

effects (Mazzarella et al, 2016; Morris et al, 2018) or other non-genetic factors, such as epigenetical changes (Trucchi et al, 2016). A specific *EDA*-genotype can further also give varied number of plates within each morph (Hansson et al, 2016). However, while there seemingly is a strong, direct selection on the plate phenotype, the *EDA*-gene also experiences additional selection, possibly from pleotropic effects of the *EDA*-gene itself, or on physically linked genes (Barrett et al, 2009b; Mills et al, 2014; Robertson et al, 2017).

When moving between salinities, the fish need to counterbalance their osmoregulation as to survive, and **Paper III** tested for putative associations between the three lateral plate morph originating in salt,- brackish and freshwater, and test their standard metabolic rate (MO_2) in the three water qualities (0‰, 15‰ and 30‰). Oxygen is essential for organisms to generate sufficient energy (ATP) to meet their metabolic demands, and the rationale for testing for an association between plate morph and plate number to oxygen consumption was that at the time, recent experiments had indicated different salinity preferences between the morphs (Barrett et al, 2008; Barrett et al, 2009b), and also potentially different morph growth rates when raised in a common salinity (Barrett et al, 2009a; Marchinko, 2009). Contrarily to our expectations, the results showed that all the plate morphs spent more or less the same amount of oxygen across the three experimental salinities, suggesting that the ability to thrive in different salinities is not under correlated selection with plate morph or plate numbers, and that the osmoregulatory robustness in threespine stickleback may somehow be evolutionary constrained. The result of **Paper III** strengthened previous findings of equal osmoreulatory demand in the gill's NA^+/K^+-ATPase activity for the same setup with saltwater and freshwater fish (Jurss et al, 1982), and similarly also when freshwater and brackish water populations were exposed to 0‰ and 20‰ (Schaarschmidt et al, 1999). Marine and non-migratory resident freshwater stickleback in yet another study did similarly not differ in standard metabolic rate, but showed significant evidence for difference in maximum metabolic rate and scope for activity (Dalziel et al, 2012). Respiration rates may vary among seasons (Meakins, 1975) and temperature regimes (Schaarschmidt et al, 1999), and stickleback populations originating from different salinities have been found to differ in oxygen consumption when tested in novel salinities (Kitano et al, 2010; Tudorache et al, 2007). The findings in **Paper III** hence supports the threespine stickleback being an osmoregulatory generalist. The ability to osmoregulate across salinities, seemingly without costs, is one of the factors that has enabled migrants to move more or less freely across salinity barriers, and thus maintaining potential for gene flow between populations, as in **Paper I**, unless other isolation mechanisms exist. The link between lateral plate morphs and osmoregulation was clearly tentative, as a set of complex metabolic and phenotypic traits could be selected simultaneously during the marine-freshwater transitions (DeFaveri et al, 2011; Jones et al, 2012b; Kitano et al, 2010), but at the same time, the stickleback must somehow regulate oppositely as to survive novel salinity exposure, and we would have expected that this would have been associated with a cost.

The threespine stickleback seem to have a large capacity for plastic adjustments. In **Paper IV** and **V,** the focus was on salinity-adaptive changes through genetic regulations, by exposing stickleback to saltwater and freshwater for nine time points, before studying the gene-expression for a few targeted genes in **Paper IV**, and through a transcriptomic approach for the six-hour exposure group in **Paper V**. Again, contrary to our predictions of opposite expression profiles in saltwater and freshwater exposed fish, the targeted osmoregulatory genes displayed no evident regulatory trends following abrupt salinity exposure across the three weeks, and

there were also surprisingly few transcriptome products that were significantly differentially regulated in the saltwater-comparison for the six-hour exposure in **Paper V**. The targeted gene-results presented in **Paper IV** were normalized by the use of two genes which were not to be affected by salinity; GADPH and EF1a, and by the first timepoint in the experimental setup, the five-minute exposure, as few regulatory changes would have made it to the mRNA-stage at that point. The transcriptomic data were not normalized in the same way, but the gene-products were filtered statistically, and many genes that would have been found to be significantly differently expressed in e.g. a GLM from the qPCR-results were filtered out based on a multiple testing and a false discovery rate of $q > 0.05$. However, when results from these two approaches are to be compared here, in this Thesis, I use the Cq-values from the qPCR-experiment in **Paper IV** as the basis for the comparison (**Figure 8**). And, as can be seen from the raw Cq-values plot, the trend in the qPCR-results changes from the published results in **Paper IV**. I will shortly exemplify the reason for this with the use of the gene *KCHN4*.

When in the qPCR, the gene-product of KCNH4 came up late (**Figure 8a, 10a**), potentially due to a failure in designing primers, but the low expression was also suggested from the transcriptomic data (**Figure 10b**). The expression-pattern for KCHN4 when the qPCR-data were normalized (as in the publication) did not express any large changes, except for the freshwater control group increasing in expression after 48 hours. However, when plotting the raw Cq-values here to compare with the transcriptomic data, the overall pattern is reversed, and the gene now has an overall larger expression in saltwater, as was also found for the transcriptomic data (however, the gene was filtered out before the analysis started due to low transcriptome numbers). Looking at the time-series plots for the control-genes (**Figure 8a**), the saltwater control has a higher value at the five-minute time point than the other groups, which were interpreted to correlate to a higher starting concentration in the saltwater -control samples, but it could also be the result of the reference-genes not being optimal (Barber et al, 2005). The effect of normalizing the Cq-values at later timepoints to the five-minute values was therefore to reduce the expression levels of the saltwater control by higher Cq-values than for the three other groups, for both reference genes (**Figure 8a**). By looking at the raw-data, the expression pattern is also more stable for the two control groups when compared to the experimental groups (**Figure 8**), a pattern that would be expected, as the controls do not change salinity. Also, the overall higher expression rates in the beginning of the three weeks in the raw-plots are perhaps to be more in line with a movement of "habitat" and raised awareness, an effect that drops of as time makes a habit of the situation the fish finds themselves in. I will discuss the results from **Paper IV** as they are presented in **Figure 10** in this Thesis, as this makes the results from **Paper IV** and **V** more directly comparable.

The overall trends when comparing the six-hour exposure for the Cq-values and the transcriptome data, is that the group-pattern is the same for the ATP1A3- and CFTR regulation, as the two genes are not displaying any major differences between any of the groups, and for KCNH4, where the saltwater fish has a higher average expression. The comparison of qPCR and transcriptomic data is more reversed for HSP70, where saltwater control goes from having the highest expression values in the qPCR-reactions, to the lowest in the transcriptomic sequencing, however, this gene was also filtered out early in the transcriptomic statistical analysis due to low sequencing numbers (signs of lower expression and less statistically significant results). Further, the fold change between the group comparisons for all four targeted genes were less than two, which is increasing the chances of non-concordant results

between the two methods; with 'non-concordant' defined as both approaches yielding differential expression in opposing directions, or one of the methods showing differential expression while the other does not (Everaert et al, 2017).

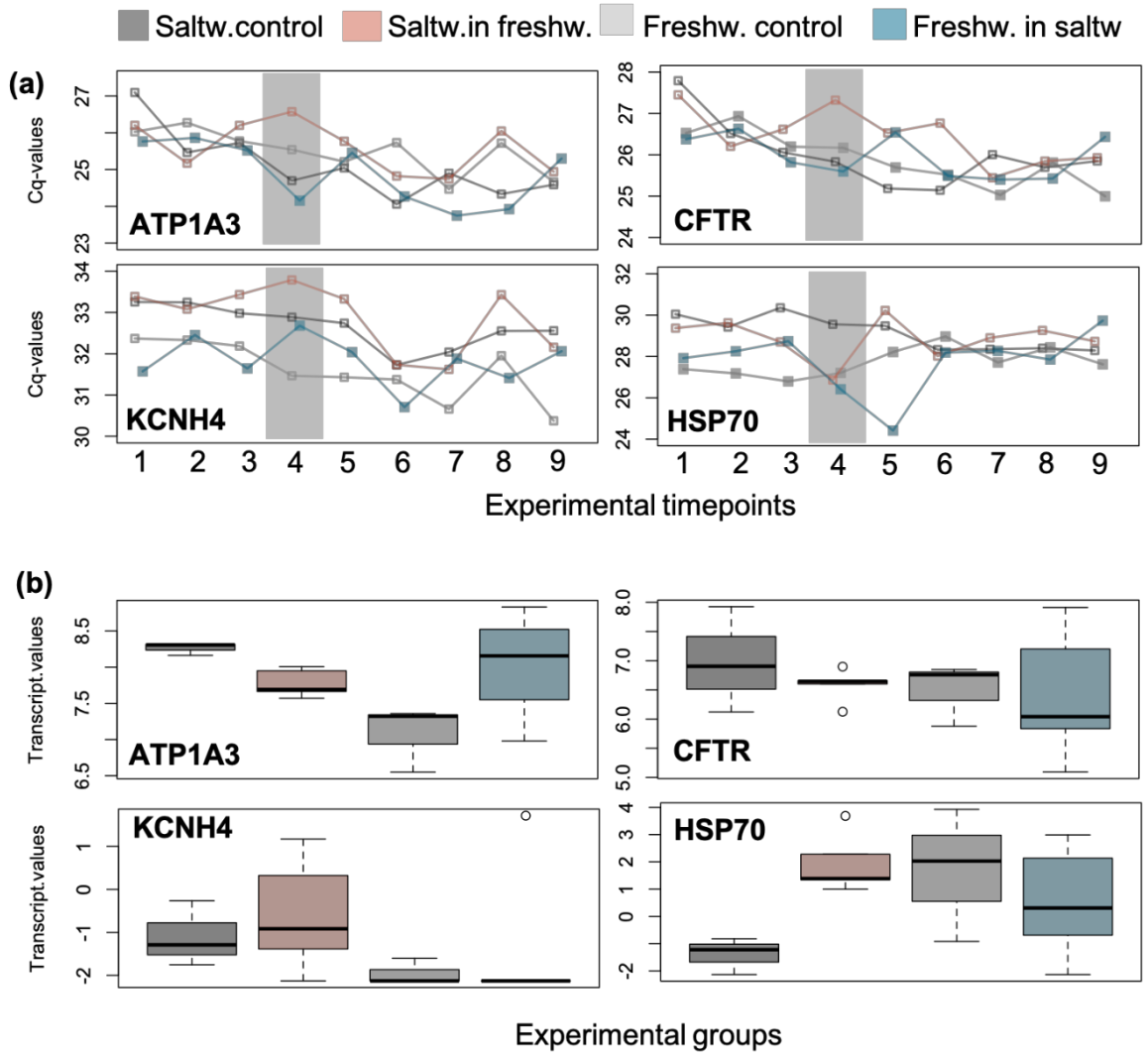

Figure 10.*Comparison of raw qPCR -values (Cq-values) and transcriptomic values for the same targeted genes as were applied in Paper IV. (a) Cq-values of mRNA-expression for the four targeted genes plotted for the four experimental groups (saltwater control, saltwater exposed to freshwater, freshwater control, freshwater exposed to saltwater). The plots express raw qPCR-values across the nine timepoints (1= 5 minutes, 2= 30 minutes, 3= 90 minutes, 4= 6 hours, 5= 24 hours, 6= 48 hours, 7= seven days, 8= 14 days and 9= 21 days).*

Overall, it was surprisingly few differences in the targeted osmoregulatory genes, and especially interesting that there were so little difference between the control groups, which in theory should have more opposite expression patterns. Choosing which genes to target in various experimental settings can to a certain extent be to pick at random- taking CFTR as an example, where results from the literature spans from CFTR not being expressed at all in Mosambique tilapia, *Oreochromis mossambicus* (Hiroi et al, 2005); diffuse in Killifish (Marshall et al, 2002); higher for lab-reared freshwater stickleback than lab-reared saltwater stickleback (McCairns

and Bernatchez, 2010); higher in transfer to saltwater for Atlantic salmon, *Salmo salar* (Singer et al, 2002) and eel, *Anguilla anguilla,* (Wilson et al, 2004); to no change in expression in striped bass, *Morone saxatilis* (Madsen et al, 2007), indicating an overall complicated involvement of CFTR in freshwater osmoregulation. However, the currently accepted model for active salt-secretion of NaCl by ionocytes in teleosts consists primarily of the cooperative action of three major ion-transporting proteins: Na^+/K^+- ATPase (NKA), the $Na^+/K^+/2Cl^-$-cotransporter (NKCC), and the CFTR (Marshall, 2011). In the model, the basolaterally located NKA transports three Na^+ outward in exchange for two K^+, creating a low intracellular [Na^+], and a highly negative charge within the cell; the Na^+ gradient is then used to transport Na^+, K^+ and two Cl^- into the cell through a basolateral NKCC; Cl^- then leaves the cells down an electrical gradient through an apically located CFTR and Na^+ is transported back outside the cells via NKA, and then leaves through a paracellular pathway between ionocytes and adjacent smaller cells known as accessory cells (Hiroi and McCormick, 2012). As CFTR is a passive transporter in this setup, with a potential to also "flip" direction in the cell-wall, the number of CFTR proteins might not be the most relevant protein to adjust for in osmoregulatory challenges, and the result of few osmoregulatory changes in **Paper III** might not be so surprising after all.

Capture, handling and crowding are all factors that can initiate stress response in fish, as can short-term fluctuations in the physical environment. Physiological responses to stressors are complex, but include increased activity of cellular defense mechanisms, such as the up-regulation of Heat shock protein-genes, part of the secondary responses to stressors (Moseley, 2000) and the involvement of HSP70 in the acclimation of fish to salinity changes has been well documented experimentally (Deane et al, 2002; Fangue et al, 2006; Larsen et al, 2008; Roberts et al, 2010). The targeted gene for HSP70 in **Paper IV** was filtered out due to low expression, but several subunits of HSP70 was significantly increased in freshwater fish exposed to saltwater in **Paper V**. One of the main cellular consequences of stress is protein damage and aggregation of unfolded proteins, and HSP70 assists in a wide range of folding processes for proteins, in membrane translocation of organelles and secretory proteins and controlling the activity patterns for many regulatory proteins (Mayer and Bukau, 2005). Further, HSP70 inhibits apoptosis by acting through a range of pathways, including increased resistance against apoptosis-inducing agents, such as tumor necrosis factor-α (TNFα), where a tumor necrosis factor alpha (tnfaip8l2b) also was upregulated in the saltwater exposed freshwater fish. On the other hand, the regulation of the heat shock factor (HSF2) was lower when compared to the freshwater control fish, which is a transcription factor that binds specifically to heat shock elements (Ostling et al, 2007), likely resulting in lower regulation of HSP-linked genes, which again could indicate that the secondary stress responses were slowing down, as expected (Barton, 2002; Tsui et al, 2012). Stress responses and genetic expression to stress is not straight forward, as was also exemplified in Knag & Taugbøl (2013), where two stages of initiated stress-responses started two stress-related expressions; glucose responded to the first stressor (confinement) and cortisol, HSP70 and HSP90 to the secondary stressor (exposure to produced water), but not with an increase in stress with the additional first stressor (confinement + produced water). The overall main take home message from **Paper IV** is that controlling for variation is only as good as the chosen housekeeping gene(s) and selecting relevant target genes is key for interesting results.

The results from **Paper V** shows that picking the right genes would not have been very easy for the saltwater comparison if the six-hour exposure are representative for the expressional

differences, as only ten transcripts were significantly differentially regulated between the saltwater control and freshwater exposure group. A significantly higher number of transcripts had altered regulations in the freshwater control and saltwater exposure group, and amongst the 1535 significant gene products, five were shared with the saltwater setup; arrdc3a, SLC16a9a, CYP1a, Dok4 and a gene with unknown function. *SLC16a9a* is a member of the monocarboxylate transporter family (MCT/ *SLC16*), and an additional two gene products of this family were upregulated in the saltwater treatment for the freshwater fish. MCT's are involved in H^+-linked transport of monocarboxylic anions (Verri et al, 2012), that again are linked to the level of carnitine and energy metabolism by the transport of long fatty acid chains, like lactate, into mitochondria for energy production and between cell-types. Fish gills are highly oxidative tissues, and oxygen requirements (*in theory*) increase with increasing salinities (Vijayan et al, 1996), which again increase the natural concentrations of both plasma and gill-cellular lactate (Mommsen, 1984; Sangiao-Alvarellos et al, 2003; Sangiao-Alvarellos et al, 2005). Lactate might hence be the primary candidate for rapid carbohydrate fuel in the gill tissue, for especially the saltwater fish in the early salinity responses towards freshwater. A total of 27 solute carrier transcripts differed in regulations within the freshwater setup, indicating that these cell-membrane transport proteins are important in the first phase of osmoregulatory changes. Also, as expected, the $Na^+/K^+/2CL^-$ cotransporter (*NKCC1*/ *SLC12a2*) had higher expression in saltwater, which also is consistent with findings in long term salinity exposures in stickleback (Gibbons et al, 2017), and other species of saltwater fish (Shaughnessy and McCormick, 2020). That *NKCC1* has a central role in salinity acclimation is further supported with no expression in gills of the salmonid grayling (*Thymallus thymallus*), which is a strict freshwater fish (Varadharajan et al, 2018).

Genetic assimilation occurs when a plastic ancestral trait becomes environmentally stable, resulting in a loss of plasticity (Lande, 1976). In **Paper V**, a transcript was categorized as assimilated if it had a significantly different expression between the two control groups, and if the exposed group did not differ from their controls. Many of the assimilated genes were linked to known osmoregulatory- and immune functions, and many gene-products linked to calcium showed assimilated expression profiles, such as several ATP6V's. Calcium availability has been implicated as one of the selective forces operating on the *EDA*-locus due to an increased demand for calcium in bone structures, and the ability to take up calcium from freshwater with a lower Ca^{2+}-concentration have likely been a selection factor in freshwater. ATP6Vs are subunits of a V-type proton, located at the basolateral membrane of mitochondria rich cells (Sun-Wada et al, 2004), where five V1 and two V0 subunits had a significantly higher expression in freshwater fish in **Paper V**, and an additional 11 subunits also had higher expression in freshwater, clearly indicating that this protein has been important for both freshwater and saltwater acclimation. Another gene linked calcium regulation that was overall upregulated in freshwater was *ANXA5,* which has an ability to bind around twelve Ca^{2+} ions and is exhibiting calcium channel activity in plasma membranes and in matrix vesicles (Lizarbe et al, 2013). ANAXA5 is also linked to pathogenic antigen responses, so it is unclear if ANXA5 has an immunological or osmoregulatory function in freshwater fish (or both).

Maintaining cell volume is critical during salinity changes and many tight junction proteins such as claudins and occludins were upregulated in both freshwater groups in **Paper V**. Aquaporin3a is typically being reported with higher expression in freshwater for euryhaline fish species (Cutler et al, 2007; Velotta et al, 2017), as was also found in **Paper V**, in addition to a slight

plastic change within the freshwater ecotype, as the saltwater fish exposed to freshwater had reduced expression. Aquaporin expression has been found to be involved in the meditation of osmoreception in the tilapia prolactin-secretion and gill chloride cell differentiation (Yan et al, 2013), and the DNA sequence for aquaporin in sticklebacks has previously been associated with positive selection between marine- and freshwater populations (DeFaveri et al, 2013b; Shimada et al, 2011), as has the gene expression patterns (Gibbons et al, 2017). Again, the results from the Norwegian setup indicates that stickleback-populations around the globe has evolved slightly different pathways to becoming osmoregulatory superfish, as *e.g.* Wang et al. (2014) identified *AQP4*, another member of the aquaporin family, as a salt-responsive gene in the kidneys of sticklebacks, whereas *AQP4* was filtered out due to low overall expression in the transcriptomic analysis in Paper V.

One of the gene ontologies (GO) with a significant number of annotated genes within the freshwater comparison was cilium organization- and assembly. The cilium is a complex organelle comprising over 600 proteins (Satir and Christensen, 2008), and primary cilia have increasingly been found to have diverse and important roles in key signalling pathways, as they function as sensory organelles that relays extracellular stimuli to intracellular signalling pathways, such as changes in osmolarity (Siroky et al, 2017). Amongst the 30 differentially expressed genes linked to this GO-term, five were intraflagellar transport (IFT) genes, genes that have been found essential for assembly, maintenance and function of sensory cilia, which was downregulated in freshwater fish exposed to saltwater, and TRPV1, which in general had higher expression in freshwater fish. In *Caenorhabditis elegans*, TRPV1 was one of two receptors important for sensing extracellular hypoosmotic stimulus localized in primary cilia (Tobin et al, 2002), and TRPV1 is also linked to influx of Ca^{2+} as a response to cellular osmotic stress (Hsu et al, 2019); to osmosensing for hormone regulation (Takei and McCormick, 2012) and forms synthetic ion channels during changes in cell volume (Sharif-Naeini et al, 2008).

Interestingly, one of the ten differentially regulated transcripts within saltwater was the enzyme GAL3ST1, that functions in catalysing the sulfation of membrane glycolipids, including the final step in the synthesis of sulfatide; a major lipid component of the myelin sheath that protect the nerve cells. GAL3ST1 is mainly found to be involved in meiosis, and in Paper VI, the freshwater stickleback's sperm was found to not activate in saltwater, whereas the sperm mobility was as high as in freshwater after the males had been acclimated in saltwater for two days. Although the comparison is speculative, since Paper V is targeting gene expression in the gills; gills also have ciliated epithelium present on the gill filaments, which are connected to nerve fibers (Jonz and Zaccone, 2009), and the sperm flagellum microtubule has structures similar to the ones found in cilia, so perhaps GAL3ST1 also could have a function in osmotic sensing and activation of salinity-dependent pathways. Details of sperm activation in stickleback are not known, and the sperm characteristics found in Paper VI seem more similar to those of internally fertilizing fishes, e.g. the sperm ceasing to move quite quickly in water regardless of salinity. In internal fertilizing fish such as ocean pout, *Macrozoares americanus L* (Alavi and Cosson, 2006) and spotted wolfish, *Anarhichas minor* (Yao and Crim, 1995), the milt contains highly active sperm that are immobilized immediately upon dilution in salt water, but stay active for days in the ovarian fluid of the females. In stickleback, the male builds a nest where the female deposits her eggs, which are covered in gelatinous ovarian fluid. The ovarian fluid contains a variety of ions (Elofsson et al, 2003b; Yao and Crim, 1995), and as the stickleback male spawns directly on the eggs, which are within the nest, the ejected sperm are exposed to

the ovarian fluid or a dilution, rather than directly exposure to the eggs/surrounding water. This is presumably increasing the longevity of the sperm cells by several hours (Elofsson et al, 2003b), clearly raising the potential fertilization success of the eggs, as successful fertilization of a stickleback egg clutch has been found to take several minutes (Bakker et al, 2006; Elofsson et al, 2006). However, it is still interesting that the sperm from freshwater males failed to activate when exposed to saltwater, but the sperm from the saltwater males could be activated in both salinities directly in Paper VI. If the results had stopped there, this could partly have explained why the numbers of low plated sticklebacks are rare in saltwater, and also why some freshwater males did seemingly not fertilize eggs well in saltwater (Marchinko and Schluter, 2007). However, the stickleback again showed off by putting on a plastic trait; as sperm from wild caught freshwater males acclimated in saltwater for only two days also could activate in both salinities directly. Phenotypic plasticity in sperm activation has also been observed in tilapia (Morita et al, 2003), where the range of osmolarity that activated sperm motility shifted higher and broadened as the acclimation salinity of the fish increased for Gulf killifish, *Fundulus grandis* (Yang and Tiersch, 2009) Spermatogenesis usually starts in adult stickleback males immediately after the reproductive season, meaning the sperm can be mature (thought to be a fixed state) for several months prior to spawning season (Morita et al, 2004). It is therefore quite surprising that the sperm of mature, wild caught freshwater stickleback had the ability to adapt to sudden changes in osmolarity and go from almost zero activation in saltwater when being adapted to freshwater, to having fully active sperm in saltwater after being acclimated to saltwater for only a few days. Therefore, sperm movement is also crossed off the list of potential barriers between completely plated saltwater and low plated freshwater populations of sticklebacks.

Conclusions

Salinity is a known barrier to gene flow for many fish species (Moyle and Cech, 1996). How the sticklebacks are able to be so plastic and how they abruptly handle everything from oxygen consumption, genetic regulation and sperm mobility when thrown into their non-native environmental salinity has still not been answered in this Thesis. Survival was high in all treatments for Paper III- V, where the variation in oxygen consumption and gene expression between the exposed fish were relatively small, especially between the saltwater fish in the two water qualities. This suggests that the capacity for osmoregulation in a wide range of salinity regimes has not been lost in either salinity population. The stickleback has assumingly originated as a marine species (Bell, 1977) and has repeatedly colonized freshwater habitat all over the northern hemisphere, clearly indicating salinity itself to not prevent gene flow between adjacent stickleback populations, as was also found to be the case in Paper I. However, as also found in Paper I and Paper II, sticklebacks have undergone phenotypic radiations after colonization of freshwater habitats (Klepaker, 1993a; McKinnon and Rundle, 2002), which has typically been linked to genetic changes (Colosimo et al, 2005; Jones et al, 2012b); an indication of selection favouring certain genotypes or traits in relation to salinity. The isolation by salinity, and the low numbers of heterozygotes between lagoon and freshwater sticklebacks, despite ample of opportunity found in Paper I, do however indicate some form of intrinsic genetic inculpabilities (Seehausen, 2006) and ongoing divergent selection, which may lead to increased reproductive isolation through reduced gene flow and ultimately to ecological speciation (Nosil,

2012; Schluter, 2000; Schluter, 2009). Adaptation to ecologically diverse environments have been found to facilitate reproductive barriers to gene flow (Rundle and Nosil, 2005), and natural selection against maladaptive hybrids favours reinforcement of premating isolation between sympatric species across taxa (Nosil et al, 2003; Singhal and Moritz, 2012), including stickleback (Rundle and Schluter 1998).

Phenotypes are often linked to environmental factors through reaction norms; a function that relates the environment to which a particular genotype is exposed and the phenotypes that can be produced by that genotype. Stickleback in Alaska were also found to diversify in phenotypic relations predicted from allometric scaling relationships, likely resulting from shared evolutionary history and constraints in the evolvability of the traits, restricting the phenotypic space for selection on traits associated with foraging, swimming ability and predator defense. However, there were small differences across salinity and lateral plate morphs in Paper II, but to what effect natural selection is operating on these traits, if indirectly or directly, is yet unknown. The fish from Paper I and II consisted of three lateral plate morphs, where the completely plated morph from the lagoon were phenotypically different from the completely plated morph in freshwater, and the low plated morphs had been present in the system since the 1960's. Fish predation is one of the theories that are at the top of the list of many stickleback researchers when naming the selective forces behind plate loss in freshwater, and fish predation could in theory also explain the dominance of completely plated fish in Paper I and II. However, the main potential fish predators in the system, Dolly Varden, *Salvelinus malma* and Coho salmon, *Oncorhynchus kisutch*, seem to rarely prey on sticklebacks (Bond, 2013; Roos, 1959; Ruggerone, 1992), and with the high abundance of stickleback in the system (Harvey et al, 1997), the overall predation pressure is likely very low. Further, most lakes across the northern hemisphere have trout or other predatory fish present, and one would therefore expect completely plated sticklebacks in freshwater to be more common if predation were the single explanation for evolutionary plate loss in these fish. The evolutionary loss of plates is also accompanied by a change in the lateral line sensory system (Mills et al, 2014; Wark and Peichel, 2010; Wark et al, 2012), suggesting that the loss of plates might be due to selection on the lateral line rather than the plates. It is thus still uncertain which selective agent(s) account for the high degree of completely plated stickleback in the freshwater system in Chignik.

Movement between environments of different salinity is considered to be highly physiologically costly and resident populations experiencing different salinity levels are predicted to be locally adapted to their native habitat, as traits promoting euryhalinity are expected to be rapidly lost if they are not under selection. It is likely that adaptation to the local environments takes some time, but a decrease in salinity tolerance for the freshwater fish would be expected since they likely colonized the sampled freshwater Glitredammen for more than 7000 years ago; giving selection between 3500 to 7000 generations, assuming a two year or one-year life cycle, respectfully. Further, recent literature does indicate that evolutionary change often is rapid (Hendry and Kinnison, 1999), and population genetic studies on stickleback have recognized salinity to be a major factor in the distribution of genotypes in systems that exchange migrants (McCairns and Bernatchez, 2008), and across high gene-flow environments such as in the Baltic ocean (DeFaveri et al, 2013b), indicating that adaption to salinity is under selection, despite not being a migration barrier.

Changes in gene expression can evolve very rapidly in fish (Roberge et al, 2008), and expression patterns of transcriptomes have been recognized as being plastic (Evans, 2015), meaning that genetically similar individuals can have different genetic expression profiles (phenotypes) as a response to environmental cues (Mäkinen et al, 2016; Papakostas et al, 2014), and could therefore play an important role in the early steps of population divergence (Wolf et al, 2010). The locally adapted saltwater stickleback in Paper V could osmoregulate in freshwater with almost no significant change in genetic expression, whereas the freshwater populations originating from marine environments back in the days, induced a large set of generegulations when exposed to saltwater. The saltwater fish in this study were collected from the Oslofjord, where they experience seasonal variation in salinity, due to periods of high freshwater influx from rivers after heavy rain and snow-melting. It is therefore even more surprising that the saltwater fish exhibited such low significant expressional plasticity when exposed to freshwater. Further, the genetic background for the saltwater fish is likely more diverse as fish can flush out from several rivers and lakes, increasing the likelihood of gene flow towards the marine environment. Having a more diverse genetic makeup can likely also lead to a higher variation in genetic expression (higher standard deviations), which again will impact the false discovery rates and estimations of significance between experimental groups when filtering on expressional differences as in Paper V. On the other hand, recent studies suggest that a reduced level of genetic diversity, likely found in the freshwater population due to genetic bottlenecs and genetc drift, can even increase the expessional diversity, and possibly buffer some of the potential loss from the adaptive potential given with a higher genetic variation (Liu et al, 2019; Mazzarella et al, 2015; Morris et al, 2014). The high ability to osmoregulate for both populations anyway indicates that for stickleback, the cost of retaining osmoregulatory plasticity is small, or that the traits promoting euryhalinity in stickleback are under strong selection or pleitropically linked to other traits under strong selection, perhaps to cilia movement and the salinity of the ovarian fluid?. However, for fish living in marine and freshwater environments, the selection pressure for osmoregulation is still opposite, indicating that the stickleback must have alternative cell-regulating mechanisms for survival in unfamiliar salinities. In Paper IV and V, the only targeted product were gene expression values extracted from gill tissue, however, the amount of mRNA does not necessarily imply equal concentrations of the functional proteins, as a number of mechanisms can limit or increase the final protein production. Protein expression profiles, also from kidney tissue, another highly relevant organ for osmoregulation, could have revealed different results. An alternative to differential expression of the could be a redistribution and reuse of proteins, as Marshall et al. (2002) illustrated movement of the protein from an apical location in saltwater to a more diffuse and basolateral location in freshwater. Structural changes like these would not have been picked up by ether qPCR in Paper IV or transcriptomic data in Paper V. Mechanical reorganizations could be how the stickleback deals with overall salinity-changes, as they express very little osmoregulatory stress (Paper III), they migrate long distances, clearly a heavy workout on its own for such a small fish (Paper I), not even including additional physiological stressors, and they also survive for a long period in their opposite water quality, where in fact the highest mortality were for freshwater fish in freshwater (Paper III). Whichever method(s) the stickleback are employing to osmoregulate so effectively, it has created a species that is incredibly adjustable to colonize new habitats regardless of the salinity they find themselves in, which has been a huge asset to this species in its spread throughout the northern hemisphere. Additional studies targeting the exact genetic and physiological mechanisms for the wide salinity tolerance in marine and freshwater stickleback are needed to understand the stickleback's incredible capacity for

alternations in ion secretion and absorption. Their ability to adapt immediately to the environmental demands, with no apparent increase in physiological stress, is as unusual as it is intriguing, especially as this has been so evolutionarily important for this widespread species.

