The effect of salinity on oxygen consumption rate in ecologically and morphologically divergent three-spined stickleback (Gasterosteus aculeatus)

M.Sc. Thesis by
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# Table of contents

Acknowledgements .............................................................................................................. 2

Table of contents .................................................................................................................. 3

Abstract ................................................................................................................................ 5

Introduction .......................................................................................................................... 6

Materials and methods ....................................................................................................... 11

Study area and sampling ................................................................................................. 11

Experimental design ....................................................................................................... 13

Experimental protocol .................................................................................................... 16

Estimations .....................................................................................................................1 7

Statistics.......................................................................................................................... 18

Results ................................................................................................................................ 19

General observations ........................................................................................................ 19

Measurements of O₂ consumption ............................................................................... 19

The general level of O₂ consumption rate................................................................. 20

Effect of fish body mass on mass specific rate of O₂ consumption ......................... 21

Test for population and lateral plate morph differences in O₂ consumption rate .... 22

Effect of the number of lateral plates on O₂ consumption rate ................................. 24

Discussion .......................................................................................................................... 27

Local adaptation to salinity in stickleback? ................................................................. 27

Effect of lateral plates? ................................................................................................. 31
Methodological issues ............................................................................................................ 32
Concluding remarks ............................................................................................................. 34
Future research .................................................................................................................... 34
References .......................................................................................................................... 35
Abstract
The osmoregulatory abilities of a freshwater, brackish water and marine population of three spined stickleback Gasterosteus aculeatus were tested in a salinity gradient, to test for adaptation to native salinity. There is evidence to suggest that a high number of lateral plates impose a higher osmoregulatory cost in freshwater, and that a low number of lateral plates lead to higher osmoregulatory costs in saltwater. I thus tested for differences in osmoregulatory abilities between, partially and low-plated sticklebacks in a salinity gradient. It is assumed that some of the osmoregulatory costs are reflected by an increase in oxygen consumption. Respirometry methods were therefore used as indirect measure of osmoregulatory costs. Fish from all populations and lateral plate morphs were tested in three different salinity treatments: 0, 15 and 35 PSU at a standardized photoperiod of 12:dark:12light. No significant difference in oxygen consumption rate was found between populations or across salinity treatments. Furthermore, there was no significant effect of lateral plate morph or in the number of lateral plates on oxygen consumption rate in any salinity treatment. There is thus no sign of local adaptation to salinity in any of the ecologically divergent stickleback populations or any evidence for a relationship between lateral plates and osmoregulatory costs.
Introduction

Adaptive divergence among populations can result in local adaptation whereby genotypes in their local environments exhibit greater fitness than genotypes in nonlocal environments (Futuyma 2005; Nuismer and Goodnight 2006; Barrett et al. 2008; Hoeksema and Forde 2008). This may occur when a new population is founded through the colonization of a formerly unused environment or resource (allopatric, sympatric, or parapatric) (Hendry 2001) and can be expressed as a divergence in morphology, behaviour and physiology (Futuyma 2005). Adaptive divergence and local adaptation has been confirmed in numerous studies of organisms introduced to new environments (Losos et al. 1997; Gilchrist et al. 2001; Haugen and Vøllestad 2001; Reznick and Ghalambor 2001). Many populations of three-spined stickleback *Gasterosteus aculeatus* founded post-glacially have shown morphological and behavioral divergence in various character traits over relatively short time scales (Bell and Foster 1994; Bell 2001; Bell et al. 2004; Bjærke 2008; Kitano et al. 2008) and offer unique opportunities to testing evolutionary hypotheses about traits favored by particular environmental factors.

The three-spined stickleback (hereafter sticklebacks) is a small (<10 cm) euryhaline fish species distributed in boreal and temperate regions of the northern hemisphere (Bell and Foster 1994). They can be found as marine, anadromous and landlocked freshwater populations; however, they are assumed to be native to the marine environment (Bell and Foster 1994). Skeletal armor structures are characteristic for this species (although there are exceptions) – the armour consists of three dorsal spines, pelvic spines, and lateral bony plates along the sides of the body. Sticklebacks further show great variation in morphology, physiology and life history (Baker 1994; Guderley 1994).

Hagen and Gilbertson (1972) introduced the terms, completely-plated, partially-plated and low-plated morphs, to describe the morphological variants of sticklebacks (figure 1). Marine stickleback populations are usually monomorphic for the completely-plated morph while estuary (brackish water) populations often display great variation in the number of lateral plates often containing low-plated individuals in higher frequencies than in the ocean (Bell and Foster 1994). Freshwater populations, on the other hand, are usually monomorphic for the low-plated morph (Bell and Foster 1994).
Figure 1. Three morphs of the three-spined stickleback as described by Hagen and Gilbertson (1972).

Completely-plated individuals have a continuous row of lateral plates along both sides of the body, forming a pronounced keel on the caudal peduncle, partially-plated individuals have reduced number of plates and a keel which is often weakly expressed. Low-plated individuals have a few anterior plates, and the keel is absent.

The populations of stickleback resident in fresh water habitats are the result of an invasion of populations native to the marine environment following the rise in sea level and isostatic rebound after the continental glaciers retreated approximately 10000 years ago (Bell 2001). Fossil evidence has suggested that stickleback have a long history of repeated freshwater colonization during postglacial periods (Bell 1977; Bell 1994; Colosimo et al. 2005). The oldest stickleback fossil has been dated back to middle Miocene (about 11Ma) and appear to be identical in morphology to extant marine completely-plated morphs (Bell 1977; Bell 1994). Bell (1977) suggested that the morphology of extant populations consisting of completely-plated individuals, represents the ancestral condition from which fresh water populations have diverged since Miocene.

One of the most intriguing aspects of stickleback evolution is the often rapid loss of lateral bone plates observed in a large number of populations that have colonized freshwater. Evidence based on the geographical position of many freshwater systems as well as genetic analysis strongly suggests parallel evolutionary events (Bell and Foster 1994; Bell 2001;
Marchinko and Schluter 2007). This gives a strong indication that the traits are adaptive, evolving as a consequence of natural selection.

Recent evidence suggests the lateral plate phenotypes are regulated by one major gene (Ectodysplasin (Eda)) with two alleles at one locus and several modifier genes (Colosimo et al. 2005; Barrett et al. 2008). The major allele coding for the reduced number of lateral plates is most likely co-dominant to the “completely-plated allele” and it is believed to have evolved once during the Miocene epoch (Bell 1994). Colosimo et al (2005) have shown that parallel evolution of stickleback low-plated phenotypes at most freshwater locations around the world has occurred by repeated selection of Eda alleles derived from an ancestral low-plated haplotype. Marine and anadromous populations carry the low Eda allele at very low frequencies (>1 %). It is therefore assumed that there has been repeated selection on the standing genetic variation in the ocean.