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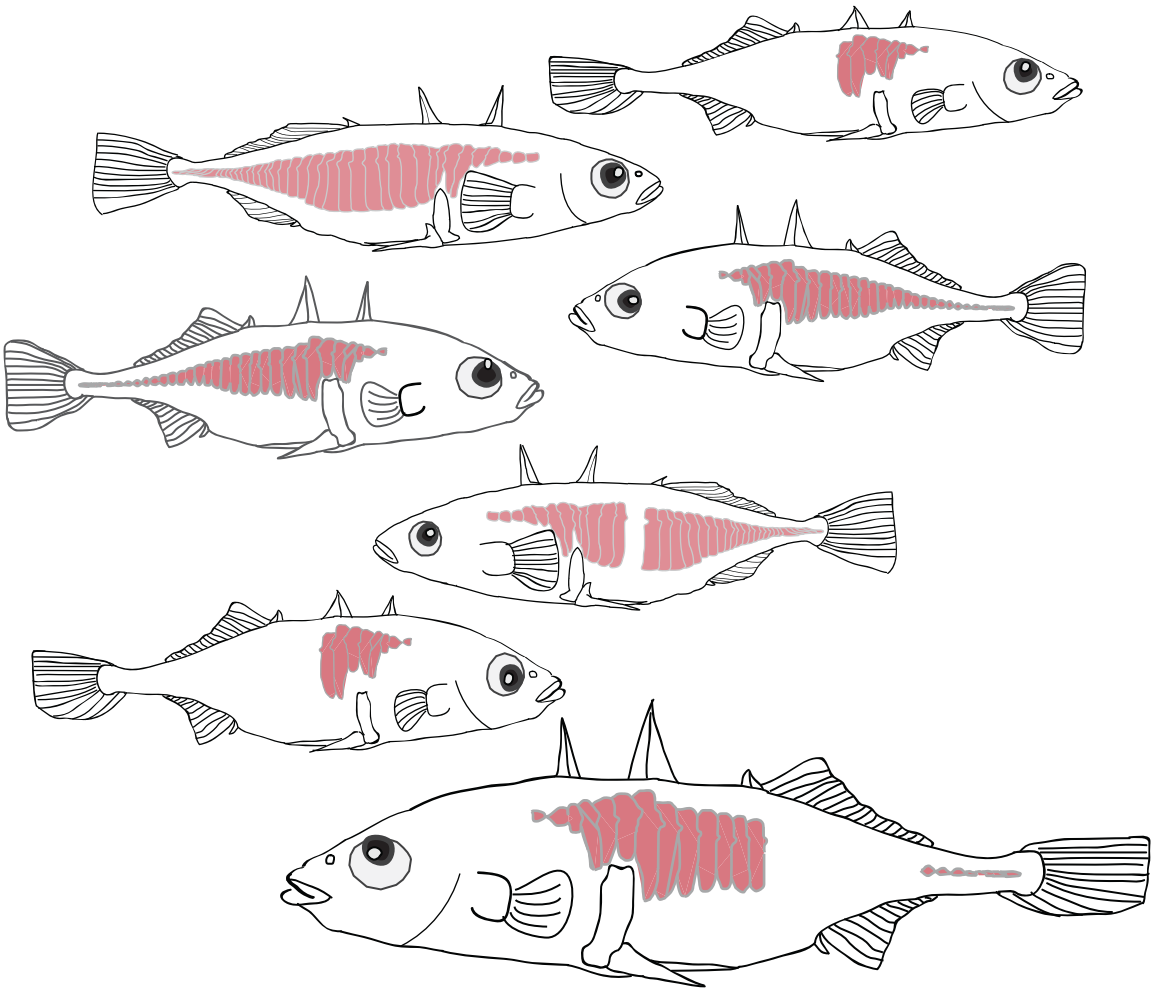

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Genetic and morphometric divergence in threespine stickleback in the Chignik catchment, Alaska

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Adaptation, hybridization, life-history polymorphism, microsatellites, phenotypic differentiation, population differentiation.

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Abstract

Divergent selection pressures induced by different environmental conditions typically lead to variation in life history, behavior, and morphology. When populations are locally adapted to their current environment, selection may limit movement into novel sites, leading to neutral and adaptive genetic divergence in allopatric populations. Subsequently, divergence can be reinforced by development of pre- or postzygotic barriers to gene flow. The threespine stickleback, *Gasterosteus aculeatus*, is a primarily marine fish that has invaded freshwater repeatedly in postglacial times. After invasion, the established freshwater populations typically show rapid diversification of several traits as they become reproductively isolated from their ancestral marine population. In this study, we examine the genetic and morphometric differentiation between sticklebacks living in an open system comprising a brackish water lagoon, two freshwater lakes, and connecting rivers. By applying a set of microsatellite markers, we disentangled the genetic relationship of the individuals across the diverse environments and identified two genetic populations: one associated with brackish and the other with the freshwater environments. The "brackish" sticklebacks were larger and had a different body shape than those in freshwater. However, we found evidence for upstream migration from the brackish lagoon into the freshwater environments, as fish that were genetically and morphometrically similar to the lagoon fish were found in all freshwater sampling sites. Regardless, few F1-hybrids were identified, and it therefore appears that some pre- and/or postzygotic barriers to gene flow rather than geographic distance are causing the divergence in this system.

Introduction

The genetic structure of contemporary populations is the result of both historical and current ecological and evolutionary processes. Habitats are often not stable over evolutionary timescales, and as environments change, organisms adapt, perish, or disperse. During the last ice age, much of the freshwater habitats in North America and Eurasia were inaccessible due to an extensive sheet of ice (the last glacial maximum was ~18,000 years ago (Clark et al. 2009)). As the ice retreated, new freshwater habitats became accessible and were colonized by fish and other freshwater organisms expanding from glacial refuges, either through migration corridors (rivers and lakes) or through coastal dispersal (Lindsey and McPhail 1986, 1986). Such coastal dispersal is also a contemporary process in some areas (Milner and York 2001; Milner et al. 2008). Dispersal through marine waters is especially prevalent for anadromous and euryhaline fishes, such as salmonids (*Oncorhynchus*, *Salmo*, and *Salvelinus* spp.) (Hendry et al. 2004) and the threespine stickleback, *Gasterosteus aculeatus*.

The threespine stickleback (hereafter stickleback) has invaded many young, postglacial habitats through coastal dispersal (Bell and Foster 1994; Klepaker 1995; Von Hippel and Weigner 2004) and is today found in a wide variety of marine, brackish, and freshwater environments (Wootton 1976; Bell and Foster 1994). Following freshwater invasion, they have diverged in many phenotypic traits compared to the ancestral marine ecotype (Bell 1977; Klepaker 1993; McKinnon and Rundle 2002), making it a model species in evolutionary biology. One phenotypic

trait that commonly differs between the marine and freshwater stickleback is body size; marine sticklebacks tend to be larger than those in freshwater (McPhail 1994), possibly resulting from a combination of environmental and genetic factors (Jones et al. 2012). Body size seems to be an important trait for mate choice for the stickleback (McKinnon et al. 2004; Conte and Schluter 2012), potentially functioning as a prezygotic barrier to gene flow between marine and freshwater fish. Another well-described divergent trait in stickleback is the number and location of lateral plates, which vary within and among populations (Hagen 1967; Narver 1969; Hagen and Gilbertson 1972; Hagen and Moodie 1982; Klepaker 1996). On the basis of the location of the plates, a stickleback can be assigned into one of three commonly recognized forms: complete-, partial-, and low-plated morphs (Wootton 1976). The different lateral plate morphs are typically found in different salinity environments, with the complete-, the partial-, and the low-plated morphs being associated with high, intermediate, and low salinity, respectively (Heuts 1947; Münzing 1963; Wootton 1976). Recent findings indicate that the repeated loss of lateral plates across different freshwater populations occurred as a consequence of parallel directional selection on one major locus, the *Ectodysplasin* (*Eda*) gene (Colosimo et al. 2005), as allele variants of this gene are strongly linked to the lateral plate morphs (Colosimo et al. 2005; Le Rouzic et al. 2011; Jones et al. 2012).

In this study, we investigated gene flow between stickleback populations inhabiting a southwestern Alaskan lagoon-river system (Fig. 1). Movement between environments differing in salinity is physiologically costly, and salinity gradients can therefore limit gene flow in fishes (Moyle and Cech 1996). Previous studies from this system showed that stickleback from the brackish lagoon were monomorphic for the completely plated morph, whereas the freshwater sites had all of the three lateral plate morphs. Further, the completely plated stickleback in the marine lagoon differed from the freshwater fish by having more lateral plates and a more developed keel (Narver 1969). The fish in the opposing environments were also reported to have different life histories; the lagoon population matured at one year of age and bred in *Zostera* (eel grass) belts in the brackish water and in the lower parts of the freshwater habitat, whereas the freshwater fish bred in freshwater habitats at age two (Narver 1969). Apparently, all these fish die after breeding as no older age classes were recorded for either population (Narver 1969). Intensive upstream spring migrations between lakes have been observed (Narver 1969; Harvey et al. 1997), indicating that there are no physical barriers to migration and hence potential for gene flow between the different environments. In this system, the differences in morphology and life history could have evolved due to reduced gene flow, in combination with different adaptations to the ecological environments as natural selection can generate phenotypic and genetic differences between populations (Schluter 2000, 2009; Nosil 2012). Alternatively, there could be one large, diverse population with some individuals that migrate between habitats, and variation in growth potential (likely higher in marine than freshwater environments) and selective predation on low plate morphs in marine waters causing the observed differences in body size and shape.

The main goals of this study were to (1) examine the genetic population structure and integrity within the study system (i.e., determine how many distinct populations are present and identify potential hybrids) and (2) determine how size and shape differ between fish from the brackish and freshwater habitats. We screened 14 neutral microsatellite markers and tested for genetic relationships in fish sampled at four different locations – a brackish water lagoon, a river, and two lakes in the Chignik system in Alaska (Fig. 1). Subsequently, we tested for phenotypic differences in body shape among the detected groups based on 30 digitized landmarks. Our analyses revealed both migrants and hybrids between two well-defined genetic populations, and it was therefore particularly interesting to test for morphological differences between these individuals and the resident, nonhybrid individuals from the freshwater and lagoon habitats.

Materials and Methods

Study area and stickleback collection

Adult threespine sticklebacks (length >4 cm, when all the lateral plates are fully developed (Bell 1981)) were collected from the Chignik Lake system of southwestern Alaska (56°25′ 40″N, 158°75′ 60″W) (Fig. 1A,B), using beach seines (35 × 4 m, 3 mm mesh), tow nets (1.8 × 2.7 m), and fyke nets (1.22 m^2 frame with 3–5 m wings). At each of four locations, the fish were collected from a single site, with sample sizes between 78 and 122 individuals. All sampling was conducted in the two last weeks of June 2009, within the breeding season for stickleback. The sampled fish were stored in 95% ethanol.

The sample locations were all areas where sticklebacks are very abundant: Chignik Lagoon, Chignik Lake, the Black River, and Black Lake (Fig. 1B). The Chignik Lagoon is a semi-enclosed estuary ranging about 12 km from Chignik Bay up to the Chignik River. Depending on the location in the lagoon and the stage of the tide, the salinity ranges from 0 to about 30‰ (Simmons et al. 2012). Tidal amplitudes that exceed 3 m can expose half the estuarine substrate, largely covered by eelgrass

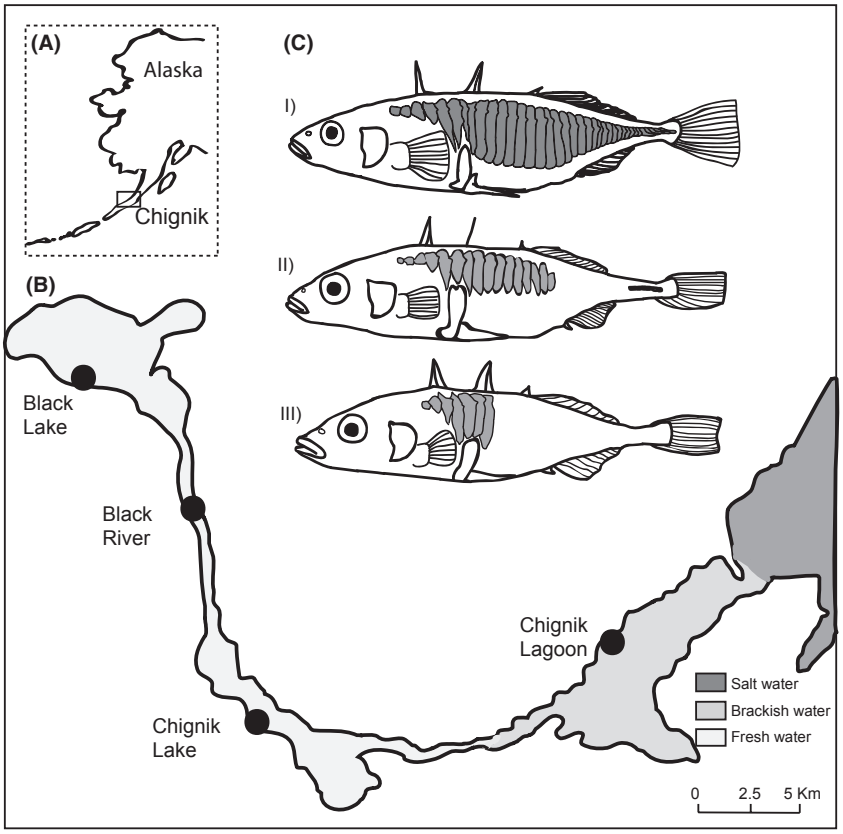

Figure 1. Study area. (A) Map of Alaska showing the position of the Chignik Lake system. (B) Locations of the four sampling sites, the graded color is according to salinity. (C) Figure of the threespine stickleback morph variation found within the Chignik system, I) completely, II) partial, and III) low plated.

(*Zostera spp*.). The sample was collected from a site in the middle of the lagoon, between the outlet of the Chignik River and the sand spit that separates the lagoon from the more oceanic Chignik Bay. The Chignik River (7.2 km long) drains Chignik Lake (22 km^2), a deep lake (maximum depth of 64 m) with a shoreline dominated by gravel. The Black River (12 km) connects Chignik Lake to Black Lake, which is larger (41 km^2) but shallower (maximum depth 4 m) than Chignik Lake. Black Lake rapidly warms up in the spring and is highly productive with abundant vegetation and provides good breeding habitat for threespine stickleback (Narver 1969). The fish communities of these two lakes are dominated numerically by threespine sticklebacks and juvenile sockeye salmon, *Oncorhynchus nerka* (Westley et al. 2010). The main fish predators are juvenile coho salmon (*O. kisutch*) and Dolly Varden (*Salvelinus malma)* (Roos 1959; Narver and Dahlberg 1965; Ruggerone 1992).

DNA extraction, PCR amplification, and genotyping

Genomic DNA was extracted from a pectoral fin from each fish using the salt-extraction method developed by Aljanabi and Martinez (1997). A total of 14 potentially neutral and two quantitative trait loci (QTL) microsatellite markers (Appendix S1) were genotyped for 389 individuals; 104 from the Chignik Lagoon, 122 from Chignik Lake, 85 from Black River, and 78 from Black Lake. This set of markers was selected to identify potential genetic structure within or across the populations and to discriminate plate morphs (stn382) and sex (idh). Each PCR had a total volume of 6 μL, where each mixture contained 1–5 ng of genomic DNA, 1 × *Q* multiplex PCR solution (Qiagen, Hilden, Germany), and 1 pmol of each primer. The forward primers were fluorescently labeled based on their fragment lengths and the complete multiplex (Appendix S1). The PCR profiles for the 14 neutral markers were divided up into three multiplexes and consisted of 95°C for 15 min, followed by 37 cycles of 94°C for 30 sec, 59°C for 90 sec, 72°C for 60 sec, an extension step at 60°C for 30 min and a final extension step at 20°C for 10 min. The PCR products were diluted, and 1 μL of that dilution was added to a mixture of 10 μL formamide and 0.125 μL allelic size standard (LIZ 500 bp, Applied Biosystems, ABI, Foster City, CA) for electrophoresis on a 3730 DNA Analyzer (ABI). The software GENEMAPPER (ABI) was used to analyze the individual alleles through visual inspections and manual corrections. Neutrality was checked for all the 14 microsatellites in LOSITAN (Beau-

mont and Nichols 1996; Antao et al. 2008), testing both the stepwise mutation model and the infinite allele model using 5000 simulations at a false discovery rate of 0.1. For two of the microsatellites, stn309 and stn319, a weak signal of positive selection was detected for both models (F_{ST} 0.053 and 0.043 for stn309 and stn319, respectively), but including or excluding these markers did not qualitatively change the results of the population genetic structure (data not shown), and they were kept in the dataset as neutral markers for all the analyses.

The two quantitative trait loci *stn382* and *Idh* were run in simplexes. 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 (Colosimo et al. 2005). The homozygous "AA" is mostly associated with the completely plated, the "Aa" mostly with the partial plated, and the "aa" mostly with the low-plated morph. The amplification reactions for this locus were performed as described in Colosimo et al. (2005). As this marker has two alleles only, with fragment lengths of either 151 ("a") or 218 ("A") base pairs (bp), the individual genotype could be visualized on a 2% agarose gel. Fragment size was verified with a size standard (Generuler, Fermentas) and internal gel controls for the three genotypes. Sex determination of the fish was carried out genetically, using the *Idh* locus (Peichel et al. 2004). Two alleles are recognized, where females are homozygous for one of the alleles (allele size 302 bp), while males are heterozygous (allele sizes 271 bp and 302 bp). The alleles were also separated on a 2% agarose gel with internal positive controls.

Population genetic structure analysis

Using sampling sites as proxies for "populations" might give a false impression of the actual population structure, especially if dispersal between sites is common or if multiple populations occupy a site. As sticklebacks have been observed migrating between lakes and rivers in the Chignik system (Harvey et al. 1997), we used a genetic self-assignment test to allocate all sampled individuals back to an unknown number of genetic clusters ("populations") using the program STRUCTURE 2.3 (Pritchard et al. 2000, 2007). By running STRUCTURE without a priori sampling information, the program clusters individuals based on their allele frequencies alone by identifying putative groups in the data that minimize departure from Hardy–Weinberg equilibrium (HWE). We first ran an initial analysis in STRUCTURE, with correlated allele frequencies and LOCPRIOR (Hubisz et al. 2009), to test for the number of separate genetic units ($K = 1$ to $K = 6$; set manually) in our total sample. The admixture model probabilistically assigns each individual to one or more clusters (K) and estimates the proportion of ancestry (Q) to each cluster (ranging from zero to one). Values of Q can subsequently be used to assign individuals to genetic clusters irrespective of their sampling locations. We ran five independent analyses for each value of K, using 700,000 iterations (following a burn-in period of 500,000) (Pritchard et al. 2000). The number of K that best fits the data is estimated by comparing the log likelihood of the data given the number of clusters ($\ln P(X|K)$) (Pritchard et al. 2007). As using $\ln P(X|K)$ criteria can lead to an overestimation of population numbers (Pritchard et al. 2007), we also examined the second-order rate of change of $\ln P(X|K)$ (ΔK), which is a more conservative approach (Evanno et al. 2005). Output files obtained from STRUCTURE were graphically summarized using R (R Development Core Team 2011). After running STRUCTURE on all the data ($n = 389$), it was evident that $K = 2$ gave the best fit, clearly separating the lagoon fish from most of the fish sampled from the freshwater sites. We also analyzed subsets of the data to further verify that $K = 2$ was the model that best fitted the data (for the three freshwater sites individually in addition to all fish from freshwater pooled).

To detect migratory individuals, the fish were separated into lagoon or freshwater fish, based on whether their sampling site was brackish or fresh, and analyzed for putative migrants and individuals with recent immigrant ancestry using the assignment test implemented in STRUCTURE 2.3 (Pritchard et al. 2000). This test is a fully Bayesian method that uses sampling location as a prior when assigning the fish as migrants or admixed (hybrid) individuals. The program assumes a user-specified probability (v) that corresponds to the likelihood of an individual being a migrant. To be conservative, we applied $v = 0.05$ to our study, which corresponds to each individual having a 5% chance of being a migrant or having mixed ancestry. The model was run under the assumption of correlated allele frequencies among populations using a burn-in of 500,000 followed by 700,000 iterations. For all subsequent analyses, we assigned individuals as lagoon, migrants, hybrids, or freshwater fish, on the basis of their Q-value and migratory assignment from the STRUCTURE cluster at K_{max}=2 (termed genetic population), in addition to using sampling sites directly for comparison.

To assess the population patterns and to characterize how differentiated the stickleback are in this region, we investigated the genetic diversity within and between the four sampling sites and the two genetically defined populations described earlier, including the individuals with recent migratory life history and putative hybrids. Genetic diversity (number of alleles per locus and sample), linkage

disequilibrium (LD) of the markers, Hardy–Weinberg equilibrium (HWE), and observed and expected heterozygosity were calculated using Arlequin (Excoffier and Lischer 2010). Tests for significant deviations from HWE were performed for each locus and population. The p-values were estimated without bias using a Markov Chain (MC) random walk, following the algorithm of Guo and Thompson (1992), implemented in Arlequin (Excoffier et al. 2005). The MC parameters were set to default values, and corrections for multiple tests were performed by applying sequential Bonferroni corrections (Rice 1989). To compare the genetic differentiation between sampling populations and sampling populations excluding the migrant individuals, we calculated pairwise F_{ST} values for all pairs of populations using 10,000 permutations, and a significant level of $\alpha = 0.05$ in the population comparison test implemented in Arlequin 3.5 (Excoffier and Lischer 2010).

Morphological analyses

Fork length was measured to the nearest mm, and the lateral plates were counted directly on both sides of the body of each fish. The fish was classified as a complete-, partial- or low-plated morph according to Münzing (1963). To better recognize and place homologous landmarks (see below), each fish was stained in alizarin red (modified protocol after Dingerkus and Uhler (1977)), and a digital photograph was taken on the left side of each individual. The photograph was taken at a standardized distance, and a ruler was placed in each photograph for scaling. Females with bulky abdomens were excluded from the shape analysis. Further, the staining method also makes the fish very stiff, and some individuals were fixed in unnatural positions, making it hard to analyze their shape. After removing such individuals, 267 fish were analyzed for geometric shape variation.

To quantify geometric body shape variation in the genetically assigned stickleback populations, we placed 30 digitized landmarks on each picture (Fig. 2) using tpsDIG2 (Rholf 2005). The digitalized landmark positions were analyzed with MorphoJ (Klingenberg 2011), and figures were plotted in R (R Development Core Team 2011). We visualized the differences between the predefined groups by the use of a canonical variates analysis (CVA). CVA is a method that first performs a principal component analysis (PCA) of the pooled within-group variation to construct a coordinate system in which the position of each group can be positioned. After rescaling the axis proportionate to the elongation of the average fish, the program solves for the direction in which the fish seems to be farthest apart in the rescaled space by performing a PCA on the group centroids, producing the canonical variates (CVs). The scores of individuals on the CVs are the projection of the individuals onto these new coordinate axes (Zelditch et al. 2004). As all deviations from the centered data are expressed in the same metrics, it is possible to quantitatively visualize the shape change associated with a given principal component using warped outline drawings. As males and females may differ morphologically (Kitano et al. 2007), we tested for variation in shape within and between sex and genetic populations by extracting and plotting the two first axes of the CVA.

Results

Fish length and plate numbers at sampling locations

There were large differences in length and plate numbers among the four sampling sites (Table 1, Fig. 3). The fish from the lagoon were significantly larger (>1 cm on average) than fish from the freshwater sites ($F_{3,\ 385} = 184$, $P < 0.001$). Further, 82% of all sampled individuals were females (92%, 66%, 80%, and 93% females in Chignik Lagoon, Chignik Lake, Black River, and Black Lake, respectively). The females were on average 1 cm longer than the males ($F_{1,\ 368} = 45.93$, $P < 0.001$), and this pattern was seen at all sites. The fish sampled in the lagoon were all completely plated, whereas all three morphs were found in freshwater. Among the completely plated individuals, the lagoon fish had more plates than those collected in fresh water (an average of 61.5 in Chignik Lagoon and 60.9 in fresh water, after adjusting for length; $F_{2,\ 255} = 76.72$, $P < 0.001$). The freshwater samples contained high proportions of completely plated fish, even at the upper-most site, (Black Lake: 61%; Chignik Lake: 58%; Black River: 46%), and there were very few low-plated fish. There was no difference in plate number between the two sexes. Further, there was a tight linkage between the three *Eda* genotypes and the lateral plated morphs (Fig. 4); 78% of the low-plated individuals were aa, 66% of the partially plated were Aa, and 95% of the completely plated individuals were AA. Analyzing the data for either variable gave similar results, and therefore, only morph information was used as an explanatory variable in the subsequent statistical analyses.

Descriptive statistics and population structure

The expected heterozygosity across the 14 neutral microsatellite loci varied between 0.582 and 0.944, with an average of 0.860, and the observed heterozygosity varied between 0.606 and 0.980, with an average of 0.827 (Appendix S2). There was no indication of linkage

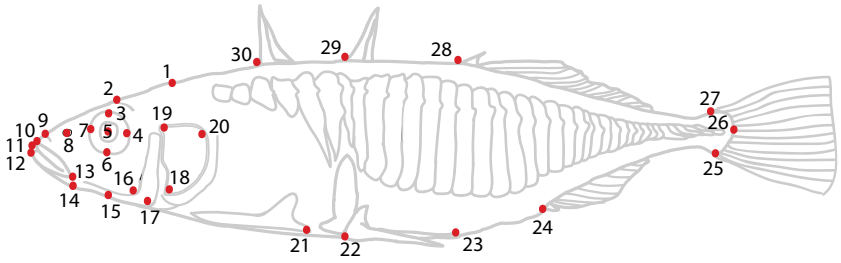

Figure 2. Outline of a threespine stickleback showing the locations of 30 landmarks (numbered circles) used to measure shape differences. The landmarks refer to: (1) Section between the frontal and the supraoccipital bone, identified as a small lowering in the head; (2) eye-brow; (3–7) eye; (8) nostril; (9) Maxilla; (10–12) anterior upper, middle, and lower lip; (13) posterior end of mouth; (14–15) outline of ventral jaw; (16) posterior jaw; (17) posterior preoperculum; (18–20) operculum; (21) posterior end of ectocoracoid; (22–23) anterior and posterior end of posterior process; (24) origin of anal spine; (25) insertion of ventral caudal fin ray; (26) posterior tail/end of vertebrae; (27) insertion of dorsal caudal fin ray; (28) origin of third spine; (29) origin of second spine; (30) origin of first spine.

Table 1. Mean length (cm) and plate number distributions for both males (M) and females (F) for completely (C), partially (P) and low plated (P) phenotypes at the four different sampling locations, in addition to their assigned genetic populations.

	Sample sites											
	Chignik Lagoon (n = 104)			Chignik Lake (n = 122)			Black River (n = 85)			Black Lake (n = 78)		
	C	P	L	C	P	L	C	P	L	C	P	L
Length												
M	7.2	–	–	5.5	4.8	5.0	6.6	5.9	6.0	6.5	–	6.0
F	8.2	–	–	5.9	5.3	5.5	7.1	6.1	5.8	7.0	6.2	6.7
#Plates												
M	67.8	–	–	65	55	14.2	67	50.5	12.7	65.2	–	14
F	66.9	–	–	65	55	14.4	65.3	51.9	13.2	65.6	51.6	12.8
	Genetically assigned individuals											
	Chignik Lagoon (n = 104)			Migrants (n = 35)			Hybrids (n = 17)			Freshwater (n = 234)		
	C	P	L	C	P	L	C	P	L	C	P	L
Length												
M	7.2	–	–	7.36	–	–	–	–	–	5.5	5.5	5.4
F	8.2	–	–	8	–	–	6.8	6.2	–	6	5.9	5.9
#Plates												
M	67.8	–	–	67	–	–	–	–	–	64.9	52.3	13.6
F	66.8	–	–	67	–	–	65.8	58	–	64	52.59	13.4

disequilibrium between any pairs of loci in any of the sample populations after Bonferroni corrections ($P > 0.05$). Four loci deviated from HWE for some sampling sites, also after Bonferroni corrections; however, the pattern was not consistent across all population comparisons (Appendix S2). Therefore, all the 14 neutral microsatellite markers were used in all analyses.

The likelihood value $\ln P(X|K)$ for each of the STRUCTURE runs without a priori sample information was highest for $K = 2$, indicating the presence of two genetic populations in this system. Visual inspection of the values indicated low variance for the replicated runs of $K = 1$, 2, and 3 and increasing variance for $K = 4$, 5, and 6 (Fig. 5). Additional evaluation of ΔK (Fig. 5), and plotting individual Q values (Fig. 6) confirmed that $K = 2$ captured the major genetic structure in the dataset.

Migrants and genetic differentiation among populations

The STRUCTURE analysis for the detection of first-generation migrants and individuals with mixed ancestry

identified 35 (32 females and 3 males) first-generation migrants from the lagoon in freshwater sites (7 in Chignik Lake, 12 in Black River, 16 in Black Lake), and 16 females with mixed ancestry (one in the lagoon, six in Chignik Lake, four in Black River, and five in Black Lake (Fig. 6)). This genetic identification of migrants and F1 hybrids was consistent with the morphological data. The length distributions of the migrants and lagoon fish did not differ, but the identified hybrids and the freshwater fish were on average 1.3 and 2.3 cm shorter than those in the lagoon, respectively ($F_{3,\ 385} = 292.7$, $P < 0.001$). All migrants were completely plated and had similar plate numbers as the lagoon fish, whereas completely plated hybrids and completely plated freshwater fish had an average of 0.8 and 1.7 fewer plates, respectively (ANCOVA with length as covariate; $F_{3,\ 253} = 60.5$, $P < 0.001$).

The STRUCTURE analysis revealed the presence of two genetic populations in the system, but F_{ST} tests indicated that the samples from the four sites were all significantly different from each other (Table 2; F_{ST} values from 0.003 to 0.046). The level of differentiation was highest between fish from the Chignik Lagoon and the Black River ($F_{ST} = 0.036$), rather than between fish from the Chignik Lagoon and Black Lake ($F_{ST} = 0.028$) as would have been expected in an isolation-by-distance scenario. When the individuals classified as migrants from the brackish environment were removed from the three freshwater samples, the level of differentiation between the lagoon sample and the respective freshwater samples increased (Table 2).

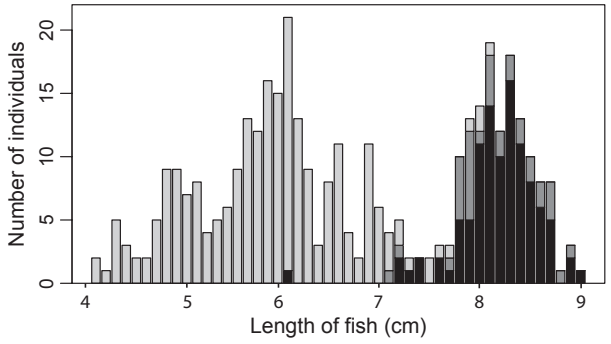

Figure 3. The length distribution for the fish sampled in freshwater (gray) and the lagoon (black). Light gray indicates a freshwater fish, dark gray indicates migrants; fish sampled in freshwater but with a genetic signature as a lagoon fish.

Geometric shape analysis

Using 30 digitized landmarks on morphological traits (Fig. 2), we extracted geometric-morphometric information for the sticklebacks. As CVA analyses the relative positions of the groups in the sample, the method requires that the individuals be grouped before the analysis begins. We grouped the fish in two sets, one set including males and females from the lagoon and freshwater, excluding the identified migrants and hybrids (Fig. 7A), and another set including only female fish classified as either being lagoon fish, migrants, hybrids, or of freshwater origin (Fig. 7B). The comparison between the two sexes coming from the lagoon and freshwater clearly separated i) the two populations on the first axis (CV1) and ii) the two sexes on the second axis (CV2) (Fig. 7A). The lagoon fish had more streamlined bodies with thinner heads, smaller eyes, and more upward-pointing mouths (Fig. 7C) compared with the freshwater fish, and the females had more shallow bodies compared with the males. Visualizing the females separated into genetic populations also showed a clear separation of fish with a

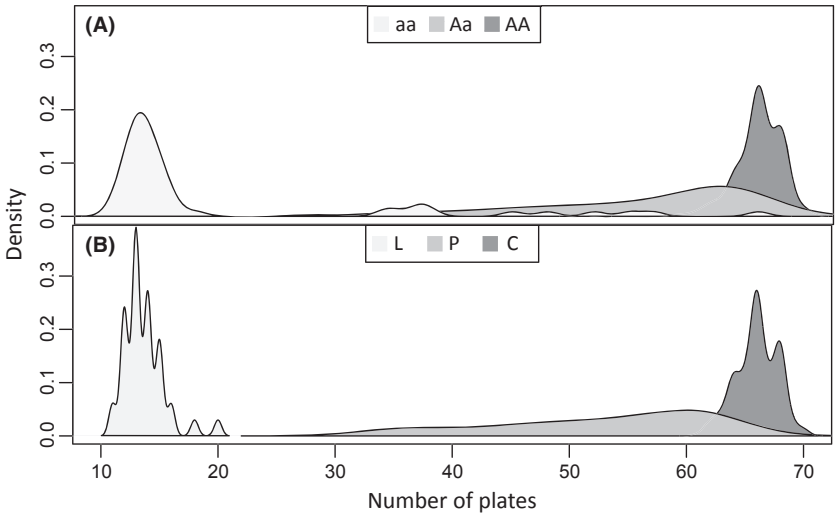

Figure 4. The frequencies of (A) the three *eda* genotypes: aa, Aa, and AA, and (B) the three morphs: complete (C), partial (P) and low (L) plated, in relation to total number of plates. As the separation of morph closely follows the distribution of the *eda* genotypes, only morph was used as an explanatory variable in the statistical analysis.

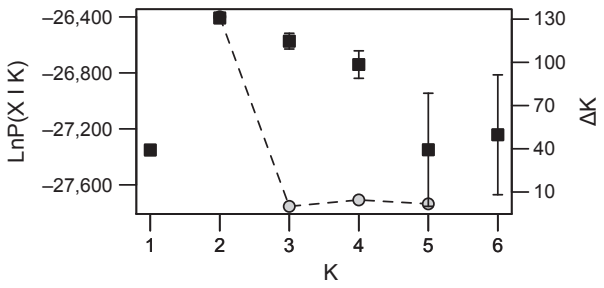

Figure 5. Interpretation of the number of genetic clusters (*K*) estimated in STRUCTURE. Both the likelihood of the data, lnP(*X*|*K*) (dark squares), and the standardized second-order rate of change of lnP(*X*|*K*), the Δ*K* (gray circles), are plotted as a function of the assumed *K* (1–6) for each run. Each *K* has been run five replicated times, and the error bars for the lnP(*X*|*K*) indicate standard deviations.

genetic signature from the lagoon and the freshwater environments (Fig. 7B) with the identified migrants grouping with the lagoon and the identified hybrids resembling both populations. The freshwater fish had larger eyes and a more bulky shape compared with the fish from the lagoon and the migrants (Fig. 7D). There was no evident separation between the three lateral plate morphs in geometric shape (results not shown).

Discussion

The threespine stickleback in the Chignik system clustered into two distinct genetic populations: one associated with the lagoon environment and the other with the freshwater environments, indicating a significant barrier to gene flow at the freshwater–lagoon interface. Fish with a lagoon genetic signature were, however, commonly found in freshwater (5% of all samples in Chignik Lake, 14% in the Black River, and 20% in Black Lake, the uppermost site), but no fish with a distinct freshwater genetic signature was found in the Chignik Lagoon. We interpret these results as indicating that the main direction of gene flow in this system mirrors the evolutionary history of sticklebacks (i.e., from marine to freshwater habitats), rather than following the passive downstream direction. However, without more extensive sampling, especially at different locations in the lagoon and at different times of the year, this conclusion is tentative.

Genetic variation and differentiation

Significant pairwise F_{ST} values were found between all four samples. However, the differentiation between the three freshwater samples was low, and when comparing all freshwater samples to the Chignik Lagoon sample, the F_{ST} values indicated very limited gene flow between these two environments. Moving between water with different salinities is costly for most fish, and salinity can therefore be a barrier to gene flow (Moyle and Cech 1996). However, the stickleback originated as a marine species (Bell 1977) and has repeatedly colonized freshwater habitats all over the northern hemisphere (Bell 1977), indicating that salinity itself does not prevent gene flow between adjacent stickleback populations differing in salinity levels (Grøtan et al. 2012). However, rapid parallel phenotypic radiations after colonization of freshwater habitats (Klepaker 1993; McKinnon and Rundle 2002) indicate that selection favors certain traits in the different environments. The differences between brackish water and freshwater stickleback observed in this study are consistent with other studies on sticklebacks. In a recent study from the Baltic Sea, absent of obvious physical barriers, the stickleback diverged in accordance with local differences in salinity (DeFaveri et al. 2013). Moreover, McCairns and Bernatchez (2008) studied threespine stickleback populations in the open St. Lawrence River system in Canada and found that the genetic differentiation (although weak) correlated more with salinity than with geographic distance. Thus, adaptation to different salinities may act as a barrier to gene flow after colonization occurs.

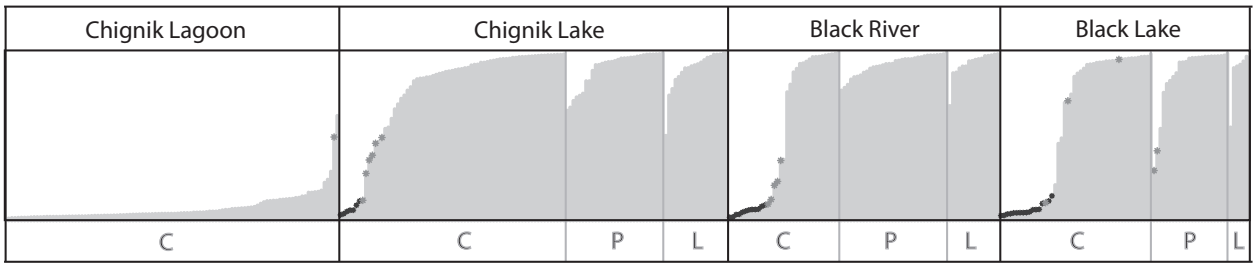

Figure 6. Summary plot of individual estimates of *Q*, where *Q* is a quantification of how likely each individual is belonging to each group (*K*) under consideration (here *K* = 2). Each vertical line is one individual where the two colors represent individual membership to each cluster *Q*. black and grey dots indicate individuals identified as a first-generation migrant and as F1 hybrids, respectively. Sample sites are shown at the bottom, and the fish have been sorted based on location, morph (complete- [C], partial- [P] and low [L] plated) and *Q*-value.

Table 2. Pairwise F_{ST} values between the sampling sites (above diagonal; dark gray) and between the sampling sites excluding the migrant individuals (below diagonal; light gray).

	Chignik Lagoon	Chignik Lake	Black River	Black Lake
Chignik Lagoon		0.034***	0.036***	0.028***
Chignik Lake	0.037***		0.005***	0.004***
Black River	0.046***	0.006***		0.004**
Black Lake	0.039***	0.003*	0.004**	

Significant pairwise comparison is indicated by ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

Morphology

Freshwater colonization events have led to many changes in morphology between ancestral marine and derived freshwater sticklebacks (Bell 1977; McKinnon and Rundle 2002). The lagoon fish were significantly larger than the freshwater fish, consistent with findings from other studies showing that marine stickleback in general are larger than stickleback in freshwater (Wootton 1976; McKinnon et al. 2004). In the Chignik system, juvenile sockeye salmon utilizing similar habitats (the lagoon and the two freshwater lakes) also show increased growth rate and larger average overall body size in the lagoon (Simmons et al. 2012). These size differences may therefore be explained by increased growth potential in the marine environment relative to freshwater environments in the Chignik system (Bond 2013). Although there might be some biases associated with the local sampling, the size differences between freshwater and lagoon samples were marked and also consistent with work in the 1960s (Narver 1969).

Sticklebacks from the two environments differed in geometric shape. There are many examples of parallel morphological evolution in sticklebacks after colonizing freshwater habitats (McKinnon and Rundle 2002; Adachi et al. 2012) where the derived morphological variation is assumed to be adaptive (Bell 1977). Resource use during ontogeny influences morphology in stickleback populations (Day et al. 1994; Day and McPhail 1996; Kristjansson 2005) as well as in other fish species (Torres-Dowdall et al. 2012) and birds (Badyaev et al. 2002). Adaptation to benthic and limnetic food resources in many different species leads to specially adapted morphotypes (Schluter

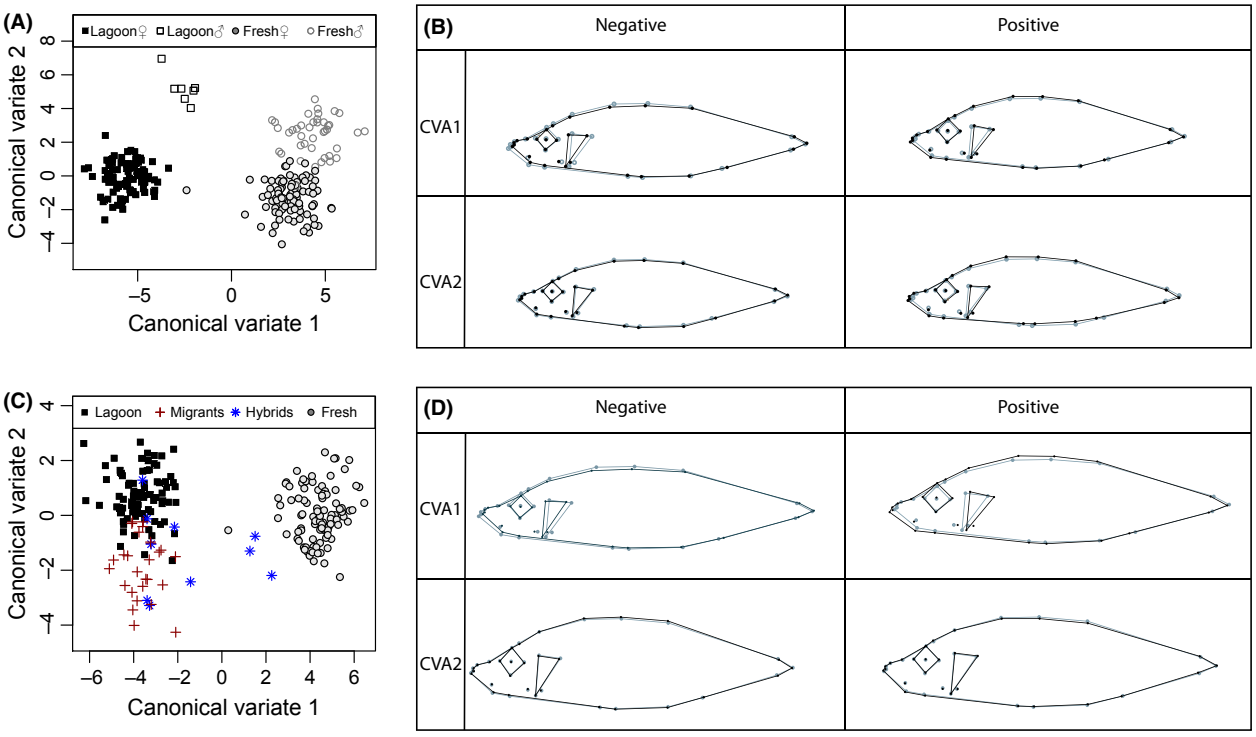

Figure 7. Geometric shape. (A) CVA scores of geometric shape for lagoon males (white squares), lagoon females (black squares), freshwater males (white circles), and freshwater females (gray circles); (B) The geometric shape changes for the two CVA axis, gray lines representing the average fish, black lines representing the landmark shifts associated with the vector values; (C) CVA scores for the genetically assigned populations, lagoon (black squares), migrants (red crosses), hybrids (blue stars), and freshwater (gray circles); D) the geometric shape changes for the two CVA axis.

and McPhail 1992; Bernatchez 2004). Benthic and limnetic morphotypes are also common in the threespine stickleback (Larson 1976; McPhail 1992, 1994), and although the differentiation along this benthic–limnetic axis is generally continuous, a few stickleback populations have diverged into sympatric populations (species pairs) that feed exclusively on one prey type or the other (McPhail 1984). We have no direct evidence that the morphological divergence of the Chignik stickleback is driven by differential adaptation to food types. However, fish from the Chignik system seem to follow the benthic–limnetic divergence as the lagoon fish were more streamlined and had smaller heads than the more bulky freshwater fish (Schluter and McPhail 1992).

The observed phenotypic variation in this system could be resulting from genetic factors (McPhail 1977; Hendry et al. 2002; Leinonen et al. 2011; Jones et al. 2012) as the two populations are genetically differentiated, by phenotypic plasticity (Pfennig et al. 2010; McCairns and Bernatchez 2012), as they inhabit different habitats, or a combination of both factors. In a similar study spanning marine and fresh water environments McCairns and Bernatchez (2012) raised offspring in reciprocal salinities and found that most of the phenotypic divergence observed in the two original populations resulted from plastic responses to the environmental salinity rather than genetic differences in body shape. While we have no data on the underlying causes of morphological variation observed in these populations, it is likely that both genetic differentiation and plasticity are causing the observed geometric-morphometric shape differentiation in the two populations.

Potential pre- and postzygotic barriers to gene flow

Divergent selection in different environments may lead to reproductive isolation through reduced gene flow and ultimately to ecological speciation (Schluter 2000, 2009; Nosil 2012). Hybridization and exchange of genes occur when allopatric species come in contact, or when reproductive isolation barriers break down between diverging species that still lack intrinsic genetic incompatibilities (Seehausen 2006). The two genetic stickleback populations in the Chignik system are differentiated morphologically, but there is potential for gene flow between populations as evidenced by individuals apparently of lagoon origin present in freshwater during the spawning period. However, the estimated level of hybridization was low; only 4.3% of the fish sampled in freshwater were genetically identified as F1 hybrids. This percentage is lower than reports from other hybrid zones; hybrid proportions of 46% and 33% were detected in the hybrid zones of Little Campbell River and River Thyne, respectively (Hagen 1967; Jones et al. 2006). However, in those studies, hybrids were identified based on lateral plate morphology alone (hybrids between completely plated marine and low-plated freshwater sticklebacks are usually partially plated), and this might underestimate the actual number of hybrids, as all the hybrids identified in the Chignik system were completely plated or overestimate the number of hybrids, as not all partially plated fish are hybrids (this study; Hagen and Moodie 1982).

The low hybridization rate observed in this study indicates the presence of pre- or postzygotic barriers to gene flow (De Cara et al. 2008). Adaptation to ecologically diverse environments can restrict gene flow between populations (Rundle and Nosil 2005), and natural selection against maladaptive hybrids reinforces premating isolation between sympatric species across taxa (Sætre et al. 1997; Rundle and Schluter 1998; Nosil et al. 2003; Singhal and Moritz 2012; Yukilevich 2012), including stickleback (Rundle and Schluter 1998). Phenotypically divergent populations inhabiting different ecological environments can experience selection against dispersers moving between them, limiting gene flow by mate preferences for similar phenotypes.

Body size (Nagel and Schluter 1998; McKinnon et al. 2004; Albert 2005; Conte and Schluter 2012) and shape (Head et al. 2013) appear to be an important trait for mate selection in sticklebacks and could be important also for the Chignik populations as they differ greatly in body size; both females and males from the lagoon were significantly larger than the freshwater fish. Positive assortative mating between conspecific members in areas where the migratory and resident freshwater forms coexist has been reported (Hay and McPhail 1975; McKinnon et al. 2004), and recent experiments indicated that body size alone functions as a mate signal between the morphologically different benthic and limnetic species pairs found in British Colombia (Conte and Schluter 2012), as could well be the case with the Chignik sticklebacks.

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Data Accessibility

Microsatellite, morphological and landmark data information: doi:10.5061/dryad.t0b62.

Conflict of interest

None declared.

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Supporting Information

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

Appendix S1. Table with descriptive statistics for the loci used to determine genetic background, *eda*-genotype and sex in this study.
Appendix S2. Table with descriptive statistics for all individuals sampled at each sampling site and for the genetically assigned populations (see Materials and methods).

A. Taugbøl, T.P. Quinn, K. Østbye & L.A. Vøllestad. 2020. Allometric relationships in morphological traits associated with foraging, swimming ability, and predator defense reveal adaptations toward brackish and freshwater environments in the threespine stickleback. Ecology and Evolution, 10: 13412-13426.

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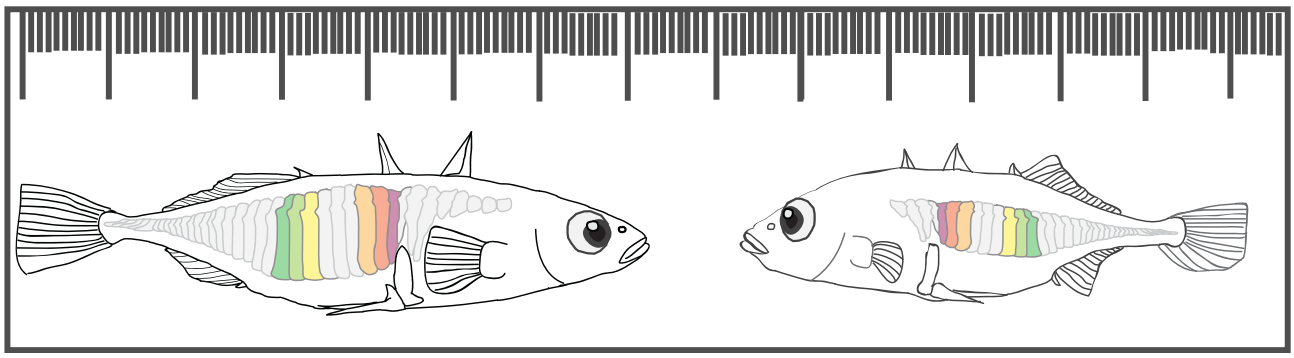

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ORIGINAL RESEARCH

Allometric relationships in morphological traits associated with foraging, swimming ability, and predator defense reveal adaptations toward brackish and freshwater environments in the threespine stickleback

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Abstract

Freshwater colonization by threespine stickleback has led to divergence in morphology between ancestral marine and derived freshwater populations, making them ideal for studying natural selection on phenotypes. In an open brackish–freshwater system, we previously discovered two genetically distinct stickleback populations that also differ in geometric shape: one mainly found in the brackish water lagoon and one throughout the freshwater system. As shape and size are not perfectly correlated, the aim of this study was to identify the morphological trait(s) that separated the populations in geometric shape. We measured 23 phenotypes likely to be important for foraging, swimming capacity, and defense against predation. The lateral plate morphs in freshwater displayed few significant changes in trait sizes, but the low plated expressed feeding traits more associated with benthic habitats. When comparing the completely plated genetically assigned populations, the freshwater, the hybrids, the migrants and the lagoon fish, many of the linear traits had different slopes and intercepts in trait-size regressions, precluding our ability to directly compare all traits simultaneously, which most likely results from low variation in body length for the lagoon and migrant population. We found the lagoon stickleback population to be more specialized toward the littoral zone, displaying benthic traits such as large, deep bodies with smaller eyes compared to the freshwater completely plated morph. Further, the lagoon and migrant fish had an overall higher body coverage of lateral plates compared to freshwater fish, and the dorsal and pelvic spines were longer. Evolutionary constraints due to allometric scaling relationships could explain the observed, overall restricted, differences in morphology between the sticklebacks in this study, as most traits have diversified in common allometric trajectories. The observed differences in foraging and antipredation traits between the fish with a lagoon and freshwater genetic signature are likely a result of genetic or plastic adaptations toward brackish and freshwater environments.

KEYWORDS
allometry, *Gasterosteus aculeatus*, lateral plates, morphology, spines

1 | INTRODUCTION

When dispersing into new environments, novel resources, competitors, and predation regimes are agents of natural selection that may lead to new phenotypic optima (Schluter, 2000; Schluter & Conte, 2009). In general, the evolutionary and phenotypic response to such new selection regimes, or environmental conditions, will likely be contingent upon the genetic diversity of the founders, gene flow, and the evolvability of traits. For aquatic organisms, the transition from marine to freshwater habitats represents a considerable change in biotic and abiotic selective forces, and few species make this shift and retain populations in both environments. A fundamental question in evolutionary biology is how morphological traits are shaped and maintained in the environment. One way of studying environmental effects is hence to compare populations of the same species inhabiting marine to freshwater environments.

The threespine stickleback fish (*Gasterosteus aculeatus*, hereafter termed stickleback) is an attractive model organism for studying adaptation to marine and freshwater environments. The stickleback inhabits a wide range of salinities and varies extensively in morphology, both within and among populations (Klepaker, 1995; Lucek et al., 2010; McKinnon & Rundle, 2002). All present freshwater populations are believed to have descended from marine ancestors (Bell, 1981), and the numerous independent postglacial invasions of freshwater habitats have resulted in parallel evolution of a set of predictable phenotypes. The best known example of these changes in the stickleback is the repeated evolutionary loss of lateral plates in freshwater populations (Bell & Foster, 1994).

The shape and size of fishes are affected by both genetic (Arnegard et al., 2014) and plastic (environmental) factors (Day et al., 1994; Wimberger, 1992). Experimental studies indicate, for instance, that salinity alone can account for a large amount of the observed morphological shape differences between marine and freshwater stickleback (Mazzarella et al., 2015). Other studies indicate that food type and habitat complexity can also explain much of the morphological divergence, as stickleback feeding on benthic prey typically have smaller eyes, larger mouths, and deeper heads (McGee et al., 2013; McGee & Wainwright, 2013). The limnetic form, however, which preys on smaller organisms such as zooplankton, has a more streamlined body (Hart & Gill, 1994; McPhail, 1984, 1992). The differentiation along this benthic-limnetic axis is usually continuous, but it sometimes results in populations or morphs of stickleback that feed almost exclusively on one or the other prey type (Gross & Anderson, 1984; Lavin & McPhail, 1986). Intermediate phenotypes tend to have reduced fitness, promoting ecological speciation (Nosil, 2012; Schluter, 1994).

The stickleback is small (<10 cm) and is preyed upon by a wide range of predators (Reimchen, 1994). As predation is a significant selective force that usually differs with salinity, it promotes multiple types of antipredator adaptions, including changes in morphology, behavior, and life history (Brönmark & Miner, 1992; Magurran, 1990). Having more bony lateral plates, as found in the marine stickleback, increases the probability of survival following an attack from a predatory fish (Hagen & Gilbertson, 1972; Reimchen, 1991, 1992). Therefore, a reduction of piscivore predation pressure in fresh water has been one of the hypotheses suggested to explain lateral plate reduction in freshwater stickleback. Freshwater populations are typically of the low-plated morph, but completely plated stickleback do occur in fresh water (Hagen & Moodie, 1982; Kitano et al., 2008; Leinonen et al., 2012), either as resident individuals, or as anadromous fish breeding there (Harvey et al., 1997; McKinnon & Rundle, 2002; Taugbøl et al., 2014). The anterior plates are seemingly under strong selection, as few populations completely lack plates in the head region, but nonplated sticklebacks do exist (Deagle et al., 2013; Klepaker, 1995; Mazzarella et al., 2016). The anterior plates protect against puncturing injuries and also buttress the dorsal and pelvic spines, that, when erect, increase the effective size of the stickleback (Hoogland et al., 1957; Reimchen, 1983). The spines probably also function as a warning to gape-limited piscivores (Hoogland et al., 1957). In areas where predators are common, the sticklebacks spines are often significantly longer than in areas where predators are absent or sparse (Hagen & Gilbertson, 1972; Zeller et al., 2012).

There are many documented examples of morphological evolution in freshwater populations after colonization by a marine ancestor, where the similar freshwater phenotypes are interpreted as a result of natural selection causing the populations to adapt to the new environmental conditions. Despite numerous studies, there is still no conclusive evidence for any major selective drivers of the phenotypic variations (i.e., shifts in diet, competition, predation, or a combination of these factors). Assuming that all freshwater lineages originated from the same ancestral population (Bell & Foster, 1994), it can be hypothesized that descendant freshwater populations inherited similar genetic architectures and developmental constraints and that there are allometric limitations to the evolvability of the traits. The stickleback included in this study is part of a subsample from a brackish water gradient in Chignik, southwestern Alaska (Figure 1), where we documented two genetically differentiated populations, one in brackish water and one in fresh water, with a few hybrids (Taugbøl et al., 2014). Individuals from the two genetically assigned populations also differed in size and geometric body shape. All individuals assigned to the brackish water population were completely plated (Figure 1), and some of these individuals were also found in fresh water as migrants. The individuals assigned to the freshwater population were either of the completely, partially, or low-plated morph. None of the assigned freshwater individuals were found in brackish water. As size and shape are only imperfectly correlated, and their correlation is determined by the allometric relationships

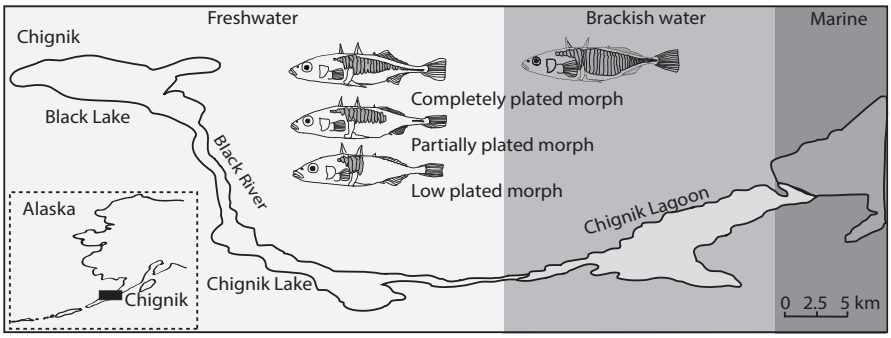


FIGURE 1 Study area and stickleback morphs. Inserted map of Alaska showing the position of the Chignik Lake system and locations of the four sampling sites as points within Chignik. The graded color is according to salinity: light gray being freshwater, gray being brackish water, and dark gray being marine salt water. The figure also illustrates the three stickleback morphs in the system, and where they were sampled. The completely plated morph has a full row of plates on both sides of the body, the partially plated have a gap in the row of plates, and the low plated has plates in the anterior region only, providing structural support for the pelvic- and dorsal spines. The illustrated fish are exemplified based on pictures of the morphs in the system

of the various body parts (Schlichting & Pigliucci, 1998), this paper aims to investigate allometric relationships in several ecologically important metric (linear) traits known to be important in the stickleback (Aguirre et al., 2008; Dalziel et al., 2012; Leinonen et al., 2006; Sharpe et al., 2008; Walker, 1997). We measured traits associated with feeding (head shape), swimming (body shape), and predator defense (size of dorsal and pelvic spines; size and width of lateral plates), to test for differences in linear traits in (a) completely, partially, and low-plated fish genetically assigned to the freshwater population, and (b) in individuals assigned to the different genetic populations and their hybrids. We did this by using principal component analyses (PCA) to extract the most important traits that separated the groups and illustrated the differences with plots of allometric scaling relationships.