Several factors have been proposed that could contribute to the armor plate reduction after colonization of freshwater, including insufficient calcium levels limiting the synthesis of lateral plates and spine number (Giles 1983) and pelvic girdle dimensions (Bell et al. 1993) and difference in predation regimes (birds, fish, crustaceans and insects) between freshwater and marine environments (Reimchen 1983;1992;1999; Rundle et al. 2003; Messler et al. 2007; Marchinko 2009; Reimchen and Bergstrom 2009).

A third and very interesting hypothesis is that there is a relationship between salinity tolerance and lateral plates (Heuts 1947). Heuts (1947) observed a decreasing tolerance for saltwater with a decreasing number of lateral plates and higher hatching success in freshwater for low-plated individuals. He suggested that salinity tolerance indirectly drive the reduction of lateral plates in freshwater while maintaining populations of armored individuals in the sea. Moreover, Colosimo (2005) revealed that the Eda gene is closely linked to three other genes, including one (Gjb1) that is possibly related to salt secretion. A study by (Marchinko and Schluter 2007) contradicts Heuts (1947) hypothesis although at the same time their results give support for a relationship between lateral plate morphs and salt tolerance.

Osmoregulation in fish is the regulation of water and ion concentrations between cells, blood plasma and the external environment (outside of the body) (Evans and Claiborne 2009). Osmoregulatory mechanisms are employed to counteract the osmotic stress experienced at salinities different from that of isoosmotic conditions (Evans and Claiborne 2009). Common for euryhaline teleost fishes are their ability to keep a relatively constant ionic composition of lymph, plasma and interstitial fluid in a broad range of environmental salinities (Evans and Claiborne 2009). The physiological mechanisms involved in osmoregulation cost energy
(Guderley 1994; Sangiao-Alvarellos et al. 2003) and changes in salinity is often reflected by changes in oxygen consumption rates (Febry and Lutz 1987; Woo and Kelly 1995; Morgan et al. 1997; Gracia-Lopez et al. 2006) and activities of various physiological parameters; two important factors being gill and kidney Na+/ K+ ATPase (Morgan et al. 1997; Sampaio and Bianchini 2002; Herrera et al. 2009; Yang et al. 2009). The main role of Na+/ K+ ATPase pumps is to ensure that the cells and blood plasma keep a relatively constant osmolality which is important for maintaining cell volume (K.Fugelli, pers.comm).

Measurements of oxygen consumption rate (respirometry) can be used as an indirect indicator of metabolism in fish (Cech 1990) and several authors (Farmer and Beamish 1969; Iwama et al. 1997; Kim et al. 1998; Morgan and Iwama 1998; da Silva Rocha et al. 2005; Tsuzuki et al. 2008) have employed respirometry methods to assess osmoregulatory costs in fish. The energetic cost of osmoregulation has been shown to influence growth rates (Boeuf and Payan 2001; Imsland et al. 2003). Some studies have supported the hypothesis that reduced metabolic cost for osmoregulation can enhance growth rates (Febry and Lutz 1987; Woo and Kelly 1995). It has further been suggested that the osmoregulatory energy demand is minimal in the environment that is natural for a particular species and life-history stage (Morgan and Iwama 1991). Moreover, Larsen et al. (2007) found interpopulation differences in costs associated with the ionic and osmotic regulations between two populations of brown trout salmo trutta inhabiting different salinity regimes.

There thus seem to be some fitness-related advantages of being adapted to local environmental salinity.

Ecologically divergent populations of stickleback living in different salinity regimes may have developed different osmoregulatory adaptations to local environmental conditions. Marine and freshwater populations likely experience more stable salinity conditions and thus would have the opportunity to become specialized (locally adapted) with regard to salinity. On the other hand, brackish water populations exposed to greater salinity fluctuations are not expected to have the same possibility for local adaptation.

Based on these assumptions I will test for differences in the osmoregulatory energy demand between a saltwater, brackish water and freshwater stickleback population in a salinity gradient, to see if their osmoregulatory energy demand is related to a population’s local environmental salinity. There is also evidence that indicates that there is a relationship between the lateral plates and osmoregulatory ability in different salinity regimes. I will therefore test completely-plated, partially-plated and low-plated individuals for their
osmoregulatory energy demand in a salinity gradient. I will also look at a finer scale and test for any effect of the number of lateral plates on osmoregulatory energy demand.

I will use respirometry methods to measure fish oxygen consumption rate at rest (fish resting metabolism) in three different salinity treatments; 0, 15 and 30 PSU (practical salinity units) as an indirect measure of the fish osmoregulatory energy demand.
Materials and methods

Study area and sampling

Stickleback were collected from three locations containing three different salinity regimes; Sandspollen (59°39’56’’N; 10°35’07’’E) close to Drøbak in the Oslo Fjord, Sandvikselva River (59°54’11’’N, 10°2 9’26’’E), and Lake Engervann (59°53’49’’N, 10°32’04’’E). Sandspollen is a near shore shallow area of the Oslo fjord and was chosen for the collection of marine stickleback. The sampling was performed during the second half of August 2008. During this part of the season the salinity is measured to be around 25.3 ± 3.6 PSU. The population resident in this area has a high frequency of completely-plated phenotypes. Preliminary genetic analysis (unpublished data; K. Østbye et al.) shows that the stickleback population in Sandspollen is genetically differentiated from both Lake Engervann and Kirkerud populations.

The Sandvikselva River stretches over several kilometers before entering the Oslo Fjord. The sampled area has an elevation of roughly 30 meters above sea level. This river contains a large freshwater population of low-plated stickleback individuals and is also an important spawning area for salmon and sea trout. Several waterfalls act as barriers to upstream gene flow from the marine and brackish water areas. However, downstream migration from the river may occur. Preliminary genetic analysis (unpublished data; K. Østbye et al.) shows that the population in Sandvikselva is genetically differentiated from Lake Engervann and Sandspollen, suggesting very low gene flow.

Lake Engervann is a brackish water lake (estuary) situated slightly above sea level. It covers an area of ca 0.18 km² and has a maximum depth of 3 meters (own observations using an echo-sounder). The lake is in connection with the fjord through the lower parts of the Sandvikselva River, and has a high seasonal and daily variation in PSU (practical salinity units) with conductivity varying from 60 to 3000 mS/m (Halvorsen et al. 2005). The lake has a large brackish water population of three-spined stickleback consisting of three lateral plate phenotypes: completely, partially and low-plated morphs (see figure 1) living in sympathy. I have estimated their frequency to be approximately 70:29:1, respectively (N=1000 fish). Preliminary genetic analysis (unpublished data; K. Østbye et al.) suggests total panmixia within Lake Engervann.

Sticklebacks from Sandspollen were sampled using a beach seine, while the populations in Lake Engervann and Sandvikselva River were caught using baited plastic fry
traps. We had an extensive sampling period (collecting several thousand fish) from 18th August 2008 to 1st February 2009. We still only managed to capture 23 low-plated individuals from Lake Engervann due to their low frequency.