2 | MATERIALS AND METHODS

2.1 | Study area and fish community

Fish were collected from one site in each of four locations from the Chignik Lake system in southwestern Alaska (56°25'40"N, 158°75'60"W) (Figure 1); Chignik Lagoon, Chignik Lake, Black River and Black Lake. The Chignik Lagoon is an estuary ranging about 12 km from Chignik Bay up to the Chignik River. Depending on the location in the lagoon and the stage of the tide, the salinity ranges from 0 to about 30 ‰ (Simmons et al., 2013). Tidal amplitudes that exceed 3 m can expose half the estuarine substrate, largely covered by eelgrass (*Zostera spp.*). The Chignik River (7.2 km long) drains Chignik Lake (22 km^2), a deep lake (maximum depth of 64 m) with a shoreline dominated by gravel. The Black River (12 km) connects Chignik Lake to Black Lake, which is larger (41 km^2) but much shallower (maximum depth 4 m) than Chignik Lake. Black Lake rapidly warms in the spring and is highly productive with abundant vegetation and provides good breeding habitat for threespine stickleback (Narver, 1969). The fish communities of the two lakes are dominated numerically by threespine sticklebacks and juvenile sockeye salmon, *Oncorhynchus nerka* (Westley et al., 2010). The main potential fish predators in the lakes are juvenile coho salmon (*O. kisutch*) and Dolly Varden (*Salvelinus malma)* (Narver & Dahlberg, 1965; Roos, 1959; Ruggerone, 1992). The main fish predator in the lagoon is Dolly Varden. Analysis of the content of 3,000 stomachs of juvenile coho salmon from a survey in the 1990s found no stickleback (Ruggerone, 1992). Similar samples of Dolly Varden in the lagoon indicated that they consume primarily invertebrates such as amphipods, and when they eat fish it is mostly sand lance, *Ammodytes hexapterus* (Bond, 2013; Narver & Dahlberg, 1965; Roos, 1959). The system is also inhabited by freshwater sculpins *Cottus aleuticus* and *C. cognatus* (Quinn et al., 2012). Although few studies have investigated whether these sculpin species prey on stickleback, their congener, the prickly sculpin, *C. asper*, feed on sticklebacks (McPhail, 2007; Miller et al., 2015), and different sculpin species tend to have very similar diets (Brown et al., 1995). There are also a number of bird species in the area (Narver, 1970), many of which may prey on stickleback (Reimchen, 1988, 1994; Whoriskey & FitzGerald, 1985).

2.2 | Stickleback collection

Adult threespine sticklebacks were collected using beach seines (35 × 4 m, 3 mm mesh), tow nets (1.8 × 2.7 m), and fyke nets (1.22 m^2 frame with 3–5 m wings) during the two last weeks of June 2009. The sampling was done during the breeding season for sticklebacks. After collection, the fish were stored in 95% ethanol. We measured fork length to the nearest mm in the laboratory and discarded fish under 4 cm as all bony plates might not be fully developed until the fish reaches this size (Bell, 1981). Also, as the fish in this study were not aged, discarding individuals under 4 cm should leave only fish that was older than 1 year of age (Rollins, 2017; Wootton, 1976). All fish were stained in alizarin red using the modified protocol after Dingerkus and Uhler (1977), and all the lateral plates were counted directly on both sides and classified to morph according to Wootton

(1976). After further discarding fish that had acquired an unnatural body curve due to storage and staining, the total sample sizes were 91 from Chignik Lagoon, 73 from Chignik Lake, 72 from the Black River, and 27 from Black Lake.

2.3 | Categorization of fish

All the sampled fish were genotyped for 14 neutral microsatellites and a sex-linked marker (see Taugbøl et al., 2014 for further details) that clearly separated the fish into two main genetic clusters; one comprised all the individuals sampled in the lagoon (hereafter called lagoon fish; $n = 92$), and one included fish sampled across the three freshwater sites (hereafter freshwater fish; $n = 136$). In addition, 33 individuals sampled in fresh water were genetically identified as belonging to the lagoon population (hereafter called migrants), and 11 individuals were identified as first-generation hybrids between the two genetic populations. None of the fish sampled in the lagoon was genetically assigned to the freshwater population. We do not know whether the lagoon fish represent marine stickleback, or whether they have a different life history and constitute their own gene pool, as we do not have any samples from local oceanic stickleback. All fish from the lagoon, the migrants, and the hybrids were completely plated, whereas in fresh water, 55 were completely plated, 57 were partially plated, and 27 were low-plated. As only 19% of the sampled individuals were males and morphological differences between sexes are known for stickleback (Aguirre et al., 2008; Kitano et al., 2007), we therefore only focus on the females (Table 1).

2.4 | Morphological measures of metric traits

Four types of morphological traits were measured: (a) traits reflecting head shape and thus important for feeding; (b) spine traits important as defense against predators; (c) traits important for swimming/movement, and (d) lateral plate traits important as defense against predators. All fish were placed on a piece of clay to reduce bending and tilting and were photographed on the left side from directly above using a digital SLR-camera (10 Mp) with a macro lens. A total of 37 landmarks were placed on the completely plated fish, whereas only 29 landmarks could be placed on the low- and partially plated fish (Figure 2) using tpsDIG2 (Rholf, 2005).

TABLE 1 Stickleback sorted in groups based on their genetic composition and morphology, the number of individuals in each group (N), length (in cm ± standard deviation, sd), and total plate numbers on the right side of the fish. The completely plated morphs in freshwater are included in both datasets

Group	*N*	Length ± sd	Plate numbers ± sd
Low plated	16	5.78 ± 0.71	6.87 ± 0.89
Partial plated	41	5.81 ± 0.69	26.64 ± 4.46
Completely plated/ Freshwater	35	5.71 ± 0.81	32.31 ± 0.82
Hybrids	8	7.13 ± 1.25	33.0 ± 1.22
Migrants	28	7.98 ± 0.40	33.5 ± 0.72
Lagoon	84	8.10 ± 0.39	33.60 ± 0.66

Information from the landmark configuration (Figure 2) was used to extract several linear measurements/traits for each fish: 15 traits for the low- and partially plated fish and 24 traits for the completely plated fish. All linear measurements were extracted using R version 3.6.1 (R Development Core Team, 2011). We extracted five traits from the head region for all fish (traits 1–5 in Figure 2a): mouth size (1), eye diameter (2), head length (3), head depth (4), and operculum size (depth) (5). To analyze for variation in traits potentially important for swimming performance (Dalziel et al., 2012), we extracted three traits (traits 6–8 in Figure 2a): body depth (6), caudal area (7), and peduncle width (8). We further extracted six traits related to the dorsal and pelvic spines from all fish (traits 9–14 in Figure 2a): length of spine one (9), distance between spines one and two (10), length of spine two (11), distance between spines two and three (12), length of the pelvic support structure (13), and length of the pelvic spine (14). For the completely plated fish, we also measured nine linear traits associated with the plates (traits 15–23, Figure 2b): the height of six plates, (traits 15–20) and the width of three combined plates (traits 21–23) (Figure 2b). We also calculated a proxy for plate coverage by dividing the length of each plate on body depth (trait 24, not illustrated).

2.5 | Data analysis

All statistical analyses were done in R (R Development Core Team, 2011). The first dorsal spine (trait 9) was excluded from all analyses as the tip of the spine often was submerged in the clay material that kept the fish stable, and the pelvic spine (trait 14) was also excluded as it was challenging to place the landmark correctly. This left 13 traits that could be compared for all fish, and an additional 9 traits that could be compared for the completely plated fish only (see Figure 2 for details on the traits). We tested for differences between lateral plated morphs in the freshwater population; between the lagoon and freshwater completely plated; and between completely plated fish genetically assigned to either population or their hybrids in three separate analysis (Table 1). As the low-plated morph differed slightly in the freshwater fish comparison, we only included the completely plated morphs in the freshwater and lagoon comparison.

Since evolutionary allometry is the log–log regression of the mean trait size on mean body length across populations, we used standardized major axis regression (Warton et al., 2006) on all traits between the different groups as a first step to test for differences in allometric scaling relationships (slope and intercept). More specifically, we used the functions “sma” and “ma” in R package “smart” (Warton et al., 2012). When the slope and/or the intercept of a trait differed significantly, we determined which groups differed from each other by mean-centering the log body length of the fish around

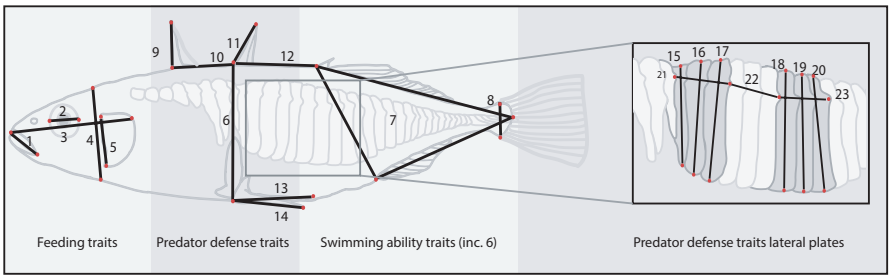
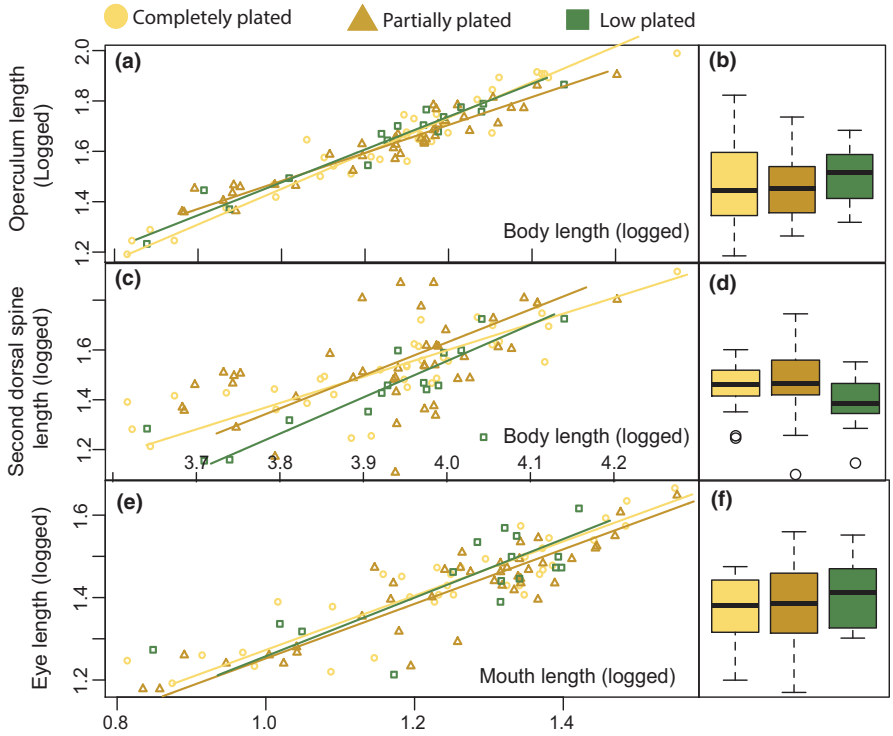


FIGURE 2 Linear measurements. Outline drawing of a threespine stickleback sampled from Chignik Lagoon illustrating distance measurements recorded from each individual with the help of digitalized landmarks (marked in red). The traits were separated into four groups: foraging, predator defense traits linked to spines, swimming ability traits, and lateral plate traits (measured on completely plated individuals only). More detailed information on the measured traits is found in the Methods

FIGURE 3 Standardized major axis regression. Allometric relationships for the freshwater morphs for 3 of the 13 investigated linear traits and their residuals from common slopes in a GLM as used in the principle component analysis (common slope not shown); a) operculum length on body length, b) operculum residuals, c) second dorsal spine on body length, second dorsal spine residuals, e) eye length on mouth length, and f) mouth length residuals. All data are logged. Completely plated is plotted in yellow circles, partially plated is plotted in brown triangles, and low-plated individuals are plotted in green squares



zero to make the intercept in the model equal to the trait mean within each treatment (mean of zero, sd = 1). This standardization enabled us to estimate the proportional trait change across morph type and genetic population as the ratio between the intercepts by using a general linear model (GLM): trait ~ bodylength × morph.

To compare between traits and groups, each trait was size-corrected by expressing it as residuals from ordinary least-squares regression on body size on logged values or on body depth for the lateral plate lengths (trait 15–20, Figure 2). We extracted the residuals using all individuals in the specific comparisons and checked the normality distribution of the data with qq-plots. To determine morphological traits that best describes intergroup differences, we applied principal component analysis (PCA) on the residual data, both grouped into feeding traits, predator defense traits and swimming ability traits, and on all traits combined. The approach of using size-corrected measures instead of defining size as the first principle component is strongly advocated when comparing several groups (Berner, 2011), as the orientation of the first principal component (PC1) also influences the orientation of the remaining PC's. As PCA provides a multivariate description of allometry for a single group, despite including data pooled from several groups, this can lead to wrong interpretations (McCoy et al., 2006), and we therefore also ran PCA analysis for each group, and each group and trait separately, and checked against the common PCA's. The resulting comparisons gave similar outcomes, and we hence only present data from the pooled PCA's. The PCA's were performed on the covariance matrix of the residuals in R by the use of two packages: FactoMiner for the analysis (Le et al., 2008) and factoextra (Kassambara & Mundt, 2020) for visualization. By using the function PCA() in FactoMiner, the program standardizes the values automatically by scaling the data to unit variance, making the variables more comparable. We screened the variables total eigenvalues and contributions and plotted the individual coordinate values for PC1 and PC2 as these are the most important dimensions in explaining the variability, in addition to

the five variables with the highest contributions as arrows. The contributions of each variable were calculated as follows: (variable. cos2*100/total cos2 of the component), where cos2 represents the quality of the representation for the variables on the factor map, as integrated in FactoMiner (Le et al., 2008). By the use of a scree plot of the percentage of explained variance by each dimension, we kept the PC's that explained most of the variability for further analysis, where we extracted the coordinate values and tested each separately for differences within morph or population using GLM; PCaxis ~ morph or population.

3 | RESULTS

3.1 | Comparing the different morphs genetically assigned to the freshwater population

The three morphs did not differ in body length ($F_{2, 91}$ = 0.104, p = .901, Table 1). All linear traits were significantly correlated with length, and length alone could hence explain between 27.8% (second dorsal spine, partially plated) and 99.1% (caudal area, completely plated) of the trait variation. We tested for differences in allometric scaling relationships for all traits (Table S1), where we documented differences in the operculum slopes only (likelihood ratio tests $_2$ = 8.11, p = .02). When testing operculum length separately on mean-centered data in a GLM, we found that the partially plated individuals differed from the completely plated by having a significant interaction between operculum and body length (T = −2.90, p = .004, Figure 3a).

There were no major significant differences between morphs when testing the morphological trait groups separately with PCA on the residual data, but the low-plated individuals were significantly different in PC3 ($F_{2, 91}$ = 3.093, p = .05) when comparing predator defense traits, having on average a shorter second dorsal spine (Figure 3c). When testing all 12 traits together in a PCA, the first PC explained 30.6%, PC2 explained 15.6%, and PC3 explained 10.6% of the variation, a total of 56.8% for the three first PCAs. PC1 was mostly related to head and mouth length, (Table 2; Figure 3e), and the main contributors to PC2 were the two lengths between the dorsal spines (trait 10 and 12). As these variables were highly correlated

TABLE 2 Coordinates for the variables for the freshwater morph dataset, the lagoon and freshwater dataset and the lagoon, migrant, hybrids, and freshwater dataset

	Freshwater morphs			Lagoon and freshwater completely plated			Lagoon, migrants, hybrids, and freshwater		
	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
Mouth length	0.7557	−0.0896	−0.1332	0.0037	−0.0616	0.0243	0.0194	0.0630	−0.0328
Eye length	0.6426	−0.1577	0.0747	0.0037	−0.0383	0.0185	0.0018	0.0398	−0.0080
Head length	0.8536	−0.0585	−0.1305	−0.0019	−0.0375	0.0213	0.0024	0.0429	−0.0135
Head depth	0.7364	0.1188	−0.0103	0.0068	−0.0132	0.0195	0.0081	0.0238	−0.0024
Operculum	0.5724	0.1447	−0.0599	0.0030	−0.0119	0.0364	0.0057	0.0302	0.0117
Body depth	0.4180	0.3162	−0.2214	−0.0269	0.0025	0.0052	−0.0358	0.0004	0.0047
Caudal area	−0.5760	−0.4197	0.3564	0.0015	0.0196	−0.0097	0.0022	−0.0181	0.0051
Peduncle width	0.5973	0.0695	0.1306	0.0023	−0.0041	0.0130	0.0070	0.0139	0.0062
Distance spine 1 and 2	−0.2941	0.7922	0.0524	0.0097	0.0157	0.0035	0.0099	−0.0121	0.0056
Second dorsal spine	0.4328	0.1697	0.6348	0.0348	0.0250	0.0093	0.0224	−0.0314	0.0018
Distance spine 2 nd 3	−0.2931	0.8440	−0.1766	−0.0011	0.0212	−0.0122	−0.0012	−0.0253	0.0062
Pelvic	0.1474	0.2729	0.7783	0.0274	0.0590	0.0627	0.0251	−0.0118	0.0693
Plate 1	-	-	-	0.0420	0.0155	0.0589	0.0464	0.0530	0.0800
Plate 2	-	-	-	0.0552	−0.0030	0.0205	0.0703	0.0243	0.0215
Plate 3	-	-	-	0.0771	−0.0041	0.0100	0.0893	0.0068	0.0063
Plate 4	-	-	-	0.1088	−0.0066	−0.0117	0.1235	−0.0115	−0.0131
Plate 5	-	-	-	0.1178	−0.0070	−0.0187	0.1307	−0.0147	−0.0170
Plate 6	-	-	-	0.1176	−0.0029	−0.0259	0.1296	−0.0216	−0.0197
Width 1	-	-	-	−0.0006	0.0353	−0.0227	−0.0065	−0.0313	0.0184
Width 2	-	-	-	−0.0140	0.0387	0.0067	−0.0100	−0.0273	0.0260
Width 3	-	-	-	0.0061	0.0214	−0.0166	−0.0053	−0.0332	0.0173

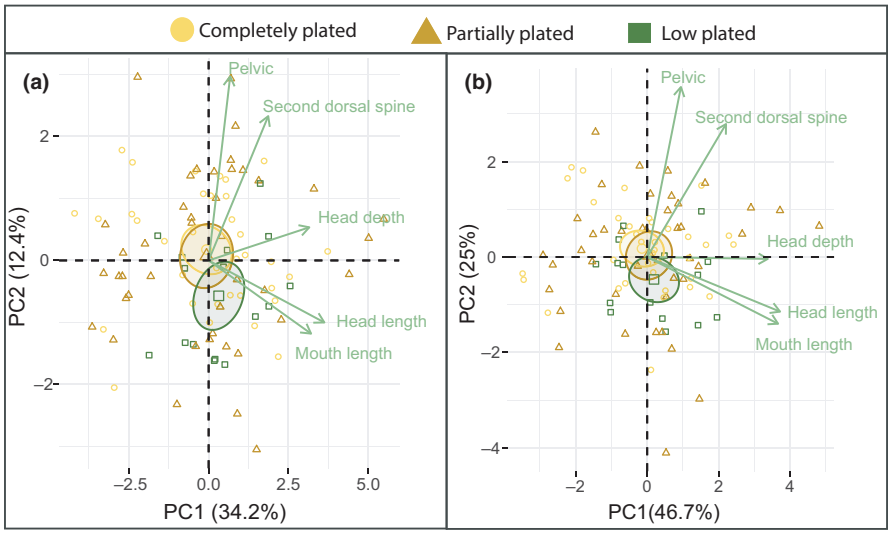


FIGURE 4 PCA for freshwater morphs. a) illustrates PC1 plotted against PC2 for all traits, and b) illustrates PC1 plotted against PC2 when only including the traits with the highest contribution from a). Both plots include the five most influential variables in green. Completely plated is plotted in yellow circles, partially plated is plotted in brown triangles, and low-plated individuals are plotted in green squares

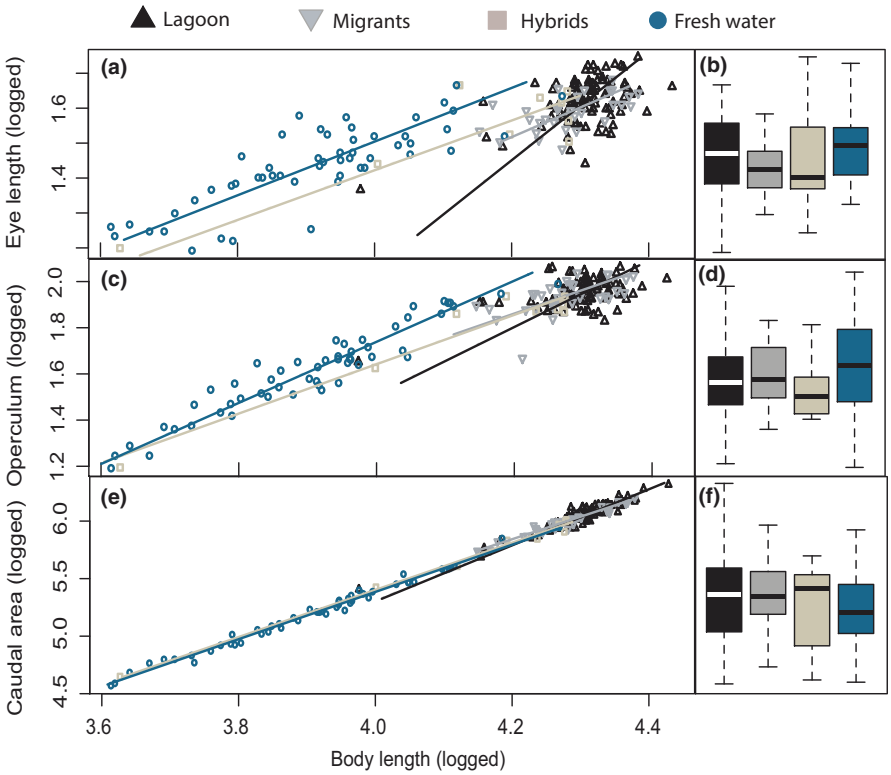


FIGURE 5 Standardized major axis regression. Allometric relationships for the lagoon, migrants, hybrids, and freshwater completely plated fish for 3 of the 21 investigated linear traits and their residuals from common slopes in a GLM as used in the principle component analysis (common slope not shown). a) Eye length on body length, b) eye length residuals, c) operculum on body length, operculum residuals, e) caudal area on body length and f) caudal area residuals. All data are logged. The lagoon fish are plotted in black triangles, the migrants in gray triangles, the hybrids in light brown squares, and the fresh water in blue circles. The plots are made in R with the use of the smatR package

(94,6%, p = <0.001), we removed trait 12 and reran the analysis (Table 2; Figure 4a). PC1 now explained 34.2% and PC2 12.4%. The low-plated individuals were significantly different from the completely plated in PC2 (Figure 4a T = −2.14, p = .035), indicating that the low-plated morphs had longer mouths and heads and shorter dorsal spines (Figure 3b, d and f). The five variables with the highest contributions to PC1 and PC2 were mouth length, head length, head depth, the length of the second dorsal spine, and the length of the pelvic (Figure 4b). By reanalyzing the data using these five variables, we could explain a total of 71,7% of the variation by the two first PC's, the low-plated morphs being marginally significantly different from the completely plated individuals (Figure 4b, T = −1.93, p = .056).

3.2 | Comparing freshwater and lagoon fish

When comparing the four groups of completely plated fish (the lagoon, migrants, hybrids, and completely plated freshwater fish, Table 1), most traits differed significantly in slope and elevation, or separately in slope or elevation (Figure 5a and c; Table S2). We

therefore tested for differences between the groups for each trait separately by standardizing the body length and used linear models for the evolutionary allometry parameter estimates. The results of the mean log eigenvalues for each genetic population are given in Table 2, where we report the proportional differences between the freshwater and the lagoon fish only and we therefore report the findings here. The freshwater fish had overall larger values in the head region, with an overall larger size (length and width), and a larger mouth (6,7%), eye radius (5.1%; Figure 5a), and operculum (6.3%) in proportion to the body compared to the lagoon fish. The swimming ability traits were more equal in size (Table 2); freshwater fish had larger peduncle width (5.3%). Caudal area had the highest coefficient of determination (R^2 = 99.01%; Figure 5c). The largest proportional trait change between the lagoon and freshwater population was the width of the three first measured plates (trait 21), which was on average 8.1% longer in the lagoon population (Table 2; Figure 8d). However, all of these results should be interpreted with caution, as the lagoon fish varied much less in body length than did the freshwater sample.

There were no major differences in PC1, PC2, or PC3 when comparing the freshwater and lagoon population's morphological trait groups separately with PCA, except for the lateral plate comparison. When only including the lateral plate traits, trait 15:23, PC1 explained 63.2% of the variation that was especially linked to plate 4–6, and the lagoon population differed significantly from the freshwater population by having on average 0.12 larger values for PC1 (T = 2.75, p = .01; data not shown). When testing all traits for the completely plated lagoon and freshwater common residuals, PC1, PC2, and PC3 explained 40.6%, 12.0%, and 10.3% of the total variation, respectively. The lagoon and freshwater fish were significantly different in PC1; the lagoon fish had on average 0.12 larger proportional trait values compared to freshwater fish (T=−2.65, p = .01), indicating larger plates and a longer pelvic spine (Figure 6a). As the plate traits were highly correlated, we also ran the analysis without the plate traits, resulting in a lower separation between the fresh and lagoon population, indicating larger values for the freshwater population in the head (Figure 6b).

3.3 | Comparing completely plated freshwater, migrants, hybrids, and lagoon fish

As we have reported on the main findings between the lagoon and freshwater populations in the previous section, we will only report main finding between the other groups here. When analyzing the head traits with PCA, the migrants were significantly different in PC3 (T=−2.98, p = .003), indicating that they had smaller eyes and narrower heads compared to the lagoon fish. When analyzing swimming ability traits, the migrants differed from the lagoon in both PC1 and PC2 indicating a narrower body (T = −0.02, p = .05; T = −0.02, p = .02, respectively). The hybrids had significantly smaller values for PC1 and PC2 when comparing the spine lengths (T = −0.01 p = .02; T = −0.1, p = .05, respectively), where the second dorsal spine had the highest contribution to the PC's. PC1 explained 64% when comparing the lateral plate traits, and the migrants were positively significantly different from the lagoon, having on average 0.12 larger plate sizes (T = 2.48, p = .01). When including all traits in the analysis, the plate traits (traits 15–20) were the measurements with the highest contributions to PC1, eye length, operculum and length of pelvic to PC2, and mouth length, pelvic, and length of plate1 for PC3 (Figure 7a, b). When excluding the plate traits from the analysis, mouth length, eye length, head length, pelvic spine, and second dorsal spine contributed the most. The different lateral plates covered from an average of 63.9% (plate 6 in freshwater) to 83.7% (plate 3 in migrants) of the body depth. Overall, the plates covered more of the body in the lagoon and the migrant individuals, when compared to the hybrids and freshwater fish (Figure 8; Table 3).

4 | DISCUSSION

The focus of the present study was to test whether morphological differences between stickleback populations in an open system could be linked to differences in functional traits important for foraging, swimming, and defense against predation. When comparing

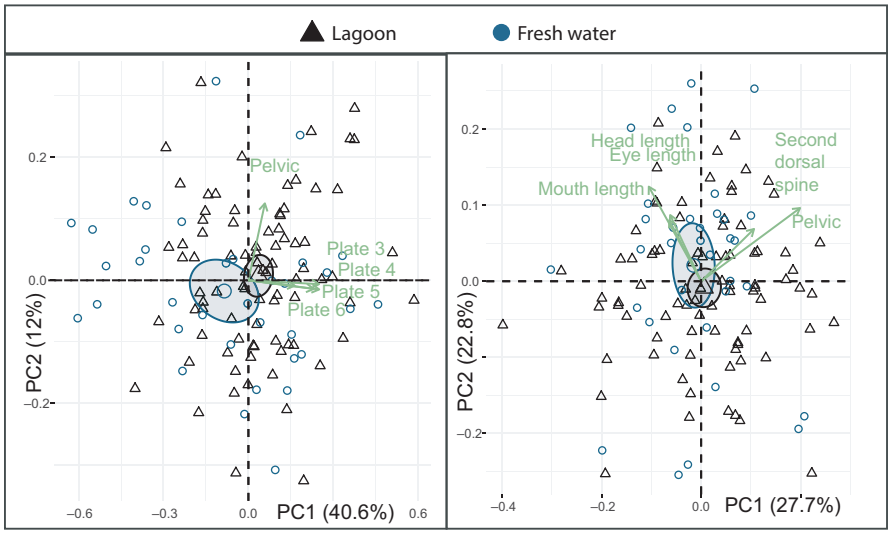


FIGURE 6 PCA for lagoon and freshwater completely plated fish. a) illustrates PC1 plotted against PC2 for all traits, and b) illustrates PC1 plotted against PC2 when excluding the lateral plate traits. Both plots include the five most influential variables in green. The lagoon fish are plotted in black triangles and the fresh water in blue circles

TABLE 3 Allometric parameter estimates for the completely plated groups

Linear trait	Mean log trait size				Proportional mean trait change
	Lag	Mig	Hyb	Fresh	
Mouth length	1.49	1.54	1.53	1.59	−6.7*
eye diameter	1.57	1.55	1.56	1.65	−5.1**
Operculum	1.91	1.88	1.86	2.03	−6.3***
Head length	2.87	2.87	2.89	2.96	−3.1***
Head depth	2.5	2.45	2.47	2.52	−0.8
Body depth	2.84	2.76	2.8	2.84	-
Caudal area	5.87	5.9	5.86	5.82	0.8*
Peduncle width	1.12	1.11	1.12	1.18	−5.3*
Pelvic	2.45	2.4	2.4	2.43	0.8
Second dorsal spine	1.9	1.82	1.77	1.79	6.1*
Distance spine 1 and 2	2.12	2.13	2.11	2.09	1.4
Distance spine 2 and 3	2.94	2.95	2.93	2.87	2.4***
Plate 1	2.19	2.03	2.11	2.16	1.3
Plate 2	2.31	2.28	2.27	2.28	1.3
Plate 3	2.37	2.33	2.32	2.31	2.5
Plate 4	2.35	2.29	2.27	2.22	5.8**
Plate 5	2.33	2.25	2.24	2.16	7.8***
Plate 6	2.28	2.2	2.2	2.12	7.5***
Width 1	1.85	1.79	1.79	1.71	8.1***
Width 2	1.68	1.65	1.65	1.64	2.4
Width 3	1.58	1.55	1.49	1.52	3.4

Note: Mean log trait size refers to the intercept of the model, proportional mean trait change is calculated as the ratio between the two trait means between the lagoon and freshwater individuals, where positive numbers indicate the lagoon fish to have larger values compared to the freshwater fish, *indicate significance; *$p < .05$, **$p < .01$, ***$p < .001$, allometric slope represents the intercept on all populations combined, SE; standard error.

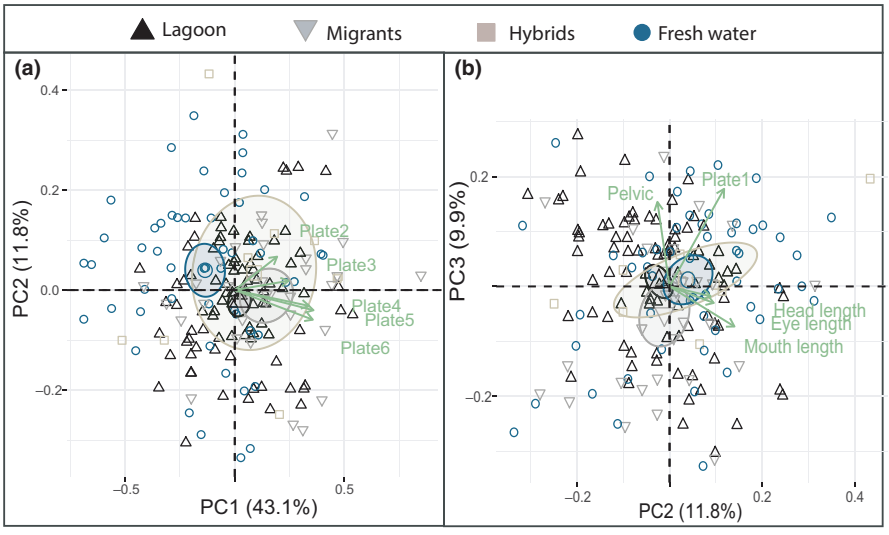


FIGURE 7 PCA for the lagoon, the migrants, the hybrids, and the freshwater population. a) illustrates PC1 plotted against PC2 for all traits, and b)illustrates PC2 plotted against PC3 for all traits. Both plots include the five most influential variables in green. The lagoon fish are plotted in black triangles, the migrants in gray triangles, the hybrids in light brown squares, and the fresh water in blue circles

the three lateral plate morphs within the freshwater population, the low-plated morph had a longer head, a larger mouth, and a shorter second dorsal spine. When comparing the four groups of completely plated fish, most traits for foraging, swimming capacity, and plate coverage had different allometric slopes and/or intercepts, implying that selection processes and/or plasticity are dividing the groups. Conversely, most traits measured for predator defense followed common allometric trajectories, likely resulting from shared evolutionary history and constraints in evolvability of the traits. The lagoon fish and migrants had overall smaller values in the head and larger values in the antipredation traits. This was especially evident in the lateral plate size and coverage of the body, where the

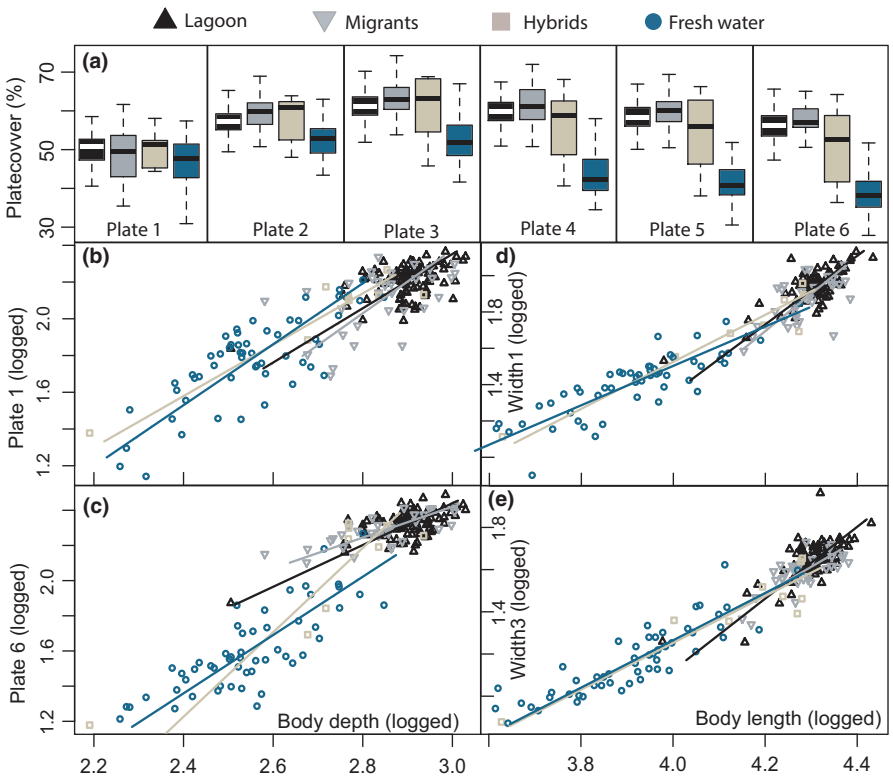


FIGURE 8 Lateral plates. a) The figure is illustrating the percent body cover for plate 1-plate 6 for the lagoon fish (black), the migrants (gray), the hybrids (light brown), and the freshwater fish (blue). The boxplots are illustrating the 25%–75% quantiles (boxes), median (black horizontal line), 95% limits (bars), and outliers (open circles). b) Allometric relationships for plate1 on body depth, c) allometric relationship for plate6 on body depth, d) allometric relationship for the width of the first three measured plates(width1) on body length, and e) allometric relationship for the width of the last three measured plates (width3) on body length. All data are logged

freshwater fish had a reduced armor coverage, likely from selection and genetic regulation.

Freshwater systems containing all three lateral plated morphs are not very common. Sympatric lateral plate morphs have been found to vary little in neutral genetic differences (Østbye et al., 2018; Pedersen et al., 2017) and have seldom been studied with respect to adaptations to foraging, swimming ability, and predator defense. Our results illustrate that most traits vary little between the lateral plate morphs in freshwater, but the low- and completely plated morphs were most different, and the partially plated morph was similar to the completely plated morph. The low-plated morph had somewhat larger mouths and longer heads. This could indicate a larger maximum gape, which again could indicate selection on feeding performance on larger prey for the low-plated morph. Low-plated morphs from a brackish water lake in Norway fed more efficiently on larger benthic prey than the two other morphs (Bjærke et al., 2010), implying that the low-plated morph in Chignik likely has a more benthic lifestyle and therefore is more adapted to freshwater habitats.

Operculum length was the only trait that had a significant different slope and elevation for the three lateral plates in freshwater, indicating a degree of ecotypic adaptation for the partially plated morph linked to size. When comparing the operculum sizes for the completely plated genetic groups (Figure 5c,d), the freshwater fish were larger, opposite to what we would expect, as other studies have found the operculum to be smaller in freshwater populations compared to anadromous ones (Kimmel et al., 2005). The size of the operculum varies between stickleback populations (Arif et al., 2009; Jamniczky et al., 2014; Kimmel et al., 2005); the larger operculum in freshwater fish in this system could indicate a more active pelagic lifestyle as more water can pass through the gills, but a larger size could also imply suction feeding ability for benthic prey (Day et al., 2015). Further, other studies have also found the ontogenetic growth of the operculum size and shape to have different allometric slopes and developmental endpoints, and the freshwater fish did not develop the full ancestral adult bone shape (Kimmel et al., 2012). This could be linked to osmoregulation, as young adult sticklebacks would continue their operculum bone development to the adult stage in salt water. Also, the skin inside the operculum in freshwater acclimated killifish (*Fundulus heteroclitus*) predominately contained chloride cells (Karnaky & Kinter, 1977), further indicating that the size differences in this study could be linked to osmoregulatory functions.

Despite being small, the observed morphological differences can have large effects in relative fitness for individuals (Parsons et al., 2011). Morphological measurements from the head indicated that the lagoon stickleback, with smaller eyes, shorter mouths, and shorter and broader heads, had somewhat more benthic features compared to the freshwater completely plated fish (Willacker et al., 2010). This is somewhat contrary to expectations, as marine sticklebacks tend to feed on planktonic prey, as inferred from gut content and gill-raker analyses (Hart & Gill, 1994; Wootton, 1976). This foraging ecology is maintained in so-called "limnetic" freshwater populations of stickleback in large, oligotrophic lakes (McPhail, 1984; Schluter, 1993; Walker, 1997). However, the habitat in Chignik Lagoon consists largely of seagrass, increasing the habitat complexity and probably also the likelihood of benthic feeding, thereby potentially selecting for a more benthic lifestyle. Further, other studies, focusing on Atlantic stickleback, also found that specimens from the

marine environment had smaller heads and eyes compared to freshwater fish (Leinonen et al., 2006; Voje et al., 2013). The size of the eye also seems to be a very plastic trait in fish (Howland et al., 2004); the size might to some extent be related to predatory regimes, where different high-predator scenarios are selecting for decreasing eye size or eye pigments (Frommen et al., 2011; Löennstedt et al., 2013; Zaret & Kerfoot, 1975), or high predatory regimes can also lead to increasing eye size (Ab Ghani et al., 2016; Miller et al., 2015). Further, larger eyes lead to improved visual sensitivity and resolution (Hairston et al., 1982) and could improve benthic feeding that is commonly associated with reduced light in the deeper parts of the lakes (Rick et al., 2012; Willacker et al., 2010). Linear allometric relationships between populations might result from genetic constraints in the founding population (Gould, 1971). The lagoon stickleback had less variation in body length and were also larger than the freshwater stickleback. McGugian (2010) found that the trait correlating the least with body length was eye diameter and head length, as bigger fish had smaller and shorter heads in comparison to their size, and Wund (2012) also found deeper bodies to correlate with a smaller eye size. These effects can be due to allometric constraints, as larger fish have allometrically smaller heads (McGuigan et al., 2010; Walker, 1997), which could partly explain the results also for these populations, as most of the fitted lines for the head-measurements are close to the common trajectories, narrowing down the possible directions of evolution in trait space.

The trait with the smallest variation between individuals, giving the tightest fit between populations in the shared common allometric trajectory, was the caudal area (Figure 5e). This similarity is likely a result of swimming performance being important for all habitats and that the trait is under strong selection. Overall, the traits measured for swimming ability were not very different between the completely plated groups, as would have been expected, especially for the anadromous group that swim over 25 Km to breed. Locomotor performance is important for a wide range of ecological processes, such as foraging, courtship, and predator avoidance (Videler, 1993). Having a smaller and more bulky body size may increase foraging performance within dense vegetation (Stoner, 1982; Webb, 1984), as is found in the lagoon, whereas a large, slim body size decreases the cost of movement and is more suited in open habitats (Webb, 1984). The migrants did have reduced body depth and peduncle width, which could be a result of selection, or more likely, from energy deficiency after a long up-river migration or a combination of these two factors.

The plate coverage was highest in the lagoon and migrant sticklebacks. This was consistent for all the measured traits, but especially for the sixth measured plate (Trait 20), where the plate covered on average 80.1% compared to 63.9% in the freshwater completely plated fish (Figure 8a). The difference suggests genetic regulation, likely from the gene GDF6 (growth differentiation factor 6), that has been linked to increased expression in freshwater fish, resulting in smaller plates (Indjeian et al., 2016). Smaller plates in freshwater might be beneficial in a number of ways, including faster burst swimming speeds (Bergström, 2002), maintenance of neutral buoyancy (Myhre & Klepaker, 2009) and metabolic demands (Grøtan et al., 2012). The plate number itself is also under strong selection. A typical trend in the stickleback freshwater invasion is that the number of bony lateral plates on both sides is reduced within few generations (Bell et al., 2004; Klepaker, 1993; Le Rouzic et al., 2011), creating a morph distribution that usually correspond to salinity, as the completely plated, the partially and the low-plated morphs associate most commonly with high, intermediate, and low salinity, respectively. The "textbook example" of a stickleback hybrid zone further includes low-plated morphs in freshwater, partially plated in the hybrid zone, and completely plated marine/anadromous fish (Bell & Foster, 1994; Hagen, 1967). The population structure between marine and freshwater stickleback in the present paper differs from most other stickleback gradients in that there is no evident hybrid zone and few identified hybrids, clearly indicating different roles of natural selection, pre-, or post-zygotic barriers between the populations. Further, the freshwater stickleback population in Chignik consists of all the three lateral plate morphs, with about 45% of the freshwater stickleback being completely plated and only 17% constituting low-plated individuals, a fraction which have been similar at least since the 1960s (Narver, 1966, 1969) and they also constitute a genetic population different from the lagoon fish (Taugbøl et al., 2014). Fish predation could in theory explain the dominance of completely plated fish in the system, but the main potential fish predators in the system, Dolly Varden and coho salmon, seem to rarely prey on sticklebacks (Bond, 2013; Roos, 1959; Ruggerone, 1992) and with the high abundance of stickleback in the system (Harvey et al., 1997) the overall predation pressure is likely very low. Further, most lakes across the northern hemisphere have trout or other predatory fish present (Reimchen, 1994), and one would therefore expect completely plated sticklebacks in freshwater to be more common if predation were the single explanation for evolutionary plate loss in these fish. The evolutionary loss of plates is also accompanied by a change in the lateral line sensory system (Mills et al., 2014; Wark et al., 2012; Wark & Peichel, 2010), suggesting that the loss of plates might be due to selection on the lateral line rather than the plates. It is thus still uncertain which selective agent(s) account for the high degree of completely plated stickleback in the freshwater system in Chignik.

In conclusion, much of the morphological variation in stickleback populations is hypothesized to be related to foraging opportunities. The Chignik system consists of a deep lake and a shallow lake. We found the lagoon stickleback population to be more specialized toward the littoral zone, displaying benthic traits such as large, deep bodies with smaller eyes compared to the freshwater completely plated morph. The lagoon and migrant fish had larger lateral plates that covered more of the body, which was especially evident for the sixth measured plate (the plate roughly above the start of the anal fin). As the freshwater population has had had a stable lateral plate morph distribution since 1960s, it seems to be a selection pressure in freshwater that sustain the completely plated morph, and at the same time selects for smaller plates.

When comparing the freshwater fish divided in three lateral plated morphs, the low-plated morph expressed values more consistent with benthic feeding and smaller antipredation traits compared to the partial and completely plated morphs, likely a plastic response to selection on habitat preference.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Annette Taugbøl: Conceptualization (equal); Formal analysis (lead); Methodology (lead); Writing-original draft (lead); Writing-review & editing (equal). **Thomas Quinn:** Conceptualization (equal); Funding acquisition (equal); Resources (equal); Writing-review & editing (equal). **Kjartan Østbye:** Conceptualization (equal); Writing-review & editing (equal). **Leif Asbjørn Vøllestad:** Conceptualization (equal); Funding acquisition (equal); Project administration (equal); Supervision (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

All the relevant data are within the paper and in the Supporting Information file 3.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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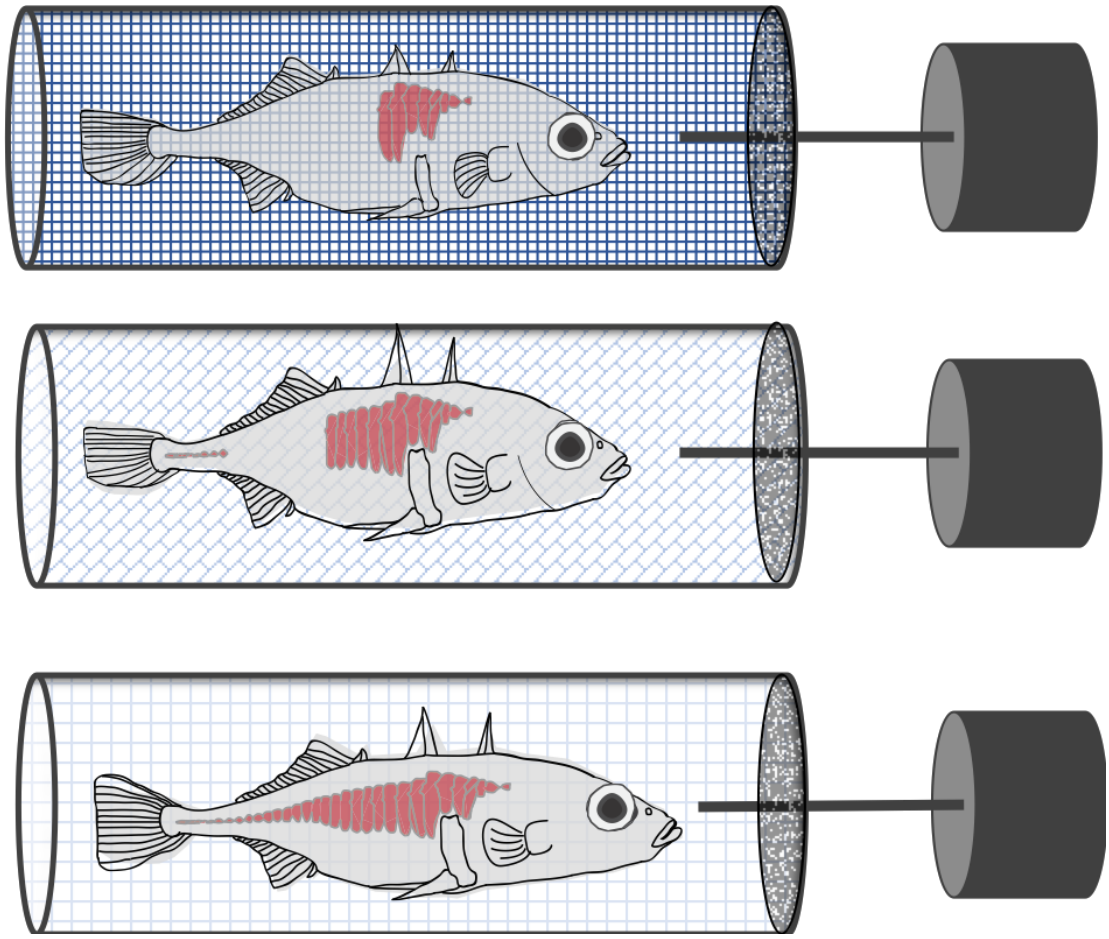
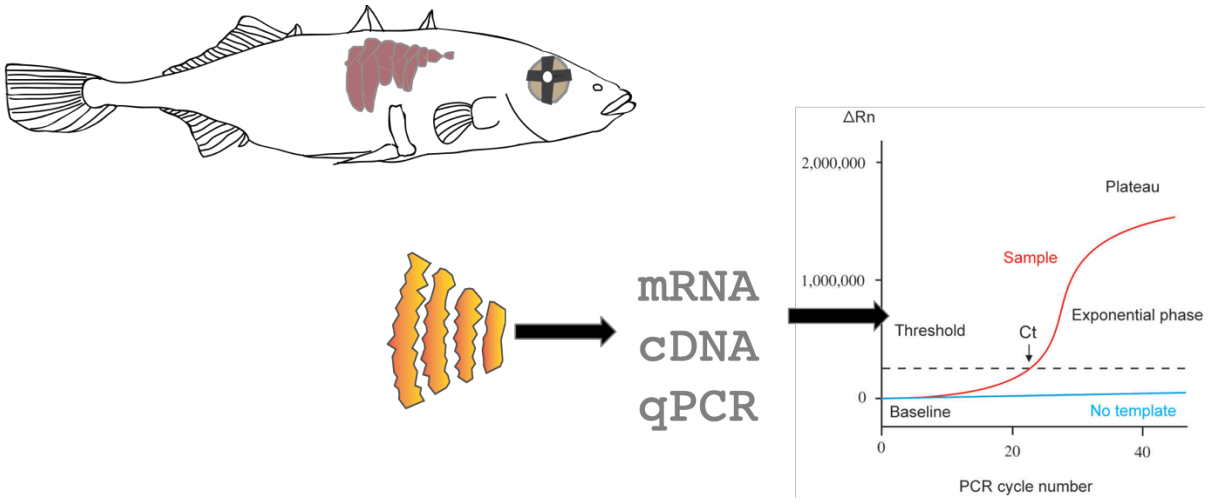
III



A.Taugbøl, T. Arntsen, K. Østbye & L.A.Vøllestad. 2014. Small changes in gene expression of targeted osmoregulatory genes when exposing marine and freshwater threespine stickleback (*Gasterosteus aculeatus*) to abrupt salinity transfers. PLOS ONE, 9 (9): 144-156

IV



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Small Changes in Gene Expression of Targeted Osmoregulatory Genes When Exposing Marine and Freshwater Threespine Stickleback (*Gasterosteus aculeatus*) to Abrupt Salinity Transfers

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Abstract

Salinity is one of the key factors that affects metabolism, survival and distribution of fish species, as all fish osmoregulate and euryhaline fish maintain osmotic differences between their extracellular fluid and either freshwater or seawater. The threespine stickleback (*Gasterosteus aculeatus*) is a euryhaline species with populations in both marine and freshwater environments, where the physiological and genomic basis for salinity tolerance adaptation is not fully understood. Therefore, our main objective in this study was to investigate gene expression of three targeted osmoregulatory genes (Na^+/K^+-ATPase (ATPA13), cystic fibrosis transmembrane regulator (CFTR) and a voltage gated potassium channel gene (KCNH4) and one stress related heat shock protein gene (HSP70)) in gill tissue from marine and freshwater populations when exposed to non-native salinity for periods ranging from five minutes to three weeks. Overall, the targeted genes showed highly plastic expression profiles, in addition the expression of ATP1A3 was slightly higher in saltwater adapted fish and KCNH4 and HSP70 had slightly higher expression in freshwater. As no pronounced changes were observed in the expression profiles of the targeted genes, this indicates that the osmoregulatory apparatuses of both the marine and landlocked freshwater stickleback population have not been environmentally canalized, but are able to respond plastically to abrupt salinity challenges.

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Introduction

The ability to respond rapidly to environmental change is beneficial in variable environments, making phenotypically plastic organisms better adapted in unstable and unpredictable environments [1]. The capacity of an organism to respond to its environment is facilitated by the environmentally induced alteration of gene and protein expression. While the evolution of plasticity depends on the trait(s) in question and the source of environmental variation, there is a general acceptance that the ability to be plastic may be constrained by a variety of costs underlying the plastic responses [2]. As such, evolutionary theory predicts loss of plasticity after periods of environmental stability, when environmental constancy eliminates or weakens the source of selection that was formerly important for its maintenance, given that the cost for the trait is high [3], or through environmentally induced genetic assimilation [4] which reduces the environmental influence on trait expression.

Phenotypic plasticity of a trait is generally assumed to be under selection when a single organism is exposed to several environments during its lifetime which each select for different trait values. Most fish species are stenohaline, living either in fresh or salt water [5,6] where they are exposed to the same type of osmoregulatory challenge during their lifetime. For fish living in marine waters, the concentration of ions is much higher in the water compared to the environment inside the cell, and surrounding ions diffuse into the cell while water is lost. The situation is reversed for a freshwater fish, where the surroundings are ion depleted, making the fish passively loose ions and gain water. In order to maintain a relatively stable internal osmotic environment, fish counteract these effects by a variety of specialized physiological mechanisms, mainly in the gills [7] and the kidney [8], and these genetic adaptations can limit movement between salinities. Only a very limited number of species are truly euryhaline [6], being able to osmoregulate in a wide variety of salinity environments. Even fewer can tolerate extreme changes in osmolality over short time scales, such as the killifishes (*Fundulus spp.*) [9] and the threespine stickleback (*Gasterosteus aculeatus*) [10,11].

The threespine stickleback (order, Gasterosteiformes, family Gasterosteidae; hereafter stickleback) is a small fish that was originally a marine species [12]. However, since the last glaciation, sticklebacks have colonized a large number of brackish and freshwater systems throughout the northern hemisphere and are now occupying an extremely wide haloniche [11,13]. Many of the newly formed freshwater populations have become landlocked due

to the isostatic uplifting of the land following deglaciation, and the stickleback in these habitats have consequently been separated from the sea for thousands of years. If the costs of having a plastic osmoregulatory machinery is high, it is expected that these landlocked freshwater stickleback populations should have lost the ability to osmoregulate efficiently in saltwater. However, studies suggest that freshwater populations of stickleback still possess the osmoregulatory machinery enabling them to handle abrupt changes in salinity [10,14], despite having been separated from the marine environment for up to 10–18 000 years [12]. This indicates that during adaptation to freshwater environments, the osmoregulatory physiology of landlocked sticklebacks has not been environmentally assimilated, or alternatively, the functionality of the osmoregulatory apparatus and its genomic architecture may not be open for selective change due to pleiotropic gene-interactions and is thus expected to remain similar in freshwater and marine populations.

One way to test if fish are adapted to a particular haloniche is to expose individuals to salinity challenges by transferring individuals from the original salinity to a test-salinity, tracking the expression of relevant genes through time. Earlier experiments show that stickleback easily tolerate transfers from freshwater to fully marine salinity, as well as the reverse [10,14]. However, it is not clear if the same osmoregulatory machinery is functioning at all times. The aim of this study was to assess the effect of experimental manipulation of salinity on the expression of genes important for osmoregulation. For this purpose, we collected adult fish from one marine and one freshwater site and exposed fish from each population to either 0 or 30 PSU (practical salinity units) for periods varying from 5 minutes to 3 weeks. The expression of four candidate genes was then followed through time (Fig. 1); three of the included genes are related to ion-pumps recognized to be under selection in marine-freshwater gradients (Na^+/K^+-ATPase (ATP1A3), cystic fibrosis transmembrane regulator (CFTR), voltage gated potassium channel gene (KCNH4)), and one is a stress related heat shock protein gene gene (HSP70), also associated with osmotic stress.

The objective of the study was to i) assess how target genes were expressed in the native salinity (assuming adaptation), ii) assess how target genes were affected when freshwater adapted fish were exposed to saltwater and iii) assess how target genes were affected when saltwater adapted fish were exposed to freshwater. As the osmoregulatory challenges are opposite in freshwater and marine environments, with ion secretion needed in saltwater and ion uptake needed in freshwater, we expect that osmoregulatory genes upregulated in freshwater will be downregulated in saltwater, and vice versa. We further expect the stress-related gene to have elevated expression levels in the beginning of the exposure for both groups due to handling and the physiological challenges associated with changing gene expression.

Materials and Methods

Fish and maintenance conditions

Adult stickleback were captured at two locations near Oslo, Norway (Figure 1a, b), during May and June 2010 and 2011. Fish from the marine population are known to breed there, and are not migratory, as many populations are known to be elsewhere [15,16,17]. The marine site (Sandspollen; 59° 39′ 58″N; 10° 35′ 11″ E) has a salinity that fluctuates between 22–29 PSU, while the freshwater pond (Glitredammen; 59° 55′ 53″N; 10° 29′ 55″ E; elevation 82.8 m above sea level) is stable at 0 PSU. The marine fish is comprised only of the completely plated morph (having a full row of lateral plates along its body flank), while the freshwater population only has the low plated morph (with lateral plates in the front region only). The two locations are geographically isolated by approximately 35 km by shortest distance through water, where about 8.5 km is through the river Sandvikselva that contains several steep waterfalls and dams. The age of the lake has been estimated at 7800 years before present using the program Sealevel32 [18]. The program uses information on postglacial land uplift and water level rise to estimate lake age. Downstream movement of fish from Glitredammen is possible, but upstream movement from the sea is impossible.

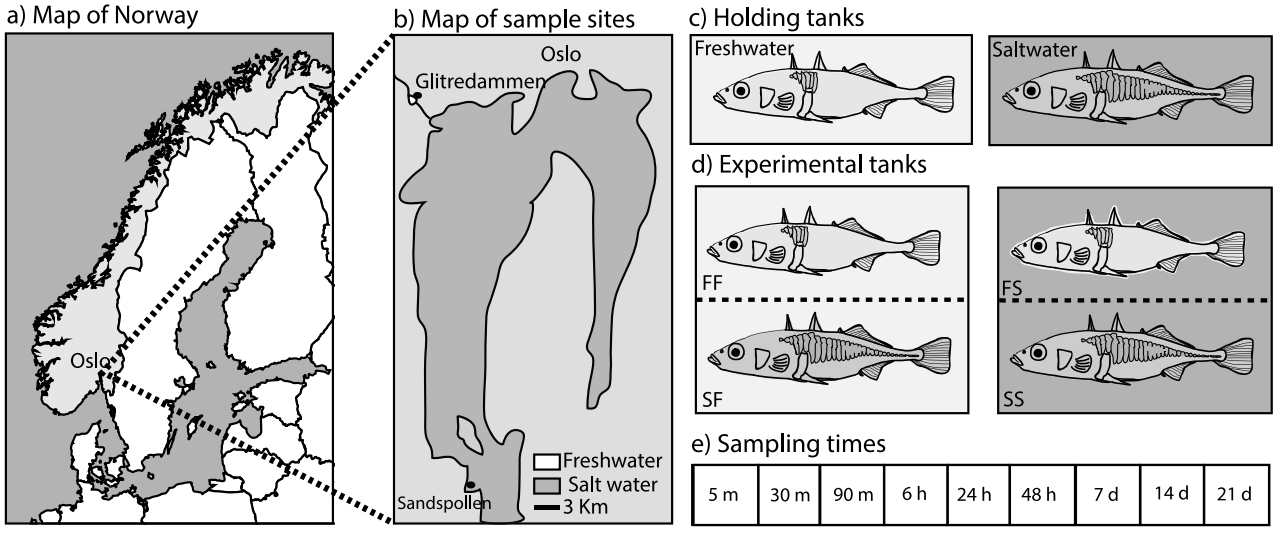

Figure 1. Study area and experimental design. a) Map of Norway showing the position of the sampling sites b) Locations of the two sampling sites, Glitredammen (freshwater) and Sandspollen (marine) c) Wild caught fish from both sampling locations were taken into the lab and placed in holding tanks of their native salinity for a minimum of three weeks. After acclimation, two groups of eight fish from both populations were exposed to either saltwater (30 PSU) or freshwater (0 PSU). The exposure tanks were divided in two by a perforated wall, so that both populations could be exposed to the same water quality at the same time d) Exposure times before the fish was collected and gill tissue was sampled.
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After capture, the fish were transported to the aquarium facility at the University of Oslo and acclimated to holding conditions in their native salinity for minimum three weeks prior to the experiment. Two glass holding-tanks (500L) with either salt (30 PSU) or fresh water (0 PSU) were used for acclimation (Figure 1c), using biologically activated canister filters (EHEIM professional 3600), and UV-filtration. The acclimation tanks were covered with black plastic in front and on the sides to reduce visual stress. Further, to reduce potential male nesting behavior, the tanks were not equipped with any environmental enrichment, leaving the tanks free of sand and vegetation. The temperature in the tanks was maintained at room temperature (approx. 20°C) and the light regime was set at a 12:12 light:dark cycle. The fish were fed two times a day with frozen red bloodworms throughout the acclimation and exposure period.

Experimental design and protocol

The experimental setup consisted of 80 L tanks; the tanks were either filled with 0 PSU water or with 30 PSU water (Figure 1c), and covered with black sheets to reduce visual stress. A grey plastic wall divided each experimental tank into two 40 L compartments, where perforation ensured water movement between compartments (Figure 1d).

At the start of an experiment, 8 fish that appeared healthy were collected from each holding tank and placed directly in either the 30 or the 0 PSU experimental tank. Saltwater fish were therefore tested in either saltwater (SS; control) or in freshwater (SF). The freshwater fish were also tested in saltwater (FS) or in freshwater (FF; control) (Figure 1d).

The fish were exposed for different time periods, lasting between 5 minutes and 3 weeks (Figure 1e). The time periods were selected to cover short-term effects as well as long-term changes in gene expression. After each experiment, the fish were quickly netted out of the experimental tanks, immediately killed by a swift blow to the head and was directly processed for tissue collection.

Ethics statement

The experiment was approved by the Norwegian Animal experimentation and care committee (permit no ID 2705) and all efforts were made to minimize suffering.