The fish were quickly transported to the facility at the University of Oslo and placed in separate 1000-litre holding tanks. Brackish water and freshwater individuals from Lake Engervann and River Sandvikselva were kept in tanks with flow through freshwater at 4°C and were kept in a room with light 24 hours a day. The aquaria were slightly shaded to reduce stress. Due to logistical constraints the marine group from Sandspollen was placed in a separate room with circulating saltwater (30 PSU). This room was also used for the experiments and had a standardized photoperiod of 12D/12L.

The fish from Engervann were further divided into groups based on lateral plate numbers (completely-, partially- and low-plated morphs (see figure 1)). This was done by carefully looking at each of the fish using a stereo microscope. The fish could then easily and quickly be assigned to the three different morphs.

All groups of fish were fed red mosquito larva six days a week.
Experimental design

A total of 145 stickleback individuals, divided into five groups representing the three populations with different salinity regimes, were used in this experiment (table 1). The five experimental groups were low-plated freshwater individuals from Sandvikselva River here called “Fresh-Low”. The three lateral plate morphs from Engervann constituting completely, partially, and low-plated morphs were called “Brack-Complete”, “Brack-Partial” and “Brack-Low” respectively. The completely-plated individuals from Sandspollen were called “Salt-Complete”.

The aim of this study was to test for differences in the oxygen consumption rate between and within groups across a salinity gradient. Ten fish from each of the five groups were tested in one of three different salinities; 0, 15 and 30 PSU respectively (see table 1). Tap water was used for the freshwater experiment. Seawater (taken from 50 depths in the Oslo fjord) was used for the saltwater treatment. Brackish water was made by mixing tap water and saltwater. The salinity was measured before the start of each measurement with the use of a handheld analogue PSU device.

When an experimental period was to start, experimental fish from each group were placed in 20-litre aquariums installed with air stones for salinity and temperature acclimation. A set of five identical 40-litre aquariums were further used to acclimatize groups to the standardized experimental conditions. These aquariums were installed with water filters and aerated using air stones. Half of the water was renewed and the filters were cleaned every four days to sustain high water quality. 20-litre aquariums were used to starve fish for 24 hours prior to the measurements to reduce metabolic activity caused by digestion (specific dynamic action).

Individuals collected from freshwater and brackish water were kept in a separate room from where the experiment was performed. Individuals that were to be tested were subsequently transferred to the experimental room and the 40 litre aquariums for acclimation before the start of a salinity treatment.

Two identical respirometry chambers were used (figure 2), both of the same size and volume (ca. 130 ml). Since the density of freshwater is 1 kg/L we determined the chamber volume by the weight of the chamber filled with water minus weight of the empty chamber.
Figure 2. Type of respirometer chamber used for the measurements of oxygen consumption rate of all the experimental groups of three-spined stickleback. The oxygen electrode head and magnetic stirrer are shown coming down from the top left chamber wall. Two respirometry chambers of this type were used enabling the measurements of oxygen consumption (reduction in oxygen partial pressure inside chambers) of two fish in parallel.

This was estimated between experiments to ascertain precise measurements. The chambers were composed of a plastic tube with a piston in one end for volume adjustment, and a lid in the opposite end making it easy to place the fish inside. They further have two openings, one in the lid to let oxygen in during acclimation of the fish, and one in the tube wall made to fit a CellOx 325 oxygen electrode. A magnetic stirrer of type RZ 300 was installed on the electrode head (see figure 2) to disrupt any gradients of oxygen inside the chamber during the measurements. The chambers were kept under water in a 30-litre aquarium to help keep a stable temperature and minimize differences in oxygen pressure outside and inside the chamber. The two respirometry chambers were cleaned every second day (using the sterilising substance vircon) and the water in which the measurements were performed was renewed between every second experiment to reduce algae growth and bacterial activity. Air stones were used to keep the water inside the chamber close to saturation prior to each measurement. A small barrier was placed between the two chambers to avoid visual contact between the fish and so that they were blocked from seeing any activity in the room. The aquarium also was slightly shaded. I left the experimental room during acclimation to the chambers and after starting each experiment to minimize fish stress.

Measurements of oxygen consumption were performed during the mornings and afternoons with a maximum of four stickleback measured each day. After about an hour into the measurements the oxygen values were checked (carefully opening the door and visually inspecting the oxygen values) for any abnormalities. If the oxygen concentration was unusually low indicating high fish stress level, the measurements of both fish were stopped.
and redone. If the fish still showed signs of stress after a second experimental run the fish was
replaced, assuming that the particular individual was too stressed to be used in the
experiment. Oxygen partial pressure and temperature was recorded by an oxi 340i handheld
oxygen meter. The oxygen meter recorded reduction in O2 partial pressure (automatically
transformed to mgO2/L) and temperature every five minutes for two hours.

Fish tested in freshwater experienced a higher average temperature (18, 1±0, 79 °C)
than fish tested in brackish and saltwater (16, 6±0, 46 °C and 16, 7±0, 49°C respectively).

This is attributed to the warmer room temperature during this period of the measurements
(higher seasonal temperature). This was taken into account in the statistical analysis.

(Table 1) Summary of mean body length, body mass and number of lateral plates in the experimental groups of
stickleback used in the experiment. The target N for my experimental groups was 10 individuals per group per
salinity treatment. (This was not achieved in the Brack-low group due to its very low frequency (>1 %) in the
brackish water population in Lake Engervann. FW = fresh water, BW = brackish water, SW = saltwater.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Length (cm)</th>
<th>Body Mass (g)</th>
<th>Number of Lateral Plates</th>
<th>N tested in FW (0 PSU)</th>
<th>N tested in BW (15 PSU)</th>
<th>N tested in SW (30 PSU)</th>
<th>Total N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh-Low</td>
<td>4.04 ± 0.30</td>
<td>0.56 ± 0.10</td>
<td>10.30± 1.20</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Salt-Complete</td>
<td>4.33 ± 0.27</td>
<td>0.61 ± 0.13</td>
<td>64.29± 1.90</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>31</td>
</tr>
<tr>
<td>Brack-Low</td>
<td>4.63 ± 0.56</td>
<td>0.83 ± 0.27</td>
<td>14.26± 4.48</td>
<td>10</td>
<td>10</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>Brack-Partial</td>
<td>4.32 ± 0.32</td>
<td>0.56 ± 0.13</td>
<td>37.41± 14.34</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>31</td>
</tr>
<tr>
<td>Brack-Complete</td>
<td>4.43 ± 0.26</td>
<td>0.67 ± 0.13</td>
<td>62.93 ± 1.89</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>30</td>
</tr>
</tbody>
</table>
**Experimental protocol**

The measurements of oxygen consumption were performed consecutively in freshwater, brackish water and saltwater. The experimental groups were acclimated in a 20-litre aquarium (table 2, sequence 1) to an experimental salinity by either increasing or reducing the salinity in daily increments of 5-10 PSU until the target salinity was reached. As an example the saltwater treatment required an acclimation period of four days at 5, 15, 25 and 30 PSU respectively for all groups except the “Salt-Complete” group. Temperature acclimation was done simultaneously with the salinity acclimation. After the groups of fish were acclimated to one of the experimental salinities they were placed in five similar 40-litre aquariums (table 2, sequence 3). The groups were then acclimated to the aquariums and the conditions in the room for one week. At the end of the acclimation period two and two fish were taken from randomly selected groups and put in separate aquariums (table 2 sequence 4) for 24-hour starvation. After the starvation period the fish were placed in two closed respirometer chambers and acclimatized for one hour. Measurements of oxygen consumption were then performed over a two-hour period.