Candidate gene expression

Candidate genes for osmoregulation were selected based on published studies on divergence in gene expression between marine and freshwater sticklebacks [19], studies identifying outlier regions in DNA sequences between marine and freshwater sticklebacks [20], and preliminary Illumina RNA-sequencing results (Table 1).

The targeted Na^+/K^+-ATPase gene, ATP1A3, has displayed salinity dependent regulation in fish when acclimated to different salinities, including killifish, *Fundulus heteroclitus* [21]. ATP1A3 is a plasma membrane protein that helps the establishment and maintenance of the electrochemical gradients of sodium and potassium ions across the plasma membrane by coupling the exchange of two extracellular K+ ions for three intracellular Na+ ions to the hydrolysis of one molecule of ATP [22], thereby ensuring a relatively constant osmolarity of cells and blood plasma. The protein is powering salt secretion in saltwater fish and salt absorption in freshwater fish [23].

The cystic fibrosis transmembrane regulator, CFTR, is an apical membrane anion channel involved in chloride secretion, and establishes an electrical driving force for trans-epithelial sodium secretion that generate the osmotic driving force for water flow, yielding an isotonic secretory product. As a candidate gene for saltwater adaptation [24], previous studies have also shown an upregulation of chloride cells and CFTR expression in a Hawaiian goby (*Stenogobius hawaiiensis*) [25] and in killifish [26] exposed to salt water.

While able to tolerate a wide range of salinity, whole genome sequencing of marine and freshwater sticklebacks have identified several chromosomal regions that have undergone parallel selection after freshwater invasion, indicating adaptive divergence and evolutionary change across the marine-freshwater boundary [20]. One identified region differing between marine and freshwater sticklebacks was an inversion with alternative functional exons of the voltage gated potassium channel gene, KCNH4, on either side, suggesting marine and freshwater specific isoforms [20]. However, although small, parallel changes in the sequences of genes may result from similar selection pressure across environments [27,28,29], it is the functional gene products and its regulation through expression that gives rise to the phenotype. Therefore, when candidate regions or loci linked to adaptive divergence have been identified, the regions should be tested in function, such as their role in gene regulation in a relevant ecological setting. The primer pairs in this study does not distinguish between marine and freshwater isoforms as the spanning ends of the inversion are identical down to a few base-pairs and the mutations are seemingly located within introns of the gene.

Both the physical handling when fish are transferred between tanks and the changes in water salinity are stressful, thus a stress-related heat-shock protein, HSP70, known to be affected by osmotic stress [30] was also included in the study.

To control for variation in expression levels not due to the experimental treatment, we used two reference genes, Elongation factor 1 alpha (EF1α) and Gluceraldehyde-3-phosphate dehydrogenase (GADPH). EF1α has been used successfully as reference gene in a similar study on sticklebacks [19] as well as in other gene expression studies on fish [26]. GADPH is a commonly used reference gene and has been stably expressed in a wide array of studies spanning predator cues [31] to exposure to offshore produced water [32].

Gene specific primers for target genes CFTR and reference genes GADPH and EF1α were previously designed and optimized (Table 1). Primers for additional target genes (ATP1A3, KCNH4 and HSP70) were designed based on genetic sequences from the Enseml genome browser and NCBI Primer-Blast (Table 1).

Tissue collection, RNA isolation, cDNA synthesis and qPCR

The gill plays an important role in the maintenance of blood ion and acid–base balance in both freshwater- and seawater acclimated fish [7,33,34]. After each fish was sacrificed, gill samples were immediately collected using sterilized tweezers and stored in RNA*later* (Ambion RNA, Life Technologies) according to the manufacturers protocol. The sampled fish were stored individually in 70% EtOH. The mRNA was isolated from the gill samples from each fish separately, using the mRNA direct kit (Invitrogen) as described by the manufacturer. The mRNA concentration and purity was quantified using Bioanalyzer (Agilent 2100 Bioanalyzer) and the Agilent RNA 6000 Pico Kit (Agilent Technologies) according to the protocol, and all samples were diluted down to 0.125 μg/μL before cDNA synthesis. The cDNA was prepared using the Superscript VILO cDNA synthesis kit (Invitrogen by Life Technologies) as described by the manufacturer, and the concentration was checked spectrophotometrically using Nanodrop (NanoDrop Teqnologies INC).

Table 1. Primers used for qPCR expression analysis of threespine stickleback genes.

Target	Gene name	Ensembl gene ID	Primer sequences	°C	E (%)	Reference
ATP1A3b	ATPase, Na+/K+ transporting, alpha 3b polypeptide	ENSGACG00000009524	F: AGCCGAGATCCCCTTCAACTCCA	60	99.07	*This study*
			R: GCTCCTTCCCCTGCACCAGGA			
CFTR	Cystic fibrosis transmembrane conductance regulator	ENSGACG00000009039	F: GCAGGCCTCTTCTTCACCAA	58	98.51	McCairns et al. (2009)
			R: TCCAGATAGAGGCTGATGTTCTTG			
KCNH4	Potassium voltage-gated channel subfamily H member 4	ENSGACG00000008648	F: CACAGTGACCTCTCTGGTGC	60	99.29	*This study*
			R: AGACATGAGCAGGGTCAGGA			
HSP70	Heat shock protein 70	ENSGACG00000013048	F: ATCGGTATTGACCTGGGCAC	60	99.20	*This study*
			R: GGTATCGGTGAACGCCACAT			
Reference						
EF1α	Elongation factor 1	ENSGACG00000002182	F: CATTGTCACTTACCTGAATCACATGA	60	99.26	McCairns et al. (2009)
			R: TGTGGCATTTAACAACATTTCCA			
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	ENSGACG00000005864	F: CAAACCGTTGGTGACAGTATTTG	60	99.9	Sanago et al. (2011)
			R: GCACTGAGCATAAGGACACATCTAA			

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The cDNA concentration was diluted down to 15 ng/μL (±1.5 ng/μL) prior to qPCR amplification after testing for optimization (standard curves, two-fold serial dilutions on pooled cDNA) and association curves for each primer pair (all primer pairs tested on dilution curves at 58 and 60°C). Primer efficiencies were calculated using the formula $E = (10^{-1/\text{slope}})$-1. All primer pairs had efficiencies between 95–100% and presented a single product, confirmed with a melting curve.

The qPCR reaction was performed on a Lightcycler 480 (Roche) using SYBR Green PCR Master Mix (Roche). Each 20 μL reaction contained 5.0 μL of the optimized concentrated cDNA, 1.0 μL of each primer, 10.0 μL of SYBR Green and 3.0 μL H2O. The thermocycle program included an enzyme activation step for 5 min, followed by 45 cycles of 95°C for 10 s, 58/60°C for 20 s and 72°C for 20 s. After the amplification phase, a dissociation curve was generated to confirm the presence of a single amplicon. The individual samples were run on duplicated plates, along with three negative reverse-transcriptase controls and an eightfold serial dilution to calibrate plate variation between runs. The obtained cycle threshold (C_q) values for the individuals were adjusted for plate efficiency, and duplicated reactions that differed by more than 0.5 C_q values were checked manually and removed from the analysis.

Statistical analysis

The target gene C_q values were normalized using the mean of the two control genes. Both the EF1α and GADPH were relatively stably expressed across the various time points but did differ somewhat between treatments. The C_q values for GADPH were slightly higher in the SS and SF treatments than in the other treatments ($F_{3,\ 237} = 6.64$, $P<0.001$), but did not differ between treatments for EF1α ($F_{3,\ 235} = 1.43$, $P = 0.236$). The relative expression levels were expressed as the individuals normalized C_q-values of the target transcript, and expressed relative to the mean values of a control group, here set to the 5 min exposure, for each treatment group (SS, SF, FF, FS). This method gives the fold change in expression relative to the control [35].

Variation in fold change of the expression of the different target genes was tested using general linear models. Each treatment group was tested at 9 different time points, where time can be classified both as a continuous variable (in minutes) as well as an ordinal factor (1–9). Preliminary analyses indicated that using continuous time was the better modeling approach and was therefore used in the model, expressed on a log-scale. To account for non-linear effects we included a squared term for time. The general model structure was thus:

$$\begin{aligned} Y = {} & \text{Treatment} + \text{Time} + \text{Time}^2 \\ & + \text{Treatment} * \text{Time} + \text{Treatment} * \text{Time}^2 \end{aligned}$$

where treatment is the four different treatment types (SS, SF, FF, FS). The best (most parsimonious) model was selected using backward selection, using the Bayesian information criterion (BIC). BIC puts a heavier penalty on parameter number than the more commonly used Akaike information criterion (AIC).

Results

In general all experimental fish handled the transfer to the experimental water qualities well, both when transferred to the control water quality (groups SS and FF) and to the treatment salinity (SF, FS). A total of 9 fish from the freshwater population died across treatments during the experiment (6 in FF and 3 in FS), whereas no marine fish died. A total of 288 sticklebacks were used throughout this study.

We used the gene expression levels at 5 minutes of exposure as the control against which all fold level changes in expression was compared. Overall there were only minor differences in C_q – levels among the various treatment groups for the target genes (Table 2, Figure 2, Appendix S1).

The model that best explained variation in fold change in ATP-expression contained the ln-time and treatment factor ($F_{4,\ 204} = 14.636$; $P<0.0001$). Overall there was a tendency for the relative expression to increase with time, and the time-adjusted

Table 2. Gene expression of four osmoregulatory genes (see Table 1) (C_q-values; mean ± se) for marine and freshwater stickleback after 5 min exposure to either salt or freshwater.

Treatment	CFTR	ATP	KCNH4	HSP70
SS	1.15 3±0.009 (7)	1.111±0.009 (7)	1.415±0.013 (7)	1.272±0.020 (7)
SF	1.133±0.013 (4)	1.121±0.010 (6)	1.429±0.014 (6)	1.257±0.022 (6)
FF	1.158±0.009 (8)	1.126±0.008 (8)	1.413±0.012	1.196±0.019 (8)
FS	1.148±0.011 (6)	1.121±0.010 (6)	1.374±0.014 (6)	1.215±0.022 (6)
	$F_{3,\ 21} = 0.836$, $P = 0.489$	$F_{3,\ 23} = 1.383$, $P = 0.273$	$F_{3,\ 23} = 2.836$, $P = 0.061$	$F_{3,\ 23} = 3.028$, $P = 0.050$

Summary statistics from an analysis of variance for each gene is also given. Treatments are: SS (saltwater fish in saltwater), SF (saltwater fish in freshwater), FF (freshwater fish in freshwater) and FS (freshwater fish in saltwater).

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mean fold change was slightly larger for the SS and FS (SS: 1.547±0.093; FS: 1.555±0.105) than for the FF and SF treatments (SF: 1.283±0.094; FF: 1.193±0.105). To further examine how the fold-change varied with treatment we re-ran the analyses grouping the fish into those tested in freshwater (FF, SF) and those tested in saltwater (SS, FS). Overall, the best model using this model-structure fit better to the data than the best model using treatment group (BIC treatment group = 478.0; BIC treatment water type = 466.0). The best model contained the interaction with time and water treatment type.

The model that best explained variation in fold change in CFTR-expression contained the ln-time and treatment factor ($F_{4,\ 206} = 13.041$; $P<0.0001$). Overall there was a tendency for the relative expression to increase with time, and the time-adjusted mean fold change was larger for the two control treatments (SS: 1.592±0.087; FF: 1.427±0.099) than for the transfer treatments (SF: 0.903±0.088; FS: 1.155±0.094). The transfer treatments and the control treatments differed significantly (Tukey HSD post hoc test, $P<0.05$).

The model that best explained variation in fold change in KCNH-expression contained the ln-time ($F_{1,207} = 14.937$; $P = 0.0001$) and treatment factor ($F_{3,207} = 14.593$; $P<0.0001$). Overall there was a tendency for the relative expression to increase with time (0.087±0.023). To further examine how the fold-change varied with treatment we reran the analyses grouping the fish into those tested in freshwater (FF, SF) and those tested in saltwater (SS, FS). Overall the best model using this model structure fit better to the data than the best model using treatment group (BIC treatment group = 527.1; BIC treatment water type = 537.6). The best model contained time ($F_{1,209} = 14.944$; $P<0.0001$ and water treatment type ($F_{1,209} = 43.995$; $P<0.0001$). The KCNH-expression was significantly elevated in freshwater (1.488±0.079), whereas it was significantly decreased in saltwater (0.756±0.077).

The model that best explained variation in fold change in HSP70-expression only contained the treatment factor ($F_{3,\ 203} = 2.688$; $P = 0.048$). Overall, mean fold change was larger than one for fish originating from saltwater (SS: 1.286±0.181; SF: 1.526±0.182) whereas if was smaller than one for the fish originating from freshwater (FF: 0.865±0.209; FS: 0.902±0.205). The fish from freshwater and saltwater differed significantly in expression level (Tukey HSD post hoc test, $P<0.05$). However, despite the expression levels being quite different between the ecotypes, the explanatory power of the model was low ($R^2 = 0.038$).

Discussion

Teleost fishes maintain nearly constant internal osmotic concentration and have osmoregulatory machinery fine-tuned to the external salinity in either salt or fresh water. However, some species can tolerate a wide range of environmental salinity, also with only short or no acclimation. Our main objective in this study was to investigate the expression of three relevant osmoregulatory genes and one stress related gene in marine and freshwater threespine stickleback, when exposed to non-native salinity for various periods. The study showed that both populations were capable of handling a direct transfer to a new and very different salinity. Survival was high in all treatments and the variation in gene expression was relatively small. This suggests that the capacity for osmoregulation in a wide range of salinity regimes has not been lost in either population. And yet, interestingly, no pronounced changes were observed in the expression profiles of the genes targeted in this study. This suggests that the ability of these fish to reverse the direction of their osmoregulation has not been canalized or lost.

Expression of ATP1A3 was elevated in saltwater

In this study, the ATP1A3 expression was lower in the FF treatment group at all time points, with a mean difference of 0.03 on an exponential scale compared to SS. Further, the SF group did show a weak down-regulation of ATP1A3 compared to SS and the expression levels stabilized around the FF values after approximately 24 hours. Comparing the FF and the FS, the FF had a consistently lower expression of ATP1A3, equally as the SS group.

As the Na^+/K^+-ATPase transporters both secrete and absorb salt in order to obtain a nearly constant internal osmotic concentration when in marine- and freshwater, respectively [7], it might be expected that the long-term expression of the protein stabilize on equal levels. Nevertheless, when a fish experiences a change in external osmolarity, the expression level is expected to shift in order to handle the novel osmotic and ionic stress: it is therefore surprising that we see so little change in the overall expression on the shorter time-scale in this study. Overall, the main finding of ATP1A3 being less expressed in freshwater is in accordance with most research on salmonid fish, where gill Na^+/K^+-ATPase activity is higher in seawater acclimated fish and decreases following migration into freshwater [36,37]; other fish species also show this pattern, including sea bass, *Dicentrarchus labrax* [38] and flounder, *Platichthys flesus* [39]. However, yet other studies have shown that the expression levels for the equivalent ATP isoform in Atlantic salmon (*Salmo salar*) did not change as a result of freshwater exposure [40], or as in killifish,

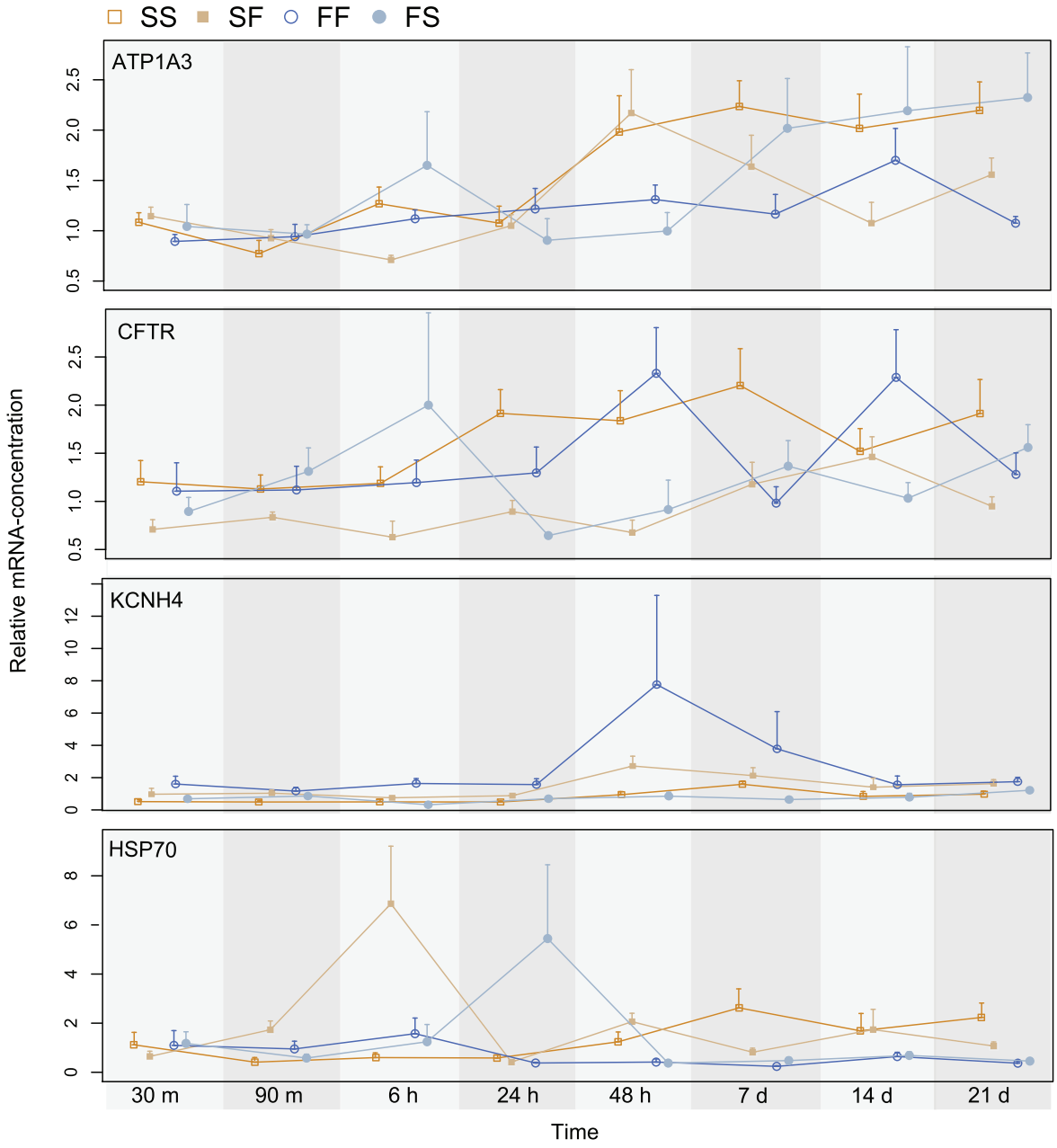

Figure 2. Relative mRNA-expression for the four targeted genes, ATP1A3, CFTR, KCNH4 and HSP70 in saltwater control (SS, dark orange), saltwater fish exposed to freshwater (SF, light orange), freshwater control (FF, dark blue) and freshwater fish exposed to saltwater (FS, light blue) for exposure periods relative to the 5 minute exposure. Values between 0–1 indicate lower expression, and values over 1 indicate higher expression relative to the expression at 5 minutes.
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where ATP1A3 was up-regulated in freshwater [21]. Overall, previous reviews on fish osmoregulation state that the role of gill Na^+/K^+-ATPase is uncertain [41], unclear [7], or that the energy required for sodium uptake can be generated only by Na^+/K^+-ATPase [42]. It should however be noted that the cellular localization of the Na^+/K^+-ATPase transporter in this study is not known, and a histological analysis of the gene localization during a salinity challenge could provide additional information on the osmoregulatory function in stickleback.

No major changes in CFTR expression between treatments

Overall, only small changes in CFTR expression were observed across the treatments in the present study. CFTR had a slightly higher expression level in SS compared to FF, but only in the early time-periods (5 min to 24 hours), where the overall expression was reduced in both treatments compared to T = 5 min. After the first day, the expression stabilizes for both groups. Transferring marine fish into freshwater (SF) lead to an overall reduced expression of CFTR compared to the SS treatment, but this trend was not

consistent across all time points. The freshwater fish transferred to saltwater (FS) had a higher expression of CFTR compared to FF for the first 6 hours, but was generally stable across all time points, indicating no major change in expression.

CFTR is thought to be central in the ion excretion at the gills of marine fish, as it is involved in the passive transport of chloride ions [24], and as such the expression is expected to decrease following freshwater acclimation. Anticipated increased expression of CFTR in apical membranes in response to transfer to saltwater have been illustrated in Atlantic salmon [43] and eel, *Anguilla anguilla*, [44]. However, contrary to expectations, another study on lab-reared stickleback observed a higher expression of CFTR in freshwater compared to saltwater [19]. Further, when comparing long-term expression levels of CFTR in saltwater exposed Atlantic salmon, the expression levels tended to decline towards the control after 30 days [43]. Although we would have expected a higher difference in the expression of CFTR, especially between the two control groups (SS and FF), there is much confounding evidence of CFTR-expression across taxa [45]; reported expression of CFTR in freshwater spans from not expressed at all in Mosambique tilapia, *Oreochromis mossambicus* [46], diffuse in Killifish [47] to no change in expression in striped bass, *Morone saxatilis* [48], indicating an overall complicated involvement of CFTR in freshwater osmoregulation [47]. An alternative to differential expression of the ion-transporter CFTR could be a redistribution and reuse of CFTR proteins, as Marshall et al. [47] illustrated movement of the protein from an apical location in SW to a more diffuse and basolateral location in FW. This could also be the case for the stickleback in this study, as a rearrangement of CFTR-proteins and hence its activity state would not be picked up by the qPCR analysis.

Much variation, but higher overall expression of KCNH4 in freshwater fish

Comparing the KCNH4-expression between the two control treatments (SS and FF), there was more temporal variation than would have been expected: KCNH4-expression decreased during the first 24 h in the SS treatment, whereas in the FF treatment the expression increased the first 48 h, before both seemed to be stabilized. The transfer of marine fish to freshwater (SF) followed approximately the same curve as for the SS treatment, and the transfer of freshwater fish to saltwater (FS) also demonstrate a down-regulated expression during the first 24 h, before expression increased reaching control levels (5 min exposure) after three weeks. Overall, the expression was higher in FF and FS compared to SS and SF. However, as there was no overall trend in the expression of KCNH4 for any of the groups in this study, this indicates that the gene is involved in other processes than osmoregulation, or that the osmoregulatory function of this transcript is located in other organs than the gill.

KCNH4 is a voltage-gated ion channel protein that is sensitive to voltage changes in the cell membrane and is known to have several functions, including regulation of cell volume, maintaining resting currents and affecting cardiac contractility [49]. Recent genome re-sequencing of 21 individual stickleback from marine and freshwater habitats across their global distribution revealed 81 loci underlying repeated parallel divergence in marine and freshwater ecotypes, including three chromosomal inversions [20]. One of the inversion sites has marine and freshwater specific 3′ exons of the KCNH4-gene, indicating parallel ecological selection on the breaking sites of the inversion, and possibly also directly on KCNH4 [20]. Although it is clear that chromosomal rearrangements can contribute to speciation [50,51,52], it is less evident how they do so and which mechanisms are involved. It is therefore important to identify whether inversion events have led to profound changes in the expression pattern of genes involved in the inversion, in relevant experimental setups.

While we do not have any information on the chromosomal arrangements in these two populations, it is still interesting to quantify the expression of the gene across different environments, as one would expect different expression profiles if the gene is under osmoregulatory selection. Chromosomal inversions are known to alter gene activity, either by causing non-functionalization of the gene, generating alternative splice sites or by altering gene regulatory networks [53,54]. In a study on development under different thermal selection regimes, one population of *Drosophila subobscura* had different expression patterns for loci located within and between inversions, where significant differences in expression tended to be more commonly found inside rather than outside the inversion [55]. The increased expression of KCNH4 in freshwater found in this study indicates a potential regulatory effect of the inversion on chromosome 1, however more studies are needed in order to disentangle the complete effect of the inversion.

Expression of HSP70 was elevated in freshwater adapted fish

HSP70 was identified as a candidate gene for detecting short-term osmotic stressful conditions in stickleback, with higher expression in freshwater compared to saltwater. When comparing SS and FF expression of HSP70, the marine fish had an overall lower expression before stabilizing after the first 24 h. Marine fish exposed to freshwater (SF) had an increased expression compared to SS the first 6 h before normalizing and freshwater fish exposed to saltwater (FS) had an overall lower expression compared to FF.

Capture, handling and crowding are all factors that can initiate a stress response in fish, as can short-term fluctuations in the physical environment. Physiological responses to stressors are complex, but include increased activity of cellular defense mechanisms, such as the up-regulation of HSP-genes [56]. The involvement of HSP70 in the acclimation of fish to salinity changes has been well documented experimentally [57,58,59]. Larsen et al. [59] observed a significant induction of HSP70 in kidney tissue of two populations of flounder, *Platichtys flesus*, when introduced to non-native salinities for both short- and long term exposures. However, the same study also illustrated tissue-specific up-regulation of HSP70, as expression in gill and liver was differentially affected by differences in salinity [59]. Other similar studies on Atlantic cod, *Gadus morhua*, illustrated expression differentiations in both gills and kidney after salinity transplantations during the first 24 hours [60], similar to the result in this study.

Conclusions and perspectives

In our study system, sticklebacks from the marine population are genetically (Østbye et al., unpublished data) and morphologically [61] differentiated from the freshwater population living in the river below the waterfall. However, as the populations are genetically differentiated, a surprising result of this study is how little variation in gene expression was observed when the fish were directly transferred to the contrasting salinity treatments, and additionally, how little it differed between the two populations in their native salinities.

Movement between environments of different salinity is physiologically costly [62], and resident populations experiencing different salinity levels are predicted to be locally adapted to their native habitat as traits promoting euryhalinity are expected to be rapidly lost if they are not under selection [63]. It is likely that

adaption to the local environment takes time, but the freshwater fish in this study have been separated from the marine populations for more than 7000 years (between 3500 to 7000 generations assuming a two year or one year life cycle), likely sufficient time for local adaptation given reasonable selection [64,65]. Further, population genetic studies on stickleback have recognized salinity to be a major factor in the distribution of genotypes in systems that exchange migrants [66,67], also across high gene-flow environments such as in the Baltic ocean [68], indicating that adaption to salinity is under selection.

Based on survival alone, the results from this study suggest that over the>3500 generations of adaptions to freshwater environments, the osmoregulatory physiology of landlocked stickleback has not been significantly canalized or experienced strong selection as they have retained their capacity for osmoregulation in saltwater. Additionally the locally adapted marine stickleback can osmoregulate in freshwater, despite originating as a marine species. This indicates that for stickleback, the cost of retaining osmoregulatory plasticity is small, or that the traits promoting euryhalinity in stickleback are under strong selection or pleitropically linked to other traits under strong selection. However, for fish living in marine and freshwater environments, the selection pressure for osmoregulation is still opposite, indicating that the stickleback must have alternative cell-regulating mechanisms for survival in unfamiliar salinities. In this study we only targeted gene expression values by quantifying the amount of mRNA extracted from gill tissue, however, the amount of mRNA does not necessarily imply equal concentrations of the functional proteins as a number of mechanisms can limit or increase the production. Protein expression profiles, also from kidney tissue, could have revealed different results. Other alternative strategies the stickleback may be utilizing could include changes in activity state of the ion-transport proteins by movement within the cell (activation/down-regulation), or by re-using the proteins by reversing the orientation in the cell-membrane, or by modulations following the interaction of other proteins [69,70,71].

Whatever method it is that the stickleback seems to be employing to osmoregulate so effectively, it has created a species that is incredibly well able to colonize new habitats regardless of the salinity they find themselves in, which has been a huge asset to this species in its spread throughout the northern hemisphere. Additional studies targeting the exact genetic and physiological mechanisms for the wide salinity tolerance in marine and freshwater stickleback are needed to understand the stickleback's incredible capacity for ion secretion and absorption. Their ability to adapt immediately to the environmental demands, with no apparent increase in physiological stress, is as unusual as it is intriguing, especially as this has been so evolutionarily important for this widespread species.

Supporting Information

Appendix S1 The Cq-values for the four targeted genes after normalization, ATP1A3, CFTR, KCNH4 and HSP70 in saltwater control (SS, dark orange), saltwater fish exposed to freshwater (SF, light orange), freshwater control (FF, dark blue) and freshwater fish exposed to saltwater (FS, light blue) for the nine different exposure periods. Higher values indicate lower expression values.
(EPS)

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Author Contributions

Conceived and designed the experiments: AT LAV KØ. Performed the experiments: AT TA KØ. Analyzed the data: AT TA LAV. Wrote the paper: AT LAV.

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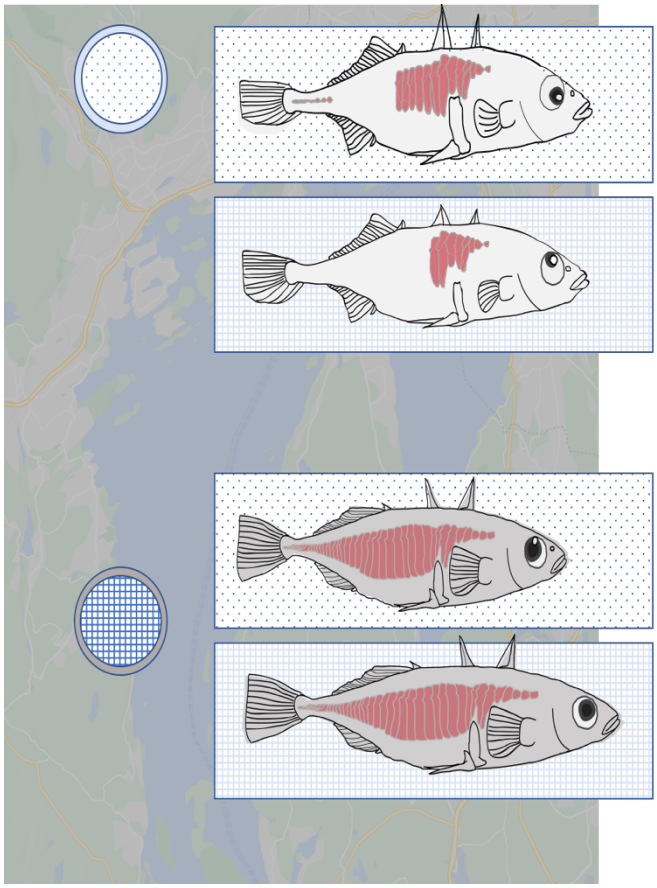

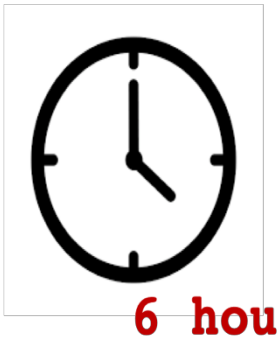

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RESEARCH ARTICLE

Salinity-induced transcriptome profiles in marine and freshwater threespine stickleback after an abrupt 6-hour exposure

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Abstract

Saltwater and freshwater environments have opposing physiological challenges, yet, there are fish species that are able to enter both habitats during short time spans, and as individuals they must therefore adjust quickly to osmoregulatory contrasts. In this study, we conducted an experiment to test for plastic responses to abrupt salinity changes in two populations of threespine stickleback, *Gasterosteus aculeatus*, representing two ecotypes (freshwater and ancestral saltwater). We exposed both ecotypes to abrupt native (control treatment) and non-native salinities (0‰ and 30‰) and sampled gill tissue for transcriptomic analyses after 6 h of exposure. To investigate genomic responses to salinity, we analyzed four different comparisons; one for each ecotype (in their control and exposure salinity; (1) and (2), one between ecotypes in their control salinity (3), and the fourth comparison included all transcripts identified in (3) that did not show any expressional changes within ecotype in either the control or the exposed salinity (4)). Abrupt salinity transfer affected the expression of 10 and 1530 transcripts for the saltwater and freshwater ecotype, respectively, and 1314 were differentially expressed between the controls, including 502 that were not affected by salinity within ecotype (fixed expression). In total, these results indicate that factors other than genomic expressional plasticity are important for osmoregulation in stickleback, due to the need for opposite physiological pathways to survive the abrupt change in salinity.

KEYWORDS
functional genomics, gene expression, osmoregulation, phenotypic plasticity, salinity tolerance

TAXONOMY CLASSIFICATION
Genetics

1 | INTRODUCTION

How organisms are able to adapt to changing environmental circumstances is still a central problem in biology (Chevin et al., 2010; Delgado & Ruzzante, 2020), as many fine-tuned mechanisms in one environment can be selected against in slightly different ecological settings. If possible, many organisms are able to use behavioral movements to avoid local stressful conditions (Sih et al., 2011; Wong & Candolin, 2015); fish can for instance avoid warm surface water by moving to deeper and colder areas, or evade fresher top layers in a fjord during snow melting in the spring. If movement is restricted, adaptive physiological responses may revert the organisms back to steady states within the new environment, in response to the new environmental cues, a phenomenon referred to as phenotypic plasticity (Pigliucci, 2001; Sangiao-Alvarellos et al., 2005). Although sounding like an optimal evolutionary force on the short scale, there is an ongoing debate whether phenotypic plasticity is hindering or facilitating genetic adaptation in the long term (Ghalambor et al., 2015); because plasticity may change the phenotypes available for selection after exposure to a new environment, and consequently influence further genetic adaptations. Furthermore, plastic responses can be either adaptive or nonadaptive (Svensson et al., 2020) with respect to the local phenotypic optimum, and is generally assumed to have an influence on evolutionary trajectories through the altered distributions of phenotypes (e.g., expressional profiles) upon which selection can act. Having a wide possibility for plastic responses is often viewed as beneficial for adaption to new environments, as this gives a higher chance of expressing a "new phenotypic optima" directly, which then can be genetically assimilated in the new environment (Levis & Pfennig, 2016; Waddington, 1961), that is, the induced expression pattern in the novel environment will become "fixated". Fixating environmentally induced plasticity through genetic assimilation should hence reduce genetic and plastic diversity in the derived population, where the rate of stabilizing selection depend on the number of loci that contribute to the additive genetic variance of the character(s) (Lande, 1976). Expression patterns of transcriptomes have been recognized as being plastic (Evans, 2015), implying that genetically similar individuals can have different transcriptome profiles (phenotypes) as a response to environmental cues (Mäkinen et al., 2016; Papakostas et al., 2014). Indeed, changes in gene expression can evolve very rapidly in many species, including fish (Roberge et al., 2008) and could, therefore, play an important role in the early steps of population divergence (Wolf et al., 2010).

For aquatic organisms such as fish, the difference between saltwater and freshwater represents considerably different selective forces. Most fish cells maintain a constant ion concentration, and few species are able to cross the salinity gradient between fresh and salt water (Delgado & Ruzzante, 2020). Most fish are therefore stenohaline, where the osmoregulatory machinery only operates within relatively narrow salinity boundaries (Hoar & Randall, 1984). Only about 3–5% of all fish species are euryhaline, meaning that they possess physiological mechanisms that allow them to adjust to a wide range of salinities (McCormick et al., 2012). Shortly, in saltwater, a fish will have a lower concentration of inorganic ions and hence a lower osmotic pressure compared to the environment, and the fish will passively gain ions and loose water (Evans et al., 2005; Rankin & Jensen, 1993). The situation for a freshwater fish is reversed, as the fish now has a higher concentration of ions when compared to the surroundings, and the fish passively gain water and loose inorganic ions. Consequently, to maintain homeostasis, saltwater fish drink saltwater, where excessive salts are actively secreted at the gills and water is absorbed in the intestine (Evans et al., 2005; Hoar & Randall, 1984), and freshwater fish actively absorb ions at their gills, minimize ion loss at their body surfaces, and actively reabsorb ions in their kidney to minimize urinary ion loss (Evans et al., 2005). Altogether, the cost of osmoregulation is highly variable, depending on salinity, oxygen, and temperature (Ern et al., 2014), where the total cost ranges from a few percent up to 30–50% of the total energy budget (Boeuf & Payan, 2001; Ern et al., 2014). In total, about 7% of the total energy budget can be spent in the gill tissue alone (Mommsen, 1984).

The threespine stickleback *Gasterosteus aculeatus* (hereafter stickleback) is a small fish known to have a wide salinity tolerance (Bell & Foster, 1994) at a seemingly low osmoregulatory cost (Grøtan et al., 2012). Originally of marine origin (Bell & Richkind, 1981), the stickleback has invaded and established populations in numerous freshwater habitats since the last glaciation in the northern hemisphere (Bell & Foster, 1994). Thus, stickleback populations are found at a wide osmotic range, spanning marine oceanic ecosystems, costal brackish water systems, freshwater rivers and lakes. Many populations have become landlocked after freshwater colonization, typically due to isostatic uplifting of the land following deglaciation, often restricting the gene flow between the founders and the derived populations. With reduced gene flow, and with freshwater habitats having a stable salinity compared to coastal waters, one would expect strong directional selection on traits that facilitate local adaptation to low salinity. Furthermore, one would also expect traits promoting a broad salinity tolerance (being euryhaline) to be selected against, due to the cost of sustaining characters that have not been required for many generations, and the low genetic variation typically found in derived populations (Schultz & McCormick, 2012). Genetic comparisons of marine and freshwater stickleback populations show signs of strong selection, and several outlier loci are identified by comparing whole genome sequences (Jones et al., 2012), SNPs (Guo et al., 2015; Hohenlohe et al., 2010; Jones, Chan, et al., 2012), and microsatellite genotypes (DeFaveri et al., 2011; Taugbøl, Junge, et al., 2014). Genetic studies further indicate that the frequency of freshwater-linked alleles can increase rapidly in newly colonized freshwater habitats (Lescak et al., 2015). However, with respect to gene expression and potential gene regulatory adaptations in response to salinity, previous experiments have either tested candidate genes (McCairns & Bernatchez, 2010; Taugbøl, Arntsen, et al., 2014); compared populations directly without exposure to non-native environments (Jones et al., 2012; Rastorguev et al., 2018); or tested for transcriptomic expression differences after a longer period of acclimatization in the non-native

environments (30 days to 3 months; Gibbons et al., 2017; Wang et al., 2014). However, less is known of the immediate expressional patterns following acute exposure to contrasting salinities. The objective of this study was to assess transcriptomic expression and compare regulatory changes in genes between marine and freshwater sticklebacks subjected to abrupt salinity transfers.

2 | MATERIALS AND METHODS

2.1 | Study sites, fish collection, and maintenance conditions

Fish used for this experiment were also part of a candidate gene study, and more methodological details can be found in Taugbøl, Arntsen, et al. (2014), Taugbøl, Junge, et al. (2014). Adult sticklebacks were captured at two locations near Oslo, Norway (Figure 1), during May and June 2010. The marine site, Sandspollen, has a salinity varying between 22‰ and 29‰, while the freshwater pond, Glitredammen, is stable at 0‰. Coastal stickleback populations in Norway are considered to be purely marine, potentially with some gene flow from nearby freshwater populations (Klepaker, 1996). Fish from Sandspollen breed locally (are not anadromous). The two locations are geographically isolated by approximately 35 km (shortest distance through water) (Figure 1b), where about 8.5 km is through the river Sandvikselva that contains several steep waterfalls and dams. This makes downstream movement of fish from Glitredammen toward the marine sampling site possible, but upstream movement from the fjord impossible. The stickleback in Glitredammen has probably been separated from marine ancestors for at least 7000 years, as the age of the lake has been estimated to be 7800 years before present using the program Sealevel32 (Møller, 2003). The lake Glitredammen is located at 82.2 m above sea level.

After capture, the fish were acclimated to holding conditions in their native salinity (30‰ or 0‰) for minimum 3 weeks prior to the experiment. To reduce potential male nesting behavior, the tanks were not equipped with any environmental enrichment, leaving the tanks free of sand and vegetation. The temperature in the tanks was maintained at room temperature (about 20°C), and the light regime was set at a 12:12 light: dark-cycle. The fish were fed two times a day with frozen red bloodworms throughout the acclimation period. More details on fish maintenance can be found in Taugbøl, Arntsen, et al. (2014).

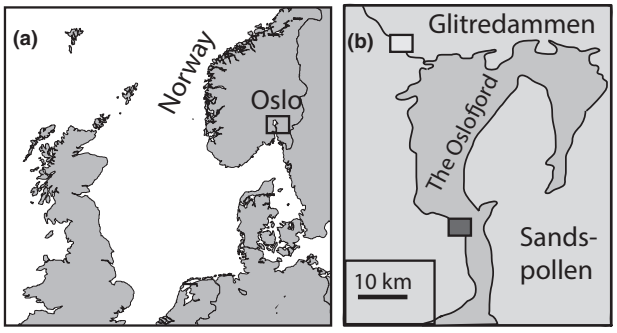

FIGURE 1 Study area. (a) map of Norway showing the position of the sampling sites just south of Oslo, (b) illustrates the two sampling locations: Glitredammen (freshwater) and Sandspollen (saltwater).

2.2 | Experimental design

The experimental setup consisted of two 80-L tanks (30‰ or 0‰), equipped with a gray plastic wall with punctures, dividing each experimental tank into two 40-L compartments. At the start of the experiment, eight randomly selected fish that appeared healthy were collected from each acclimation tank and placed directly in separate, freshly prepared, experimental compartments. Each salinity/ecotype was tested in their native salinity; saltwater control (SwC) and freshwater control (FwC), and in their non-native salinity; salt water ecotype exposed to freshwater (SwFw) and freshwater ecotype exposed to salt water (FwSw) (Figure 2). The fish were kept in the experimental tanks for 6 h before they were quickly netted out, immediately killed by a swift blow to the head, and directly processed for gill tissue sampling. Gills were used as they play an important role in the maintenance of blood ion and acid–base balance (Evans et al., 2005; Hoar & Randall, 1984). The experiment was approved by the Norwegian Animal experimentation and care committee (permit no ID 2705) and all efforts were made to minimize suffering. Large and sudden changes in salinity can influence survival and growth (Bachman & Rand, 2008); however, no fish died during the 6-hour exposure, and results from the same experimental setup demonstrate very low mortality rates also when exposing fish for up to 3 weeks (Taugbøl, Arntsen, et al., 2014). Also, the same stickleback populations did not express differences in oxygen consumption rate after 14 days of exposure (Grøtan et al., 2012), indicating that long-term salinity change has reverted the organisms back to steady states through adaptive physiological responses.

2.3 | Tissue collection, RNA isolation, library preparation, and sequencing

After each fish was sacrificed, gill samples were immediately collected using sterilized tweezers and stored in RNA*later*® (Ambion® RNA; Life Technologies™). Of the 8 fish that were exposed in each experimental group, a total of three fish from each control (SwC and FwC) and a total of five fish from each exposure group (SwFw and FwSw) were processed for sequencing. For these 16 samples, messenger RNA (mRNA) was isolated from the gill tissue from each sample separately, using the mRNA direct kit with dynabeads (Invitrogen) as described by the manufacturer. The mRNA concentration and purity were quantified using an RNA 6000 Pico Kit on an Agilent 2100 Bioanalyzer (BioRad) according to the protocol, and all samples were diluted down to 0.125 µg/µl before cDNA synthesis. Libraries

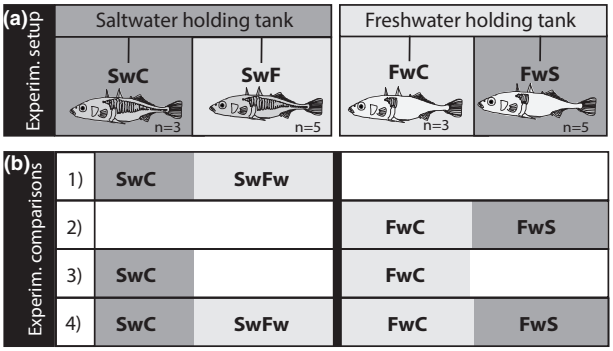

FIGURE 2 Experimental design. (a) Wild caught fish from saltwater and freshwater (see Figure 1) were taken into the laboratory and placed in holding tanks of their native salinity for a minimum of 3 weeks. After acclimation, two groups of eight fish from both populations/holding tanks were exposed to either saltwater (30‰; dark gray) or freshwater (0‰; light gray) for 6 h. The exposure tanks were divided in two by a perforated wall, so both populations could be exposed to the same water quality at the same time (SwC and FwSw shared tank, as did FwC and SwFw). (b) the four comparative analytical setups for which we compared the gene expression patterns: Comparison (1) saltwater control (SwC) compared to saltwater fish exposed to freshwater (SwFw); (2) freshwater control compared to freshwater fish exposed to saltwater (FwSw); (3) the two control groups; SwC compared to FwC; and (4) ecotype independent of salinity.

for RNAseq were prepared using the TrueSeq™ RNA low-throughput protocol (Illumina), where all samples were fragmented for 4 min to obtain the required size distribution. All libraries were sequenced as 100 bp paired-end at the Norwegian Sequencing Centre on the Illumina HiSeq 2000.

2.4 | Aligning the gill transcriptome to the reference genome, filtering, and normalization of transcripts

The reads were mapped toward the *Gasterosteus aculeatus* genome with corresponding gene annotation (BROAD S1 Ensembl release 90, 2017) using bowtie2 v2.2.2 and tophat2 v2.0.14, and assembly of transcripts and expression performed using cufflinks v2.1.1. according to the Trapnell et al. protocol (Trapnell et al., 2012). All steps were done on the high-performance computing cluster Abel at the University of Oslo (now replaced by the computing clusters hosted by Sigma2).

All analyses were run in R 4.0.1. (R Development Core Team, 2020) using the package edgeR (Robinson et al., 2010). First, the number of expressed reads for the individuals was compared as if an individual by chance was sequenced at greater depth, which might give this individual an unnaturally high gene expression count. The number of expressed reads before filtering varied from 3.832.109 to 25.515.142 (with an average of 8.756.949). The library sizes were adjusted by transforming the raw-scale libraries to logged counts per million (CMP), before filtering out the genes with low expression values, as low read counts are a significant source of measurement error in differential expression analyses (Robinson & Smyth, 2007). Filtering out insignificant genes also increases detection power of significant discoveries (Bourgon et al., 2010). Therefore, a total of 597 genes (2.87%) that were unexpressed across all samples were excluded. We further excluded genes that was expressed at low levels across the individuals by the use of the function "FilterByExpr"; keeping genes with a CMP-value of >0.68, and being expressed in at least three individuals, as that was the lower group size (Figure S1).

As small differences in expression of highly expressed genes between samples can give the appearance that many of the low-expressed genes are differentially expressed between treatments, we normalized the read counts among libraries with the function "calcNormFactors". This function uses the method of trimmed means of m values (TMM; Robinson & Oshlack, 2010) and normalizes the data by removing the extremely lowly and highly expressed genes, and also removes the genes that are very differentially expressed between samples, by keeping genes that was expressed at least six–seven times in the smallest sample, and being expressed in at least two of the libraries. The "calcNormFactors" then compares the total count for this subset of genes between the two samples and calculates a set of scaling factors for the library size that minimizes the log-fold changes between samples for most genes, as the method assumes that the majority of genes are equally expressed between any two samples. The scaling factors in this study varied from 0.764 to 1.281 (Table S1). The calculated effective library size is then used as the original library size in all downstream analyses (Figure S2). Out of the 20.789 genes retrieved from the stickleback genome (Ensembl version 90), a total of 16.211 genes (77.9%) were kept for further analysis.

As the variance in RNA-seq measurement of gene expression is typically overdispersed, a negative binominal distribution is used to model the variance. We calculated the common dispersion, using the same value for dispersion when modeling the variance for each gene, with the function "estimateCommonDisp," and found the biological coefficient of variation to be 0.364, meaning that the true abundance for each gene can vary up or down by 36.4% between replicates. Each gene likely differs in dispersion, and the common dispersion model was extended to model the mean variance relationship between genes and the dispersion estimation per gene was calculated, shrinking the dispersion toward the trended dispersion due to low sample sizes, by the function "estimateGLMTagwiseDisp". The results are visualized with a multidimensional scaling (MDS) plot, using the pairwise biological coefficients of variation as a distance measure to visualize the overall expressional relationships between individuals, and a principal component analysis (PCA) of the gene expression profiles using the *prcomp* function in R on $\log_2$-transformed data.

2.5 | Differential expression analysis between experimental groups

Differences in normalized transcript abundance levels were tested using a generalized linear model (glmQFit and glmQLFTest), with

log_2-transformed transcript abundance as the response variable with a genome-wide false discovery rate (FDR) of 0.05 with the function "*Toptags*". The results of the gene expression differences are presented as centered and scaled heatmaps, plotted with gplots (Warnes et al., 2020). The groups that were compared was: comparison (1) saltwater control (SwC) versus saltwater ecotype exposed to freshwater (SwFw); comparison (2) freshwater control versus freshwater ecotype exposed to saltwater (FwSw); comparison (3) controls (FwC- SwC) and comparison (4) transcripts that are significantly different between controls, and similarly expressed within ecotype; all freshwater ecotypes versus all saltwater ecotypes (FwC_FwSw vs. SwC_SwFw) (Figure 2b). Comparison 3 identifies transcripts that are differentially expressed between the control groups, and to a lesser extent between comparison 1 and comparison 2, but where expression patterns *within* ecotype and salinity change do not differ *significantly*. Comparison 4 identifies genes that are similarly expressed within salinity of origin (the *ecotype*) independently of treatment. The transcripts in comparison 4 were extracted from the transcripts identified in comparison 3, by calculating the average expression for each transcript within each group (SwC, SwFw, FwC, FwSw) and extracting the genes that had <0.4 CMP differential expression between the respective control and exposed group. Overlapping genes in the four comparisons were identified and extracted with the package Venn diagrams (Gao, 2019).

2.6 | Functional analysis of the differentially expressed genes: Gene ontology analysis

To identify potential biological functions that were overrepresented in the expressed genes, the differentially expressed up- and downregulated transcripts were extracted and tested for enriched Gene Ontology (GO) terms with the R package topGO 2.30.1 (Alexa et al., 2006) for biological processes (BP). The GO terms were extracted from the stickleback genome in Ensembl (n = 1829 GO-terms that were linked to the 16.111 transcripts). Checking for significance, a classical Fisher test on GO-terms with 10 or more annotated genes were used as a cut-off. The top 10 results are presented as tables on sorted weighted values.

3 | RESULTS

In this study, we performed transcriptome sequencing of individuals from two ecotypes of stickleback (saltwater and freshwater) being exposed to abrupt changes in salinity compared to their native environments. To investigate their overall response, we analyzed four different comparisons (outlined in Table 1): one for each ecotype, one between ecotypes in their control experimental salinity, and one aimed at finding transcripts that were equally expressed within ecotype regardless of salinity, through gene expression patterns.

TABLE 1 Number of transcripts for the different experimental comparisons, separated in up- and downregulated patterns (regulation), and the number of transcripts that were annotated to genes.

experimental Comparison	Regulation	Number of transcripts	Number annotated (%)
(1) SwC-SwFw	Up in SwFw	3	3 (100)
	Down in SwFw	7	3 (42)
	Total	10	6 (60)
(2) FwC-FwSw	Up in FwSw	691	569 (82.3)
	Down in FwSw	844	671 (79.5)
	Total	1535	1240 (80.7)
(3) SwC- FwC	Up in FwC	755	549 (72.7)
	Down in FwC	559	401 (71.7)
	Total	1314	950 (72.2)
(4) Ecotypes	Up in FW	329	265 (80.5)
	Down in FW	173	137 (79.2)
	Total	502	402 (80.0)

Abbreviations: SwC, saltwater control; SwFw, saltwater fish exposed to freshwater; FwC, fresh water controls; FwSw, freshwater exposed to saltwater.

3.1 | Overall gene expression

When comparing all the 16.211 transcripts that were kept after filtration and normalization, the first axis of the multidimensional scaling (MDS) plot separated saltwater fish (SW) from freshwater fish (FW), whereas the second axis in part separated the exposure groups from their native salinity (Figure 3a). The Principal Component Analysis (PCA) on logged values of all expressed genes after filtration and normalization also separated the groups based on original salinity and treatment, where PC1 explained a total of 88.29% of the variation, and PC2 explained 1.88% (Figure 3b). Both the MDS and PCA separated the samples according to their original ecotype indicating that the overall gene expression pattern is highly linked to "original" environment.

In total, we identified 2717 unique transcripts that were significantly differentially expressed in all the analyzed contrasts (Table 1, Figures 4, 5, Table S1). Interestingly, comparing saltwater fish in salt- and freshwater only reported 10 differentially expressed transcripts (Figure 4, 5a). The contrast between freshwater fish in fresh- and saltwater gave the highest number of differentially expressed transcripts (~1500, Figures 4, 5b,c), followed by a comparison of the two controls (~1300, Figures 4, 5c,d) and differences in eco-transcripts in salt and freshwater (~500; also being differentially expressed between the controls in comp. 3, Figures 4, 5e).

3.2 | Genes differentially expressed in response to salinity (comparison 1 and 2)

When contrasting SwC and SwFw (comparison 1), a total of 10 transcripts were significantly differentially regulated, six which were annotated. Of the 10 transcripts, 3 transcripts were significantly

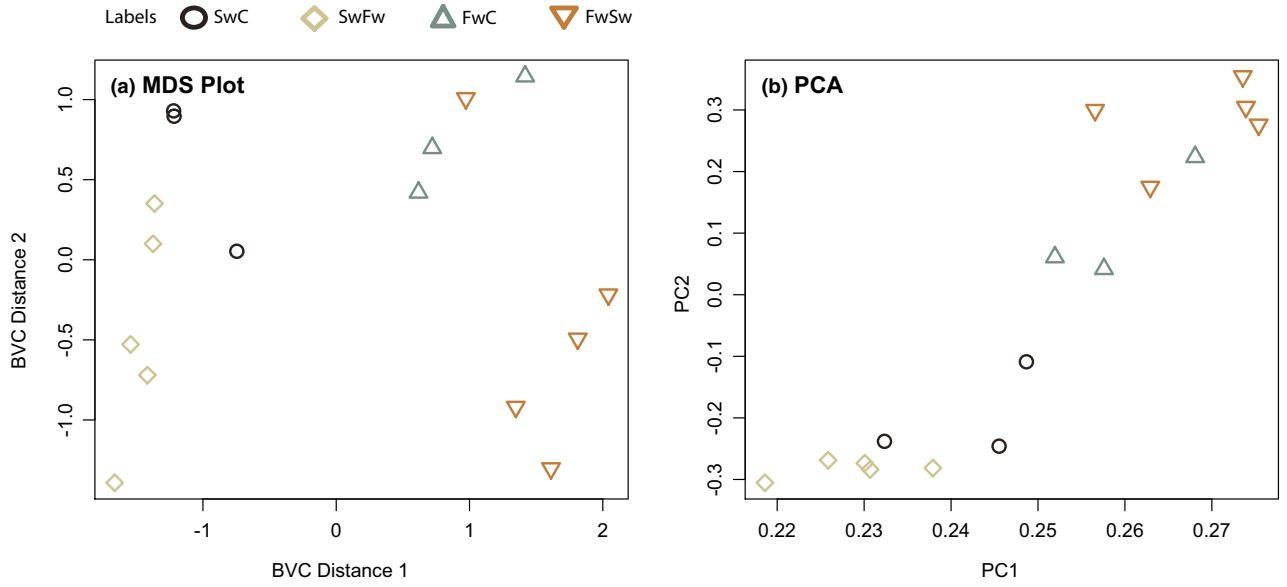

FIGURE 3 (a) MDS plot. Multidimensional scaling (MDS) plot generated with edgeR, where each point represents one sample and the distance between individual samples reflects the leading fold-change (logFC) of the corresponding RNA samples. The leading logFC is the average (root mean square) of the 500 largest absolute logFCs for genes between those two samples (default plotting parameter) (two SC individuals are overlapping on the top), (b) principal component analysis (PCA) of gene expression profiles for all genes after filtering and normalization. FC, blue triangles pointing up; FS, orange triangles pointing down; SC, black circle's; SF, beige diamonds (see Figure 1 for abbreviations for groups).

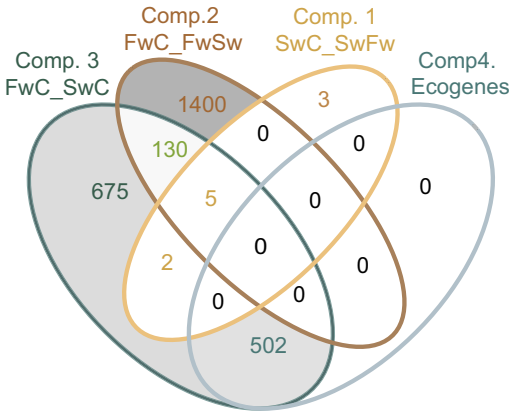

FIGURE 4 Venn diagram illustrating the total number of differentially expressed transcripts for the four different comparisons, see Figure 2 for abbreviations for groups.

different only for comparison 1 (Figure 4). Two of the transcripts were annotated; a gene predicted to be involved in the elongation factor process (*si:ch211-13k12.2*) and the enzyme Galactosylceramide sulfotransferase (*GAL3ST1*). Five transcripts were shared with comparison 2: an arrestin domain (*arrdc3a*), a solute carrier (SLC16a9a) (Figure 6a), a cytochrome P450, family 1 (*CYP1a*) (Figure 6b), a docking protein (*dok4*), and a novel gene, *ENSGACG00000019379*, which share sequence homology with TBC1 family member 24 (BlastP GenBank) that has a potential function in intracellular trafficking. Two additional transcripts, which were also not annotated, were shared with comparison 3 (Figure 4).

Of the 1535 genes reported in comparison 2, between freshwater ecotypes, a total of 691 transcripts (569 annotated) were upregulated in FwSw when compared to FwC (Table 1). The upregulated gene list included several ion transporting ATPases; ATPase Na+/K+ transporting subunit beta 1a (*ATP1b1a*), ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1 (*ATP2a1*, Figure 6b), and ATPase phospholipid transporting 11C (*ATP11c*); two ABC transporter genes were upregulated in FwSw (*ABCf2a* and *ABCb6a*) which belong to a family of proteins that utilize the energy of ATP binding and hydrolysis to transport various substrates across cellular membranes. A total of 20 different solute carriers were also upregulated in FwSw, and two genes involved in vesicular trafficking; transmembrane emp24 trafficking protein 2 (*tmed2*, Figure 6b) and 10 (*tmed10*). Genes linked to stress included five Heat Shock Protein (HSP) genes (*HSPa4b*, *HSPa5*, *HSPa8* (Figure 6b), *HSPa8b*, *HSPd1*) and seven ubiquitin specific peptidase (USP) genes. The *HSPa8* and *HSPa8b* encodes members of *HSP70*, and four DnaJ homologs which are co-chaperones of the HSP70 family were also upregulated in FwSw (*dnaja1*, *dnajb1a*, *dnaja2a*, *dnajb9b*). Gene ontology enrichment of the upregulated genes involved processes such as peptide biosynthetic process, cellular response to xenobiotic stimulus, and glutathione metabolic process (Table 2).

A total of 671 annotated genes were downregulated in FwSw when compared to FwC (Table 1). Several known genes had reduced expression for freshwater fish when in saltwater; six genes related to ion-transporting ATPases, including five related to phospholipid transporting (*ATP8a1*, *ATP8b2*, *ATP9a*, *ATP9b*, and *ATP10d*), and one linked to metallopeptidase and ATP synthase assembly factor

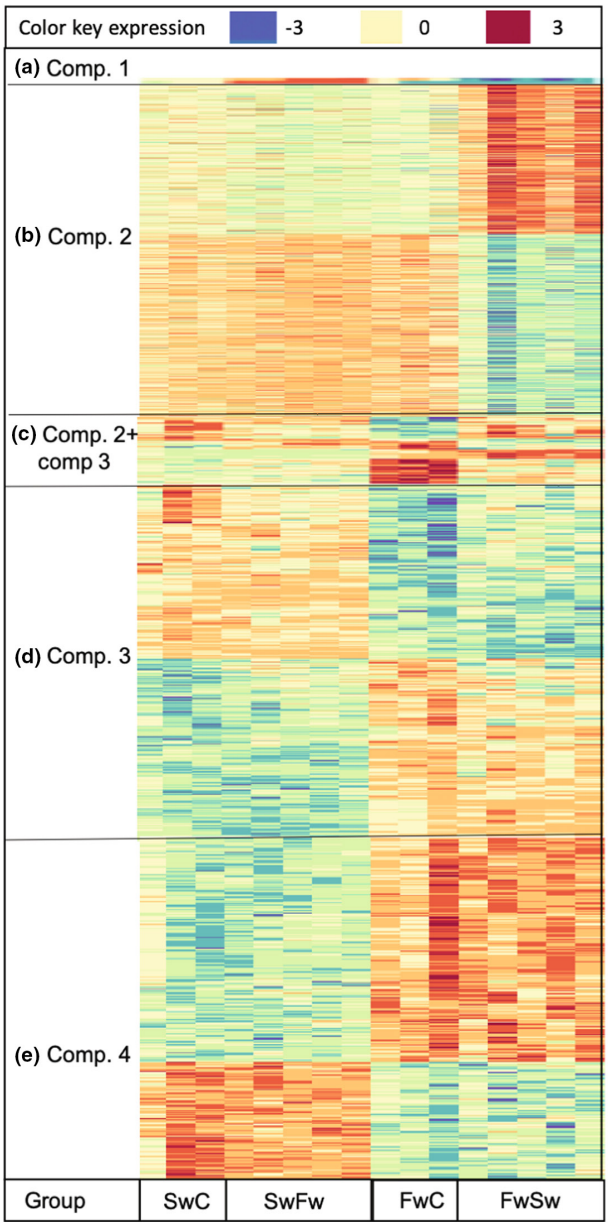

FIGURE 5 Heatmaps of the differentially expressed annotated genes from the different comparisons (comp), see Figure 2 for experimental comparisons and Figure 4 for the number of overlapping transcripts between the analyzed contrasts: (a) shows the results of all 10 transcripts that are significantly different in comparison 1, including the transcripts that overlap with comparison 2 and comparison 3; (b) shows the 1400 transcripts only significantly expressed in comparison 2; (c) shows the 130 transcripts that are significantly differentially expressed between both comparison 2 and comparison 3; (d) shows the 675 transcripts differentially regulated between comparison 3; and (e) shows the 502 transcripts that are different in comparison 4, the ecogenes. Blue color indicates less expression, red color indicates a higher expression.

homologs (*ATP23*). Furthermore, seven different solute carriers were downregulated, as were genes linked to the potassium voltage-gated channel (*KCNd3*), the potassium inwardly rectifying channel (*KCNj2a*), a potassium channel tetramerisation domain (*KCTd12b*) and the chloride channel, voltage-sensitive 6 (*CKCn6*). Two genes linked to calcium regulation; an EF-hand calcium-binding domain (*EFcab7*) and a transient receptor potential cation channel gene (*TRMP7*), and two genes linked to thyroid hormone signaling, thyroid hormone receptor interactor 10a (*trip10a*) and thyroid hormone receptor interactor 4 (*trip4*). Related to osmosensing, and involved in the activation and differentiation of immune cells, five interlukins all had similar expressions across groups, except for FwSw where they were downregulated (*ILf3a*, *ILf3b*, *IL113ra2*, *IL15*, *and IL19*). No HSP genes were downregulated, but one ATP-binding cassette (*ABCh1*), three DnaJ homologs (co-chaperones of the HSP70- family: *dnajc7*, *dnajc9*, and *dnajc11b*), together with three USP genes (*USP31*, *USP40*, *and USP45*) all had lower expression in FwSw. When testing all downregulated genes in this comparison, transcripts for GO enrichments processes such as DNA repair, nucleosome assembly, cilium assembly were included on top of the list (Table 2).