Table 2. The protocol carried out for all experimental groups of stickleback. The protocol was executed for all three salinity treatments.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Aquariums/chambers (L)</th>
<th>Protocol/description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1000</td>
<td>Holding tanks</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>Gradual acclimation to temperature and salinity treatment</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>Further acclimation to the experimental conditions (1 week).</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>Fish starved for 24 hours</td>
</tr>
<tr>
<td>5</td>
<td>0.130</td>
<td>Measurements of oxygen consumption</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Fish were euthanized</td>
</tr>
</tbody>
</table>

All fish were euthanized with an overdose of benzocain. Fish weight and volume was determined before being preserved individually in 70 % alcohol. In the lab fish fork length was measured and lateral plates were counted on both sides of the fish.

I observed dramatic differences in mortality between stickleback from freshwater and stickleback from brackish water (much higher mortality for brackish water individuals) in the 1000 litre holding tanks containing freshwater (4°C) used to store fish. The holding tanks had approximately the same number and density of individuals. However, no counts were
undertaken so no estimates of mortality rates between the two populations were performed. No mortality was observed for the marine individuals stored in the 1000-litre holding tanks containing saltwater.

Some mortality was also observed during the acclimation of the groups to the salinity treatments; however, there were no differences between groups (one individual in the “Brack-Complete” group and one in the “Fresh-Low” group).

**Estimations**

The oxygen electrode has a built-in compensation for temperature and was calibrated for freshwater (0 PSU). Since oxygen solubility in water decreases with an increase in dissolved solids, a model (formula 1) developed by Pijanowski (1973) was used to correct for the increase in salinity (experimental water qualities 15 and 30 PSU).

\[
D_{adj} = D_1 \times 0.998 e^{-0.001 \left( \frac{S_2}{0.1455 + 1.255 \times 10^{-3} T} \right)}
\]  

\(1\)

\(D_1\) = the actual measured value of dissolved oxygen in mgO\(2/\)L.

\(D_{adj}\) = The corrected value in mgO\(2/\)L.

\(S_2\) = the salinity of sample being measured by a temperature compensated instrument at temperature \(T\), calibrated at salinity \(S_1\) (\(S_1\) is 0 PSU in our experiment)

To estimate mass specific oxygen consumption rate (mgO\(2/\)g/hour) we used the equation from Steffensen (1989):

\[
MO_2 = \left( [O_2]_0 - [O_2]_1 \right) \times \frac{V}{t \times BW}
\]  

\(2\)
MO₂ = mass specific oxygen consumption rate (mg O₂/g/hour)

[O₂]₀ = oxygen concentration at time t₀ (mg O₂/L)

[O₂]₁ = oxygen concentration at time t₁ (mg O₂/L)

V = respirometer volume minus volume of experimental animal in litre

t = t₁ – t₀ (hour)

BW = body weight of experimental animal (g)

For easier comparisons with other studies the fish oxygen consumption rate was converted to μmolO₂/g/hour.

When calculating oxygen consumption rate ([O₂]₀ – [O₂]₁) I always used linear 10-minute intervals giving the lowest value of oxygen consumption rate (it was assumed to be the interval closest to the fish state of rest). This was done by visually inspecting the trajectory with the help of a ruler. I always used values above 5 mg O₂/L (156 μmolO₂/L) to calculate the interval since too low O₂ partial pressure can have an influence on consumption rate (G.Nilsson, pers. comm).

**Statistics**

All the statistical analyses were performed using JMP 8(SAS. 2008) statistical software.

A linear regression was performed to estimate the effect of size on mass specific oxygen consumption rate in our experimental groups.

Analysis of covariance (ANCOVA) was performed to test for among-treatment and among-group (see table 1) variation in oxygen consumption rate (as proxy of osmoregulatoric energy demand). I started with a full model by creating interactions between all main effects (treatment and group) and covariates (temperature and body mass). I then performed backward removal of the non-significant interactions while always retaining main effects and covariates.

An ANCOVA was also performed to test for any relationship between the number of lateral plates and oxygen consumption rate across salinity treatments. The model was constructed in a similar manner as explained above; however; I exchanged the effect of group with the covariate “number of lateral plates”.

We used log transformed values of oxygen consumption and body length to reduce heterostocastity. This transformation also reduced the number and effect of suspected outliers (excluding outliers had no significant effect on the results).

Temperature and body mass were kept as covariates in all the ANCOVA models.
Results

General observations

*Measurements of O₂ consumption*

Most of the measurements of oxygen consumption were successfully completed with the individual stickleback showing little sign of stress. The visual inspections showed that most of the fish quickly fell to rest inside the chamber. This was also reflected by a steady and linear trajectory of reduction in oxygen concentration inside the respirometry chamber (see figure 2 A). A few fish, however, showed clear signs of stress. One example is illustrated in figure 2 B. The steep trajectory for this individual indicated a very high oxygen consumption rate (later calculated to be 44.20 µmolO₂/g/hour). This was detected when visually inspecting the respirometry data recorder during the measurements.

![Figure 2](image-url)

*Figure 2. Plots of reduction in oxygen concentration per unit time inside the respirometry chamber of two individual stickleback. Oxygen concentration was measured every five minutes. The trajectory in figure 2 A is of a freshwater individual (0.64 g) tested in saltwater. Its trajectory is an illustration of how the majority of trajectories for all the experimental individuals looked like. The trajectory in B is an example of a stressed freshwater individual (0.46 g) tested in saltwater that was excluded from the statistical analysis.*
As inferred from the trajectory in figure 2 B, the oxygen concentration inside the respirometry chamber reached the lower limit (156 μmolO2/L) set to be used for the calculations after only 40 minutes of measurements. The individual representing the trajectory in figure 2 B was therefore excluded from the statistical analysis. A total of 3 individuals behaved in this way and were excluded from the statistical analysis.

**The general level of O2 consumption rate**

The overall level of mass specific oxygen consumption rate (MO2) was very similar between groups and salinity treatments (table 3). All groups across all treatments had a total mean MO2 of 13.28 ± 3.82 μmolO2/g/hour which is close to the mean MO2 within groups and across treatments. Moreover there was large variation in mean MO2 within groups especially for those tested in the saltwater treatment. An ANOVA comparing group means within treatments and within groups across treatments found no difference between groups or across treatments. This analysis does not take differences in size or temperature into account and is mainly for obtaining an overview of the general MO2 level.