3.3 | Genes differentially expressed between ecotypes (comp 3 and 4)

In comparison 3 we only discuss differences between the two controls (Figure 2). Genes that were upregulated in SwC compared to FwC included the Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (*ATP2a1*), an enzyme that catalyzes the hydrolysis of ATP with the translocation of calcium from the cytosol to the sarcoplasmic reticulum lumen; several stress-related cytochrome P450 (*CYP1a* [also overlapping with comparison 1 and 2], *CYP1c1*, *CYP1c2*, *CYP2y3*, *CYP7a1*), seven genes in the solute carrier family (including *SLC16a1b*), one myosin (*myh11a*), where the class XI myosins are associated with various organelles/vesicles (Tian et al., 2021). Genes upregulated in FwC compared to SwC included Aquaporin 3a (*AQP3a*), several ATPs (*ATP6V0C*, *ATP6V0C*, *ATP6v1d*, *AP6v1e1b*; Figure 7), arrestin domain containing 2 (*arrdc2*), 11 solute carriers, and prolactin Receptor type a (*PRLRa*) had a higher expression in freshwater, and a slight regulation within saltwater, as SwFw had a lower expression than SwC (Figure 6c).

Of the 502 transcripts that had apparently similar expression within ecotype despite salinity change, 173 had a higher expression in the saltwater ecotype, and included one gene linked to ATP transport, ATPase phospholipid transporting (*ATP8a2*), two linked to calcium transport; calcium channel, voltage-dependent (*cacnb1*, Figure 6d) and anoctamin 1, calcium-activated chloride channel (*ano1*, Figure 6b), NADPH oxidase 1 (*nox1*), one solute carriers (*SLC6a1like*), interleukin 11 receptor (*IL11ra*) and genes linked to tyrosine kinase proteins, which in turn has been found to function as activators of several ion-pumps; protein kinase 7 (*PTK7*), protein tyrosine phosphatase nonreceptor (*PTPN14*) and receptor type (PTP*rfa*), and erb-b2 receptor tyrosine kinase 3b (*erbb3b*). A total of 329 transcripts had a higher expression in the freshwater ecotype, and included several ATPs (*ATP6V0e1*, *ATP6V1A*, *ATP6V1c1a*, *ATP6V1f*, Figure 7), where one is involved in T-cell regulation

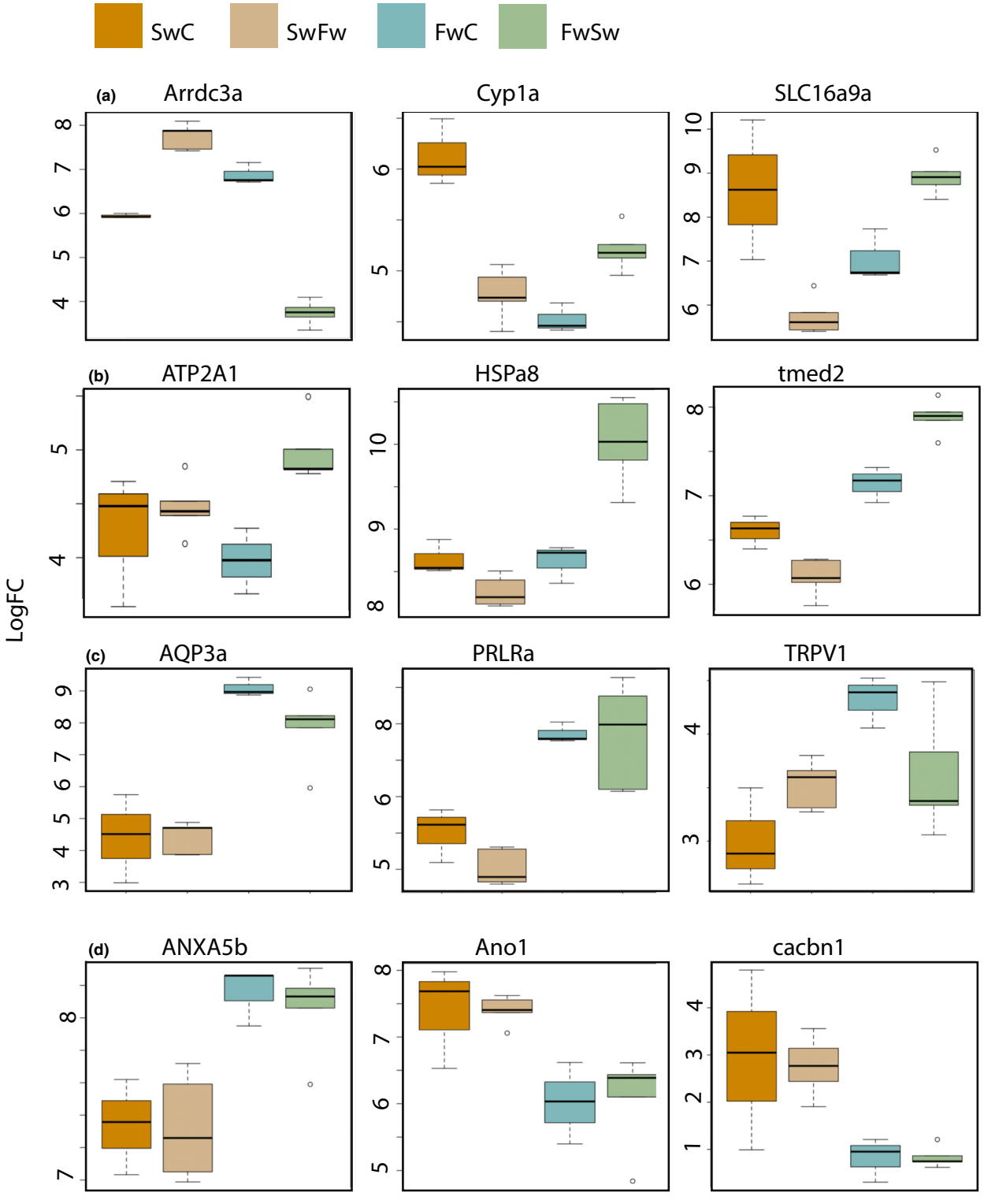

FIGURE 6 Examples of differentially expressed genes for the four different groups (SwC, SwFw, FwC, FwSW) plotted as boxplots, of (a) genes differentially regulated in comparison 1; (b) genes differentially regulated in comparison 2; (c) genes differentially regulated in comparison 3, and (d) genes differentially regulated in comparison 4. The boxplots show the 25–75% quantiles (boxes), median (black horizontal line), 95% limits (bars), and outliers (open circles).

TABLE 2 The top 10 gene ontology terms with significant number of annotated transcripts separated in upregulated and downregulated terms for biological processes

	GO.ID	Term	Annotated	Significant	Expected
Upp in FwSw	GO:0043043	Peptide biosynthetic process	283	15	12.54
	GO:0071466	Cellular response to xenobiotic stimulus	20	6	0.89
	GO:0006749	Glutathione metabolic process	28	7	1.24
	GO:0006364	rRNA processing	69	11	3.06
	GO:0044272	Sulfur compound biosynthetic process	47	8	2.08
	GO:0044262	Cellular carbohydrate metabolic process	57	9	2.52
	GO:0030835	Negative regulation of Actin filament de...	26	4	1.15
	GO:0019725	Cellular homeostasis	87	9	3.85
	GO:1901568	Fatty acid derivative metabolic process	23	5	1.02
	GO:0043065	Positive regulation of apoptotic process	25	5	1.11
Down in FwSw	GO:0006281	DNA repair	180	28	9.04
	GO:0006334	Nucleosome assembly	26	9	1.31
	GO:0060271	Cilium assembly	127	20	6.38
	GO:0034204	Lipid translocation	23	6	1.16
	GO:0090305	Nucleic acid phosphodiester bond hydroly...	105	11	5.27
	GO:0015914	Phospholipid transport	34	6	1.71
	GO:0006865	Amino acid transport	26	5	1.31
	GO:0033044	Regulation of chromosome organization	26	5	1.31
	GO:0016570	Histone modification	125	14	6.28
	GO:0006914	Autophagy	77	9	3.87

Note: The table shows results from comp. 2, were freshwater control (FwC) are compared to freshwater fish exposed to saltwater (FwSw) and in which direction the terms are regulated based on the FwSw fish. All terms significant with *p*-values <.01.

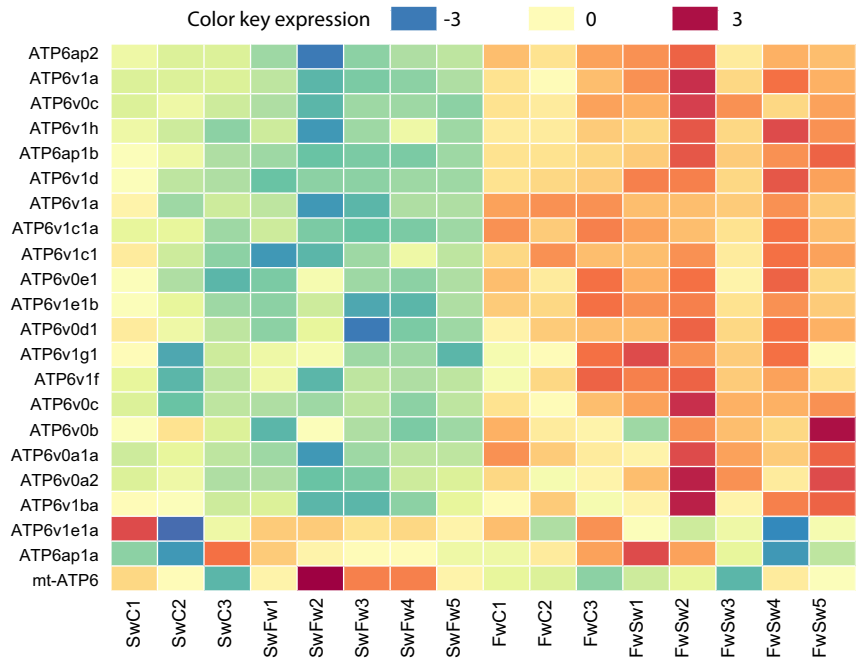

FIGURE 7 Heatmap of ATP6V0 and ATP6V1. For these two gene families, almost all transcripts showed signs of differential expression between the two ecotypes regardless of exposure treatment. The heatmap sums up individual fish on the X-axis; Swc, saltwater control fish ($n = 3$); SwFw, saltwater fish exposed to freshwater ($n = 5$); FwC, freshwater control fish ($n = 3$) and FwSw, freshwater fish exposed to saltwater ($n = 5$).

(*tcirg1a*); four annexin genes, which are linked to salinity stress in plants (*ANXAa1*, *ANXA3b*, *ANXA5b* [Figure 6d] and *ANXA11b*), two linked to calcium: calcium homeostasis modulator family (*calhm5.2*) and mitochondrial calcium uptake (*micu2*) and one stress-related UDP-glucose (*ugp2b*). The freshwater ecotype also had a higher expression of claudins (*cldn1*, *cldn7b*, *cldnf*) and occludins (*ocInb*), proteins that are involved with tight junctions and reduction of ion efflux from the cells.

4 | DISCUSSION

Here, we have investigated the transcriptomic response of an acute change in salinity for an allopatric freshwater and a saltwater ecotype of the threespine stickleback in Norway that have previously shown little energetic costs of salinity transfer (Grøtan et al., 2012). Within the 6-hour exposure in this study, very few genes were significantly differentially expressed in saltwater stickleback transferred to fresh water (comparison 1), whereas about 1500 transcripts were differentially regulated in freshwater stickleback transferred to saltwater (comparison 2). Furthermore, over 1300 transcripts differed between the controls, including ~500 transcripts that did not express significant changes in regulation within salinity, but between ecotypes. These results indicate that the ability to adjust following a change in salinity is maintained by both ecotypes, but the gene expression cost of the transition seems much larger for the freshwater ecotype. As there were so few genes with significant plastic expression in contrast 1, we could not compare expression patterns toward higher or lower salinity for these ecotypes (salinity and ecotype interactions), and we could hence not find dominating gene groups equally important for abrupt salinity transfers in either direction. Many of the genes found to be differentially regulated within freshwater and between ecotypes in this study are known to be critical for ion regulation, as they facilitate transport through energy conversion, or are directly involved in building ion channels, ion pumps or suppress passive ion diffusion. Gills consists of different cell types, and some differentially expressed genes are involved in restructuring the gill tissue, through, for example, tightening the junctions between chloride cells in freshwater and likely increasing the density of the chloride cell type itself in saltwater (Perry, 1997). By also moving the control fish over to a new aquarium, the observed stress responses in this study should hence only relate to the salinity changes and the handling itself. Many genes in the HSP family, in addition to other stress-related genes, were found to increase when freshwater fish were exposed to saltwater, but not for the saltwater fish when they were exposed to freshwater. Taken together, contrasting results between the two ecotypes strengthens the theory of many different evolutionary pathways to physiological freshwater adaptations in stickleback (DeFaveri et al., 2011; Gibbons et al., 2017) and other fish species (Velotta et al., 2017). Many genes linked to complex physiological regulatory mechanisms showed evidence of adapted expression profiles between the two ecotypes, supporting evolutionary adaptation via genetic assimilation and overall genomic reduction in phenotypic plasticity within the gill transcriptome, similar to findings in other fish species (Velotta et al., 2017).

4.1 | Plastic gene expression profiles within ecotypes

A fish adapted to a particular salinity needs to have complex physiological regulatory mechanisms, at both the organismal and cellular level, in order to maintain water homeostasis. Changes in salinity will induce alterations in the nature and direction of ion transport, and genes linked to the maintenance of water homeostasis, cell signaling and structural permeability of cell membranes and stress responses, are likely targets of short-term salinity responses. To start a physiological response, the fish must be able to recognize osmolarity change, where input from the osmolarity sensors also need to encode the magnitude, direction and ionic basis of the perceived change. Of the few genes with significant regulatory differences in both comparison 1 and 2, arrestin and SLC-genes are linked to early osmosensory signal transduction. The solute carriers are membrane transport proteins mostly located in the cell membrane where they facilitate movement of small solutes across cell membranes in response to chemiosmotic gradients. A total of 27 SLC-related transcripts were linked to salinity changes within the freshwater ecotype (contrast 2), including the Na^+/H^+-exchanger (*SLC9a2*) with a higher expression in freshwater that also have been found in long-term studies of freshwater acclimation in stickleback (although different isoforms; Gibbons et al., 2017). As expected, the $Na^+/K^+/2CL^-$ cotransporter (*NKCC1*/ *SLC12a2*) had higher expression in saltwater, which also is consistent with findings in long-term salinity exposures in stickleback (Gibbons et al., 2017) and other species of saltwater fish (Shaughnessy & McCormick, 2020). That *NKCC1* has a central role in salinity acclimation is further supported with no expression in gills of the salmonid grayling (*Thymallus thymallus*), which is a strict freshwater fish (Varadharajan et al., 2018). Three members of the monocarboxylate transporter family (MCT/*SLC16*) were upregulated in saltwater (Figure 6a), where *SLC16a9a* was one of the few genes differentially regulated in contrast 1. MCT's are involved in H^+-linked transport of monocarboxylic anions (Verri et al., 2012), that again are linked to the level of carnitine and energy metabolism by the transport of long fatty acid chains, like lactate, into mitochondria for energy production and between cell types. Fish gills are highly oxidative tissues, and oxygen requirements increase with increasing salinities (Vijayan et al., 1996), which again increase the natural concentrations of both plasma and gill-cellular lactate (Mommsen, 1984; Sangiao-Alvarellos et al., 2003, 2005). Lactate might hence be the primary candidate for rapid carbohydrate fuel in the gill tissue, especially for the saltwater fish in the early responses to reduced salinity.

Arrestin (*arrdc2*, *arrdc3a*, and *arrdc3b*) was downregulated in saltwater and *arrdc3a* was also included among the ten transcripts differentially regulated with salinity in saltwater fish (Figure 6a). Arrestins have been found to be involved in the modulation of diverse cellular processes through their adaptor functions, facilitating the localization and function of other proteins. Arrdc3a is linked to the GPCR regulation of the adrenergic signaling pathway, which again is linked to cellular Na^+ regulation (Kumai et al., 2012), to increased insulin and glucose metabolism in mice livers (Batista et al., 2020), to growth in plants under salinity stress (Colaneri et al., 2014), and to stress and phosphorylation of the actin-cytoskeleton in a soil amoeba, *Dictyostelium* (Habourdin et al., 2013). Arrestins have also been linked to positive activation of MAP kinases (Lefkowitz & Shenoy, 2005), a family of enzymes involved in osmosensory signal transduction (Fiol & Kültz, 2007),

several of which are differentially regulated between comparisons 2 and 3 in this study. Arrestins have also previously been linked to other short time osmoregulatory experiments, being downregulated in turbot (*Scophthalmus maximus*) livers, when turbot acclimated to 30‰ salinity was exposed to 5‰ for 24 h (Cui et al., 2020), and four homologs of Arrestin was upregulated in the shrimp (*Halocaridina rubra*) (Havird et al., 2019) and crab (*Portunus trituberculatus*) (Lv et al., 2016) when they were transferred from 32‰ to 15‰ salinity, also for 24 h (similar results as in this study). That arrestins could have an important role in being "osmosensory genes" is also supported by DNA sequences for the arrestin gene *arrb2b*, as the sequences for stickleback and another euryhaline fish, the tiger pufferfish (*Takifugu rubripes*), were found to be more diverse from their alpha counterpart, *arrb2a*, than for several other fish species, which likely is a result of directional selection (Indrischek et al., 2017).

Several cytochrome P450 genes were upregulated in saltwater, including *CYP1a* that was one of the ten transcripts with differential regulation in contrast 1 (Figure 6a). *CYP1* is a superfamily of enzymes that catalyzes the oxidation of many reactions, and is widely used as an indicator of environmental pollution, also for stickleback (Knag & Taugbøl, 2013). The historic focus on *CYP1* as "only" a pollutant biomarker might have constricted the assessments of many related results to other potential pathways (Evans et al., 2005), as recent findings indicate a more direct link to general stress- and immune responses (Lenoir et al., 2021). The translation and expression pattern of *CYP1a* is being regulated by the aryl hydrocarbon receptor, *AHR*, which after heterodimerizing with *ARNT*, also is functional in immune cells of Atlantic salmon (*Salmo salar*) (Song et al., 2020), and when overexpressed, *CYP1a* has been found to actively suppress the expression of interferon type 1 (*IFNI*) (but *not IRF7*) in grass carp (*Ctenopharyngodon idella*), interferons that are secreted by infected cells (Chu et al., 2019). In previous salinity treatment experiments including fish, *CYP1a* has been found to be both upregulated and downregulated with salinity; Wang et al. (2014) found sticklebacks to have the highest expression in their original water quality (salt- and freshwater) when compared to freshwater fish in both 11‰ and 34‰ after 30 days of exposure (saltwater fish was only exposed to saltwater in this study), whereas *CYP1a* was found to be upregulated in tiger puffer after 30 days of exposure in the low salinity group (Jiang et al., 2020), and opposite, to increase with increasing salinities in coho salmon (*Oncorhynchus kisutch*) (Lavado et al., 2014) and rainbow trout (Leguen et al., 2010), as is similar to this study.

Maintaining cell volume is critical during salinity changes. Tight junction proteins such as claudins and occludins were upregulated in freshwater (comparison 4), similar to a long-term salinity study on stickleback (Gibbons et al., 2017). Aquaporin 3a (*AQP3a*), a water channel protein linked to cell volume regulation and sensing, also had higher expression in freshwater, which is commonly found in euryhaline fish (Cutler et al., 2007; Velotta et al., 2017). In this study, AQP3a had a slight plastic change within the freshwater ecotype, as FwS had lower expression than *FwC* (Figure 6c). Aquaporin expression has been found to be involved in the meditation of osmoreception in the tilapia prolactin secretion and gill chloride cell differentiation (Yan et al., 2013), and the DNA sequence for aquaporin in sticklebacks has previously been associated with positive selection between marine- and freshwater populations (DeFaveri et al., 2013; Shimada et al., 2011), as has the gene expression patterns (Gibbons et al., 2017). Interestingly, in a purebred stickleback cross-fostering experiment in 20‰ and 5‰, the expression pattern for *AQP3a* was equally expressed in the freshwater ecotype, and the saltwater ecotype had a higher expression with increased salinity (Hasan et al., 2017). This is the opposite pattern of what was found here. Wang et al. (2014) identified *AQP4*, another member of the aquaporin family, as a salt-responsive gene in the kidneys of sticklebacks, although significant differences were only observed for freshwater fish in fresh- and saltwater (saltwater fish was only exposed to saltwater in Wang et al., 2014). In the present study, *AQP4* was filtered out due to low overall expression, but did have increased expression in the saltwater ecotype (data not shown).

Slow-working hormones are involved in rearrangements during long-term acclimation, by altering the abundance of ion transporters and cell proliferation, and differentiation of ionocytes and other osmoregulatory cells (Takei & McCormick, 2012). Prolactin is one of the slow-working hormones, since long known to have a function in salinity acclimation (Pickford & Phillips, 1959). The concentration of Prolactin (PRLR) is typically increased following freshwater acclimation (Takei & McCormick, 2012) and more directly, prolactin has been linked to the chloride cell regulation; if injected with prolactin, the number of chloride cells in the gills of seawater acclimated tilapia decreases to the levels characterized by freshwater acclimated tilapia (Yan et al., 2013). Prolactin consists of two receptors; PRLRa and PRLRb, for which the expression patterns in gills have been found to be individually linked to salinity in tilapia (Fiol et al., 2009). Similar to this study, Fiol et al. (2009), we found the expression of PRLRa to be overall higher in freshwater (Figure 6c), with FwC expression being significantly different from both SwC and SwFw. In contrast, the PRLRb receptor had an increased expression in saltwater, but the difference was only significant in comparison2 (increased in FwSw). Specific activation of the two PRLR receptors was also found to activate different downstream signaling pathways, likely activating alternative routes leading to osmoprotection of gill cells during the period of active restructuring of gill epithelium in response to salinity stress (Fiol et al., 2009).

4.2 | Salinity genes with contrasting ecotype expression profiles

Genetic assimilation occurs when a plastic ancestral trait becomes environmentally stable, resulting in a loss of plasticity (Lande, 1976). Many of the environmentally expressed genes were linked to known osmoregulatory and immune functions. Intracellular levels of calcium play an important role in responses to osmotic stress and functions in volume regulation of the cells (Erickson et al., 2001), and the ability to take up calcium at low Ca^{2+} concentration have likely been

selected for in freshwater. Genes that were differentially expressed in salt- and freshwater linked to the uptake of Ca^{2+}, and to a certain degree also for Na^{+} (Horng et al., 2007), included different ATP6Vs, subunits of a V-type proton located at the basolateral membrane of mitochondria-rich cells (Figure 7). Consisting of two main parts, the ATP6V1 comprise at least eight and the ATP6V0 include at least five different subunits (Sun-Wada et al., 2004), where five V1 and two V0 subunits had a higher expression in freshwater fish in this study (contrast 3 and 4), clearly indicating that this gene has been important for both freshwater and saltwater acclimation, as was also found for killifish (*Fundulus heteroclitus*) (Whitehead et al., 2012). Other genes related to calcium were ano1 and cacbn1 (Figure 6d), both with an increased expression in saltwater fish.

Pathogen diversity in freshwater is often found to be higher than in salt water (Wang et al., 2012), and in teleosts the skin, gills, and gut are continuously exposed to the external aquatic environment and are, therefore, the main mucosal surfaces that represent potential entry ports for pathogens (Gomez et al., 2013). Many of the mechanisms for antigen sampling in the mucosal epithelium of teleost fish are mostly unknown, as they lack many of the mammalian molecules for transporting pathogens across the epithelia. Recent evidence suggests that two specific antigen-sampling cell types exist in the gill, where one is expressing protein tyrosine phosphatase receptor type C (*PTPrc*) and IL-1β (Kato et al., 2018). The PTPs catalyze the dephosphorylation of protein tyrosine kinases (*PTKs*) directly or through their downstream targets, and play key regulatory roles in multiple signal transduction pathways, where most are expressed in immune cells (Mustelin et al., 2005). In this study, increasing expression of PTPs were linked to high salinity, as two members of the PTP family were found to be differentially upregulated in the saltwater ecotype (*PTPN14* and *PTPrfa*), and three were upregulated in comparison 2 (FwSw; *PTPN2a*, *PTPN21*, *PTPrna*). In contrast, IL-1β had overall higher expression in the freshwater ecotype. The second significant type of teleost antigen-sampling cell types that were recently identified in gills was a microfold cell (M-cell), expressing Anexin5 (*ANXA5*, Figure 6d) (Kato et al., 2018), a gene that has been linked to apoptosis by its ability to be recruited to the cell surface and co-localize with phosphatidylserine; the "eat-me" signal for macrophages (Lizarbe et al., 2013). In the present study, *ANXA5b* was significantly upregulated in the freshwater ecotype, which could indicate that the two different antigen sampling cell types might have been under directional selection in the opposite environments. *ANXA5* has also been linked to changes in calcium concentration, as they can bind around 12 Ca^{2+} ions and exhibit calcium channel activity in plasma membranes and in matrix vesicles (Lizarbe et al., 2013), so it is unclear if ANXA5 has a immunological or osmoregulatory function in freshwater fish (or both).

4.3 | Concluding remarks

The saltwater fish in this study were collected from the Oslofjord, where they experience seasonal variation in salinity, due to periods of high freshwater influx from rivers after heavy rain and snowmelting. It is, therefore, even more surprising that the saltwater fish exhibited such relatively low significant expressional plasticity when exposed to freshwater, although theoretical studies have shown that fluctuating environments can reduce plasticity (Leung et al., 2020). Furthermore, the genetic background for the saltwater fish is likely more diverse. Having a more diverse genetic makeup can likely also lead to a higher variation expression (higher standard deviations), which again will impact the false discovery rate and estimations of significance between experimental groups when filtering on expressional differences as in this study. However, recent studies suggest that a reduced level of genetic diversity can even increase the expressional diversity, and possibly buffer some of the loss of adaptive potential given with a higher genetic variation (Liu et al., 2019; Mazzarella et al., 2015; Morris et al., 2014). More comprehensive studies on physiological changes and osmoregulatory transcriptional responses are needed to understand how the stickleback, especially the saltwater stickleback, are able to tolerate short-term salinity changes. The low genetic expressional differences for the saltwater fish in this study indicates that they invoke some alternative strategy than gene regulation to handle changing salinities, like reversing the orientation of the proteins in the cell membrane (Hartmann et al., 1989), changing the activity state and/or function of cells and cell types, or proteins after interacting with other proteins (Pertl et al., 2010; Szczesnaskorupa et al., 1988), and/or mitochondrial activity/morphology or numbers (Austin & Nowikovsky, 2021); as short-term cellular adjustments are needed in order for the cell volumes to remain stable when moved abruptly from 30‰ to 0‰.

AUTHOR CONTRIBUTIONS

Annette Taugbøl: Conceptualization (equal); formal analysis (lead); methodology (equal); project administration (lead); visualization (equal); writing – original draft (equal). **Monica H. Solbakken:** Data curation (equal); formal analysis (equal); writing – review and editing (equal). **Kjetill S. Jakobsen:** Conceptualization (equal); project administration (equal); writing – review and editing (equal). **Leif Asbjørn Vøllestad:** Conceptualization (equal); project administration (equal); supervision (lead); writing – review and editing (equal).

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DATA AVAILABILITY STATEMENT

All data are available as supplementary material on the online version of this manuscript (Table S2), or by emailing the first author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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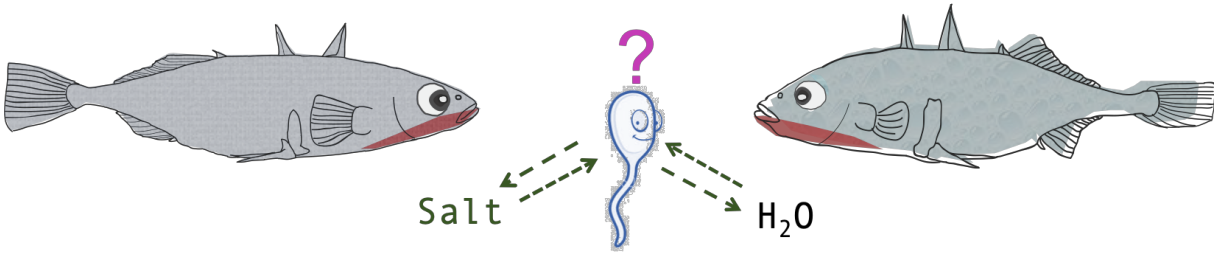

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