Table 3. Showing means and st.dv of mass specific oxygen consumption rate for all the experimental groups (rows) of stickleback across all salinity treatments (columns); Freshwater (FW), brackish water (BW) and saltwater (SW). An ANOVA was performed to compare group means within salinity treatments and within group means across treatments.

<table>
<thead>
<tr>
<th>Treatment /Group</th>
<th>Salt-Complete</th>
<th>Brack-Complete</th>
<th>Brack-Partial</th>
<th>Brack-Low</th>
<th>Fresh-low</th>
<th>F-ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW (0 PSU)</td>
<td>13.23 ± 3.27</td>
<td>12.59 ± 2.81</td>
<td>14.72 ± 3.77</td>
<td>13.13 ± 2.08</td>
<td>14.94 ± 3.13</td>
<td>1.12</td>
<td>0.35</td>
</tr>
<tr>
<td>BW (15PSU)</td>
<td>13.97 ± 3.03</td>
<td>12.79 ± 2.89</td>
<td>13.31 ± 4.04</td>
<td>11.66 ± 1.85</td>
<td>13.72 ± 3.26</td>
<td>0.84</td>
<td>0.50</td>
</tr>
<tr>
<td>SW (30 PSU)</td>
<td>12.63 ± 3.01</td>
<td>14.77 ± 6.71</td>
<td>13.76 ± 5.90</td>
<td>11.71 ± 4.29</td>
<td>12.37 ± 5.09</td>
<td>0.66</td>
<td>0.35</td>
</tr>
<tr>
<td>F-ratio</td>
<td>0.47</td>
<td>0.64</td>
<td>0.40</td>
<td>1.27</td>
<td>2.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.62</td>
<td>0.53</td>
<td>0.67</td>
<td>0.30</td>
<td>0.14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Effect of fish body mass on mass specific rate of $O_2$ consumption

There were some differences in size between individuals (see table 1). The largest individuals, constituting mainly low-plated brackish water individuals, were more than three times the size of the smallest individuals included in the experiment. Due to the low frequency of low-plated brackish water individuals they could not be excluded from the analysis.

Overall, there was a weak tendency for the mass specific oxygen consumption to decrease with an increase body mass ($r = -0.14$, $N = 145$, $P = 0.09$) (Figure 3).

Figure 3. Relationship between body mass and mass specific oxygen consumption in individuals of the three spined stickleback included in the experiment.
Test for population and lateral plate morph differences in $O_2$ consumption rate

I tested for among-treatment and among-group (populations and lateral plate morphs) variation in MO$_2$ using a full-factorial ANCOVA with fish mass and experimental temperature as covariates. After backward removal of non-significant terms (starting with the higher-order interaction level, but always retaining the main effects and covariates) the final model could explain only 9% of the variation in MO$_2$ (Table 4). Overall, there was no significant effect of either treatment or experimental groups on MO$_2$. However, there was a significant treatment * temperature interaction effect.

Table 4: Summary test statistics for a covariance analysis (ANCOVA) with mass specific oxygen consumption (MO$_2$) as response variable. Testing for differences in MO$_2$ between the five experimental groups of stickleback (Salt-Complete, Brack-Complete, Brack-Partial, Brack-Low and Fresh-Low) within treatments (saltwater (30 PSU), brackish water (15 PSU) and freshwater) and within groups across treatments. DF=degrees of freedom, SS=sum of squares. ($R^2 = 0.09$, $p = 0.16$.)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>F Ratio</th>
<th>p- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>0.029</td>
<td>0.20</td>
<td>0.81</td>
</tr>
<tr>
<td>Group</td>
<td>4</td>
<td>0.091</td>
<td>0.33</td>
<td>0.85</td>
</tr>
<tr>
<td>Temperature ($^\circ$C)</td>
<td>1</td>
<td>0.001</td>
<td>0.02</td>
<td>0.87</td>
</tr>
<tr>
<td>Log Body Mass</td>
<td>1</td>
<td>0.065</td>
<td>0.95</td>
<td>0.33</td>
</tr>
<tr>
<td>Treatment * Temperature ($^\circ$C)</td>
<td>2</td>
<td>0.478</td>
<td>3.44</td>
<td>0.034</td>
</tr>
<tr>
<td>Error</td>
<td>134</td>
<td>9.30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
To further investigate the treatment * temperature interaction effect, I analyzed each of the three salinity treatments separately using an ANCOVA. The models were built with the highest order of interaction. After backward removal of non-significant terms all models were left with the covariates (temperature and body mass) and group (table 5 A, B and C) in the final models. There was no difference in MO2 between groups within any of the salinity treatments. There was a significant effect of temperature and body mass in the freshwater treatment (table 5A), the final model explaining 42 % of the variance ($R^2=0.42$). Within the brackish water and saltwater treatments the final models could only explain 13 % and 7 % of the variance respectively.

Table 5: Summary of test statistics for a covariance analysis within each salinity treatment; A = freshwater treatment, B = brackish water treatment, C = saltwater treatment. DF=degrees of freedom, SS=sum of squares.

**A. Freshwater treatment** ($R^2 = 0.42$, $P = 0.0004$)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>F Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>4</td>
<td>0.14</td>
<td>1.10</td>
<td>0.36</td>
</tr>
<tr>
<td>Temperature</td>
<td>1</td>
<td>0.34</td>
<td>10.44</td>
<td>0.0024</td>
</tr>
<tr>
<td>Log Body Mass</td>
<td>1</td>
<td>0.31</td>
<td>9.56</td>
<td>0.0035</td>
</tr>
<tr>
<td>Error</td>
<td>43</td>
<td>1.50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B. Brackish water treatment** ($R^2 = 0.12$, $P = 0.19$)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>F Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>4</td>
<td>0.34</td>
<td>1.76</td>
<td>0.153</td>
</tr>
<tr>
<td>Temperature</td>
<td>1</td>
<td>0.02</td>
<td>0.61</td>
<td>0.437</td>
</tr>
<tr>
<td>Log Body Mass</td>
<td>1</td>
<td>0.22</td>
<td>4.69</td>
<td>0.035</td>
</tr>
<tr>
<td>Error</td>
<td>43</td>
<td>2.09</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**C. Saltwater treatment** ($R^2 = 0.07$, $P = 0.80$)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>F Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>4</td>
<td>0.39</td>
<td>0.68</td>
<td>0.60</td>
</tr>
<tr>
<td>Temperature</td>
<td>1</td>
<td>0.02</td>
<td>0.16</td>
<td>0.68</td>
</tr>
<tr>
<td>Log Body Mass</td>
<td>1</td>
<td>0.05</td>
<td>0.35</td>
<td>0.55</td>
</tr>
<tr>
<td>Error</td>
<td>43</td>
<td>1.50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Effect of the number of lateral plates on O₂ consumption rate

There was a large and continual variation in the number of lateral plates among individuals constituting the three lateral plate morphs (figure 4). The overall effect of the number of lateral plates on MO₂ (all treatments included) was not significant ($r = 0.021$, $n = 84$, $p = 0.30$).

Figure 4. Illustrating the relationship between the number of lateral plates and mass specific oxygen consumption measured over all treatments. (Only counts of lateral plates of completely, partially and low-plated stickleback individuals within the brackish water population are shown).
In the following ANCOVA I further wanted to investigate for any effect of lateral plates on MO$_2$ in a finer scale by testing for the effect of the number of lateral plates on MO$_2$ across the experimental salinity gradient.

As previously, I started with a full-factorial ANCOVA with fish body mass and temperature as covariates. I performed backward removal of non-significant terms (always maintaining main effects and covariates). The final model could explain only 10 % of the variation in MO$_2$ (Table 6). There were no significant effects of any interactions or parameters. The treatment * number of lateral plates interaction was the only effect close to significant.

Table 6: Summary of test statistics for a covariance analysis (ANCOVA) with fish mass specific oxygen consumption rate (MO$_2$) as response variable. Testing for the effect of the number of lateral plates on MO$_2$ within and across three salinity treatments; saltwater (30 PSU), brackish water (15 PSU) and freshwater. This model only includes counts of lateral plates on stickleback individuals from the brackish water population. N=84. DF=degrees of freedom, SS=sum of squares. (R$^2$ = 0.10, p = 0.30)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>F Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>0.004</td>
<td>0.026</td>
<td>0.97</td>
</tr>
<tr>
<td>Log Body Mass</td>
<td>4</td>
<td>0.0008</td>
<td>0.010</td>
<td>0.91</td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>1</td>
<td>0.047</td>
<td>0.608</td>
<td>0.43</td>
</tr>
<tr>
<td>Number of Lateral Plates</td>
<td>1</td>
<td>0.160</td>
<td>2.051</td>
<td>0.15</td>
</tr>
<tr>
<td>Treatment * Number of Lateral Plates</td>
<td>2</td>
<td>0.473</td>
<td>3.027</td>
<td>0.054</td>
</tr>
<tr>
<td>Error</td>
<td>76</td>
<td>5.94</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

I wanted to further investigate the treatment * number of lateral plates interaction effect and therefore performed an ANCOVA in each treatment separately (table 7).

There was no lateral plate associated increase in MO$_2$ in any of the treatments. As before there was a significant effect of temperature and body mass within the freshwater treatment. The final model within this treatment explained 33% of the variance.
In the brackish and saltwater treatments there was no significant effect of body mass or temperature. The final models explained only 6 % and 16 % of the variance respectively. There was, however, a close to significant effect of number of lateral plates within the saltwater treatment.

Parameter estimates show that there is a slight positive effect of the number of lateral plates in the saltwater treatment and a non-significant negative effect in the freshwater and brackish water treatment. This could contribute to the low p-value in the treatment * number of lateral plates interaction effect.

Table 7. Summary of test statistics for a covariance analysis within salinity treatments. Testing for the effect of the number of lateral plates on mass specific oxygen in each treatment. The test only includes counts of lateral plates on stickleback individuals from the brackish water population. DF = degrees of freedom, SS = sum of squares. A = freshwater treatment, B = brackish water treatment, C = saltwater treatment.

A: Freshwater treatment (R^2 = 0.33, P = 0.01)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>F Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Body Mass</td>
<td>1</td>
<td>0.16</td>
<td>4.90</td>
<td>0.03</td>
</tr>
<tr>
<td>Temp</td>
<td>1</td>
<td>0.17</td>
<td>5.09</td>
<td>0.03</td>
</tr>
<tr>
<td>Number of Lateral Plates</td>
<td>1</td>
<td>0.01</td>
<td>0.59</td>
<td>0.44</td>
</tr>
<tr>
<td>Error</td>
<td>26</td>
<td>1.50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B: Brackish water treatment (R^2 = 0.06, P = 0.62)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>F Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Body Mass</td>
<td>1</td>
<td>0.054</td>
<td>0.93</td>
<td>0.34</td>
</tr>
<tr>
<td>Temp</td>
<td>1</td>
<td>0.023</td>
<td>0.40</td>
<td>0.53</td>
</tr>
<tr>
<td>Number of Lateral Plates</td>
<td>1</td>
<td>0.006</td>
<td>0.11</td>
<td>0.73</td>
</tr>
<tr>
<td>Error</td>
<td>26</td>
<td>1.51</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C: Saltwater water treatment (R^2 = 0.16, P = 0.29)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>F Ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Body Mass</td>
<td>1</td>
<td>0.07</td>
<td>0.49</td>
<td>0.48</td>
</tr>
<tr>
<td>Temp</td>
<td>1</td>
<td>0.02</td>
<td>0.17</td>
<td>0.67</td>
</tr>
<tr>
<td>Number of Lateral Plates</td>
<td>1</td>
<td>0.51</td>
<td>3.42</td>
<td>0.07</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>2.97</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion

I found no difference in mass specific oxygen consumption rate (MO2) between groups (population and lateral plate morphs) or across salinity treatments. There was also no significant effect of lateral plate morph or in the number of lateral plates on MO2 within or across treatments.

Local adaptation to salinity in stickleback?

In contrast to the predictions the results suggests that the marine population (completely-plated individuals), brackish water population (completely, partially and low-plated individuals) and freshwater population (low-plated individuals) require the same amount of energy in all the experimental salinity treatments: freshwater, brackish water (15 PSU) and saltwater (30 PSU). There is thus no indication that any of the experimental populations are locally adapted with regard to salinity.

Gutz (1970), however, measured a significant increase in MO2 in marine (completely and partially-plated) individuals (laboratory-raised offspring of marine parents) when transferred from freshwater to saltwater (35 PSU) at 20 °C (after 7 days of acclimation) and short day length (16Dark: 8Light). The osmoregulatory energy demand thus seemed to be higher in the marine individual’s native environmental salinity which is contrary to what one would expect if the marine stickleback had been locally adapted to seawater. When Gutz (1970) tested freshwater individuals (low-plated, laboratory-raised offspring of freshwater parents) using the same experimental conditions as for the marine individuals, no significant increase in MO2 was found, which is in concordance with the results I have for all my experimental groups. The freshwater individuals of Gutz’ (1970) study displayed a cell-volume regulatory response by increasing the pool of free amino acids within the cells as opposed to the energetic costly active ion-regulation displayed by the marine individuals. Gutz (1970) also performed similar respirometry experiments during long day length photoperiod (8Dark:16Light) in which he tested individuals with fully developed gonads. Under this photoperiod and at 20 °C the marine individuals did not show an increase in MO2 when transferred from freshwater to saltwater. Moreover, marine individuals displayed the same increase in amino acids as the freshwater population. The energetic costly active ion pumping thus seems to be held at a constant low level in the stickleback individuals of Gutz’ (1970) study regardless of salinity during the long day light experiment. Although no measurements of amino acids was performed in my experiment an osmoregulatory strategy
of increasing the pool of free amino acids with increasing salinity (could be stimulated by the 12Dark:12Light and high average temperature) could explain the non-significant increase in MO₂ across salinity treatments, as it is a less energetic costly mechanism. Increasing (or decreasing) the concentration of free amino acids with changes in salinity is a cell-volume regulatory mechanism found in organisms ranging from eubacteria, marine invertebrates, cyclostomes fishes and vascular plants (Somero 1986). This mechanism has the advantage of not perturbing macromolecular structure and function as would changes in inorganic ion concentration (Guderley 1994).

In support of my results Jurss et al. (1982) found no significant difference in the osmoregulatoric energy demand (measuring gill Na⁺K⁺-ATPase activity as proxy for osmoregulatoric energy demand and salt uptake and excretion ability) between a freshwater and marine stickleback population tested in freshwater and saltwater at 30 PSU and 15 °C (16Dark:8Light). Schaarschmidt et al. (1999) tested for differences in physiological parameters associated with osmoregulation (among them gill Na⁺K⁺-ATPase activity) between individuals from a freshwater population and two brackish water populations in freshwater and brackish water (20 PSU) at both 4 °C and 15°C (12Dark:12Light). Freshwater individuals had the same level of gill Na⁺/K⁺-ATPase across treatments. In concordance with my own, Jurss et al. (1982) and Gutz (1970) results this suggests that freshwater individuals do not increase their osmoregulatory energy demand with increasing salinity (at least in the experimental salinity gradients used). Partly in support of Gutz (1970) the freshwater individuals in Schaarschmidt et al. (1999) had higher levels of free amino acids (taurine being the most important osmolyte) in the brackish water treatment (20 PSU), however, only in the low temperature experiment (4°C). Schaarschmidt et al. (1999) further found that in brackish water (20 PSU) at 15 °C the fish from the two brackish water populations had significantly lower osmoregulatory energy demand (lower Na⁺/K⁺-ATPase activity) than freshwater individuals. The authors discuss this as brackish water sticklebacks possibly having smaller ion and water permeability in the gills than freshwater sticklebacks, and that as a result a smaller amount of sea water is taken up, thus reducing their energy demand to hyperosmotic conditions (salinities higher than isoosmotic conditions). However this argumentation is rather counter-intuitive since low gill permeability would be of even more of adaptive value for a freshwater fish living in an even higher osmotic gradient (K. Fugelli, pers. comm.) Interestingly, when freshwater and brackish water individuals in Schaarschmidt et al. (1999) study were transferred from 10 PSU to 35 PSU at 15 °C they showed a similar increase, becoming significant after 29 days of acclimation, in the osmoregulatory energy demand (gill
Na⁺K⁺-ATPase activity) while at the same time adjusting and maintaining plasma osmolality at near identical levels. This is partly in contrast to my results as I found no increase in osmoregulatory energy demand when transferred from freshwater to saltwater (30 PSU). It is also in sharp contrast to Jurss et al. (1982) as he found no difference in gill Na⁺K⁺-ATPase activity between brackish water individuals and freshwater individuals after 29 days of acclimation to saltwater (30 PSU) also used the same proxy (gill Na⁺K⁺-ATPase activity).

However; importantly, Schaarschmidt et al. (1999) also give support to my results in that he found no major difference in the osmoregulatory abilities between sticklebacks from the freshwater and brackish water population. Schaarschmidt et al. (1999) results also indicate that the sticklebacks from both salt regimes needed a long time before being fully acclimated to seawater (35 PSU). Acclimation period might thus be of great importance for inferring the actual long term cost of osmoregulation. This has been shown to be the case for studies on Mozambique tilapia *Oreochromis mossambicus* where different salinity acclimation schedules gave different results about their salinity tolerance (Kueltz et al. 1992; Iwama et al. 1997).

McGibbon (1977) tested freshwater stickleback individuals in a salinity gradient (at 12 °C) and found they had to be exposed to a salinity of 32.3 PSU before showing a significant increase in MO₂. It might thus be postulated that the highest experimental salinity of 30 PSU in my study and also in Jurss et al. (1982) is too low to initiate a significant increase in the energy demanding active ion transport in the sticklebacks (at least for the freshwater individuals). However, this is not supported by Gutz (1970) as his freshwater individuals showed no increase in the osmoregulatory energy demand (MO₂) when transferred from freshwater to saltwater (35 PSU).

All in all it seems that there is no fundamental difference in the osmoregulatory energy requirement between the ecologically divergent stickleback populations across a wide range of salinities. Postglacial evolution of freshwater stickleback and thousands of years of isolation from the sea has thus not reduced the freshwater population’s ability to osmoregulate in higher salinities. There is no sign of local adaptation to salinity in any of the experimental populations.

Fish form landlocked freshwater populations of species within the family salmonidae have shown to still possess a high degree of hypo-osmoregulatory ability and thus, saltwater tolerance despite being isolated from their anadrome counterparts for thousands of years (Foote et al. 1992; Foote et al. 1994; Margolis et al. 1995). Furthermore, photoperiod and temperature has been found to be of great importance for the level of salt tolerance in both
anadromous and landlocked salmonid species (Foote et al. 1992; Foote et al. 1994; Schmitz 1995).

It is important to note that I observed dramatically higher mortality in the brackish water sticklebacks kept in the holding tanks containing freshwater at 4 °C (although I did not perform counts and have no estimates on mortality rates) compared to freshwater sticklebacks under the same conditions. Schaarschmidt et al. (1999) also observed greatly increased mortality in brackish water individuals kept in freshwater at 4 °C. The authors discuss this as a breakdown of osmoregulation caused by high permeability of branchial epithelia and renal tubules. At low salinities the reduction in permeability is the dominant factor controlling ionic loss (Evans 1984). In contrast, Gutz (1970) noted greatly increased mortality in freshwater individuals kept in saltwater (35 PSU) at 4 °C, indicating a breakdown of the osmoregulatory apparatus in the freshwater individuals at high salinities and low temperatures. Interestingly, this was reversed when thyroxin was injected into the fish, indicating that this hormone is vital for saltwater adaptation in sticklebacks.

The high mortality observed for the brackish water individuals at low temperatures at both high salinities (Gutz 1970) and low salinities (my own observations and also in Schaarschmidt et al. (1999) study) indicates that low temperatures enhance the osmotic stress to the fish. Moreover it might also imply that there are genetic differences between marine, brackish water and freshwater populations in their osmoregulatory response to different salinity and temperature interactions. Larsen et al. (2007) found that at low temperature and high salinity led to significantly higher osmoregulatory stress in an anadromous brown trout population. Moreover, the same authors found intraspecific differences in the osmoregulatory abilities (differences in Na+/K+-ATPase activity and heat shock proteins) between two populations of brown trout inhabiting different salinity regimes which indicate adaptation to local environmental conditions in these populations.

The respiratory responses to changes in salinity in various euryhaline fish species seem to vary greatly; Morgan and Iwama (1991) found that juvenile rainbow and steelhead trout *Oncorhynchus mykiss* and fall chinook salmon *O. tshawytscha* had lowest oxygen consumption rates in freshwater and that the consumption rate increased with increasing salinity. Ron (1995) found that seawater reared Mozambique tilapia from yolk-sac fry had significantly lower oxygen consumption rates compared to those reared in fresh water. Isoosmotic salinity has been found to impose the lowest oxygen consumption rate in sea bream *Sparus sarba* (Woo and Kelly 1995), Nile tilapia *O. niloticus* (Farmer and Beamish 1969) and rainbow trout *Oncorhynchus mykiss* (Rao 1968; Altinok and Grizzle 2003). This is
consistent with the idea that isoosmotic conditions should require the least energy for osmoregulation. This was not, however, found in my experimental populations (15 PSU is close to isoosmotic) as they all showed similar rates of oxygen consumption across salinity treatments.

**Effect of lateral plates?**

Since no difference in MO$_2$ was found between completely-plated, partially-plated and low-plated morphs, I found no evidence of any relationship between osmoregulatory costs and the lateral plate morphs. Furthermore, the non-significant effect of the number of lateral plates on oxygen consumption rate in all treatments, indicate that the number of lateral plates has no influence on the osmoregulatoric energy demand in any of the experimental treatments.

In light of Heuts (1947) and Colosimo et al. (2005) findings, and based on the ecologically divergent distribution of the three lateral plate morphs one would expect that completely-plated individuals performed better in saltwater than low-plated individuals and vice versa.

Marchinko and Schluter (2007) revealed that juveniles of low-plated marine individuals reared in freshwater had significantly higher growth rate compared to juveniles of low-plated marine individuals reared in saltwater (30 PSU). Furthermore, juveniles of completely-plated marine individuals reared in freshwater had significantly lower growth rate compared to juveniles of completely-plated marine individuals reared in full saltwater. Moreover, when reared in freshwater, juveniles of low-plated individuals of both freshwater and marine parents had significantly higher growth rate than juveniles of completely plated individuals from both populations. However, they found no difference between juveniles of marine low-plated and completely-plated individuals in saltwater. In addition no differences were found between juveniles of similar parental phenotypes when tested in freshwater. Unfortunately, due to lack of data (high mortality rates of freshwater individuals in saltwater) there were no measurements of growth on juveniles of freshwater individuals of either lateral plate morph. This might imply, at least in Marchinko and Schluter’s (2007) investigated populations, that there is a differentiated cost of osmoregulation between lateral plate phenotypes in saltwater and freshwater. Although my results indicate that there is no differentiated cost of osmoregulation between lateral plate phenotypes, a lateral plate linked salt-tolerance gene could explain why the marine low-plated individuals in Marchinko and Schluter’s (2007) had higher growth rate in freshwater than saltwater although this is not supported by my results. It could be however, that possible differences in osmoregulatory
abilities between lateral plate morphs in my experiment is too small to be inferred from respirometry methods. Also there might be regional differences between populations in the putative Eda associated salt-secretion gene.

It is important to note that during the juvenile stage stickleback individuals start to produce lateral bone plates which become fully developed when individuals are about 30 mm in size (K, Østbye, Pers. comm.). Based on the studies by Giles (1983) and Bell (1993), Marchinko and Schluter (2007) discuss that the major difference in growth rate between lateral plate phenotypes in freshwater lye in the energetic benefits of not having to produce a high number of lateral plates, especially in calcium poor freshwater habitats. This has found support from a more recent growth experiment study (Barrett et al. 2008). My results showed no effect of lateral plates on MO2, and gives support to the growth-advantage hypothesis as the main contributing factor to the recurrent evolution of low-plated phenotypes in freshwater habitats.

**Methodological issues**

The overall mass specific oxygen consumption rate (MO2) at rest (standard metabolic rate) in my experimental populations is similar to what has been measured in a study by (Gutz 1970). Calculations based on the MO2 values of stickleback in Gutz (1970) study showed that they had an average MO2 of 14.00 µmolO2/g/hour in freshwater at 20 °C. Stickleback in my freshwater treatment exposed to an average of 18 °C had an average MO2 of 13.77 µmolO2/g/hour.

The significant effect of temperature in the freshwater treatment might be due to the larger variation in temperature within in this treatment compared to the brackish water and saltwater treatments. Also the freshwater and brackish water treatments showed a significant effect of body mass which similarly may be attributed to a larger variation in body mass within these treatments.

Other respirometry studies on fish metabolic rate have used different time scales when acclimating fish to the chamber. The time scale of one hour used for acclimating the fish in the present study might not be sufficient to calm the fish to a resting state. Preliminary results showed that after 24 hours of acclimation the fish had a significant lower oxygen consumption rate. If I had adopted such a time scale the experiment could not have been carried out due to time limit imposed on this thesis.

The process of sealing the chamber prior to the measurements was thought to trigger a stress response after the acclimation period. I therefore measured several fish for 3-4 hours,
which was thought to be the maximum affordable time per fish for this project, to see if there was any change in the trajectory decline in O₂ consumption above a measuring period of 2 hours. Since I did not find any further decline in consumption I therefore used a 2-hour time period.

The experimental studies discussed above have either used respirometry methods or physiological parameters to infer osmoregulatory cost and a direct comparison is to be done with caution. Moreover, oxygen consumption rates do not necessarily reflect osmoregulatory costs (McCormick et al. 1989; Morgan and Iwama 1998).
Concluding remarks

I found no difference in the energy required for osmoregulation across the experimental salinity gradient, or between populations inhabiting different salinity regimes. The euryhalinity of the three-spine stickleback seems unaffected by living in stable salinity regimes over long periods of time. Hypo-osmoregulatory abilities are remarkably persistent in many landlocked salmonid species physically isolated from their anadrome counterparts. This seemingly very conservative trait might indicate that the proximate mechanisms involved in saltwater osmoregulation still have functional properties and thus possess some adaptive value in these landlocked populations. However, the persistence of the ability to osmoregulate in saltwater might also be due to the relatively short evolutionary time scale since the last ice age.

I found no evidence for any relationship between lateral plates and osmoregulatory costs. The evidence for such a relationship in other studied stickleback populations may be due to regional differences in populations genes associated with the Eda locus. Nevertheless, respirometry methods are a very indirect measure of osmoregulatory costs and other more fine-tuned methods are probably better at inferring the cost of osmotic and ionic regulation.

Future research

It would be interesting to further investigate the importance of lateral plates in stickleback evolution. One could perform growth experiments of stickleback of known genotypes with varying number of lateral plates (lateral plate morphs) and test them in a salinity gradient. It would be important to include a freshwater control group containing minerals important for the synthesis of lateral bone plates. Performing growth experiments at isoosmotic conditions compared to freshwater and saltwater could reveal possible differences in the osmoregulatory energy demand associated with the lateral plate morphs. It could also reveal the actual cost of synthesizing lateral plates. These experiments would only disclose the possible energetic benefits of a particular phenotype in a particular physical environment. To infer their importance in the evolution of landlocked stickleback populations it would be important model fitness of particular phenotypes in relation to their interactions with important predators, habitat structures and the chemical properties (salinity, turbidity, hardness etc) of the habitat.
References


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