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Metabolic costs associated with seawater acclimation in a euryhaline teleost, the fourspine stickleback (*Apeltes quadracus*)

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ABSTRACT

The cost of osmoregulation in teleosts has been debated for decades, with estimates ranging from one to 30 % of routine metabolic rate. The variation in the energy budget appears to be greater for euryhaline fish due to their ability to withstand dynamic salinity levels. In this study, a time course of metabolic and physiological responses of the euryhaline fourspine stickleback (*Apeltes quadracus*) acclimated to freshwater (FW) and then exposed to seawater (SW) was examined. There was 18% mortality in the first 3 days following exposure to SW, with no mortalities in the FW control group. Gill Na^+/K^+-ATPase (NKA) activity, an index of osmoregulatory capacity, increased 2.6-fold in SW fish peaking on days 7 and 14. Gill citrate synthase activity, an index of aerobic capacity, was 50–62% greater in SW than FW fish and peaked on day 7. Tissue water content was significantly lower in the SW fish on day 1 only, returning to FW levels by day 3. Routine metabolic rate was decreased within 24 h of SW exposure and was maintained slightly (8–22%) but significantly lower in SW compared to FW water controls throughout the 2-week experiment. These results indicate that elevated salinity resulted in increased SW osmoregulatory and aerobic capacity in the gill, but with a reduced whole animal metabolic rate to this euryhaline species.

1. Introduction

Environmental salinity impacts physiological processes in organisms, and strongly influences species and life-stage distribution of fishes (Nelson, 1968; Baker, 1971; Audet et al., 1985a; Glover et al., 2012). Highly soluble ions, such as Na^+, K^+, Cl^-, and Ca^{2+}, affect hydration, blood pH, and function of all cellular processes (Laurent and Perry, 1991; Glitsch, 2001). Teleost fishes have evolved an osmoregulatory strategy in which blood osmotic pressure is maintained at approximately one-third the concentration of seawater. Most teleosts are stenohaline and therefore physiologically restricted to a narrow range of salinity values, either freshwater (FW) or seawater (SW). Euryhaline fishes, accounting for approximately 5 % of all teleosts, tolerate a large range of salinity, often ranging from FW to full-strength SW (Schultz and McCormick, 2013).

Regulating internal ion content requires mechanisms to counteract passive fluxes and maintain water and ion homeostasis within the body. In fishes, the gills, kidneys, and intestines are the three major osmoregulatory tissues, which have different function in FW and SW (McCormick et al., 1989b). Freshwater fishes live in hyposmotic conditions, maintaining blood osmolality above the levels found in the environment (~300 mOsmol kg^{-1} versus 0 mOsmol kg^{-1}, Edwards and Marshall, 2013). To maintain these concentrations, multiple osmoregulatory strategies in FW teleosts are utilized, including increased ion uptake through the gills (Foskett et al., 1983; McCormick, 2001), reabsorption of filtered ions in the kidney, and reduced drinking (Foskett et al., 1983; Wood and Marshall, 1994). In contrast, marine teleosts are in a hyperosmotic environment (~320 mOsmol kg^{-1} versus 1100 mOsmol kg^{-1}, Edwards and Marshall, 2013). Osmoregulation in SW occurs via an increased drinking rate (Foskett et al., 1983; Wood and Marshall, 1994; McCormick, 2001), uptake of ions and water in the intestine, reduced glomerular filtration in the kidneys (Takvam et al., 2021), and an active secretion of ions by the gills (Foskett et al., 1983).

Ionocytes in the gill, also known as "mitochondrion-rich" or

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"chloride" cells, use a number of transporter and channel proteins, including Na^+/K^+-ATPase (NKA), to create concentration and electrical gradients that facilitate ion movements in both FW and SW (Wood and Marshall, 1994; Evans et al., 2005). The energy needed to produce the conformational changes in NKA that result in translocation of Na^+ and K^+ across the cell membrane is made primarily through aerobic metabolism. For most (but not all) species of teleosts examined, the levels of gill NKA and mitochondrial abundance are greater after acclimation in SW compared to FW (Edwards and Marshall, 2013), suggesting that the metabolic costs of the gill may also be greater in SW. Citrate synthase (CS) is the rate-limiting enzyme of the citric acid cycle and has been widely used as an index of respiratory capacity following exposure to SW, with some species showing little to no change (i.e. Atlantic salmon, *Salmo salar*, McCormick et al., 1989b; mummichog, *Fundulus heteroclitus*, Marshall et al., 1999), while others have seen significant increases (Mozambique tilapia, *Oreochromis mossambicus*, Tseng et al., 2008).

It has long been argued that osmoregulation must be metabolically demanding since it requires cellular energy reserves for synthesizing and activating enzymes and transport proteins (Whittam, 1962; Tseng and Hwang, 2008; McCairns and Bernatchez, 2010; Ern et al., 2014). Energy spent on osmoregulation is unavailable for alternative uses, and some investigators have argued that this cost can affect the growth and fitness of teleost fishes (e.g. Wood and Marshall, 1994; Boeuf and Payan, 2001; Sampaio and Bianchini, 2002; Altinok and Grizzle, 2003). Theoretical calculations have estimated the cost of ion transport to be quite low, only 0.5–1.6% of routine metabolic rate (Kirschner, 1993; Ern et al., 2014). In contrast, direct measures of the effect of salinity on metabolic rate are often much higher, ranging from undetectable up to 30% of routine metabolic rate (Boeuf and Payan, 2001; Ern et al., 2014). Ern et al. (2014) have suggested that metabolic costs of osmoregulation are highly species dependent; for most species, metabolic rate is lowest in the habitat in which they normally reside or at isosmotic salinity conditions. For instance, Mozambique tilapia captured in brackish water and acclimated to FW had metabolic rates approximately 2.3-fold greater than fish acclimated to SW (Iwama et al., 1997). Alternatively, measures of metabolic costs of osmoregulation in FW rainbow trout (*Oncorhynchus mykiss*) account for approximately 20% of the energy budget whereas in SW they are approximately 27%, perhaps reflecting that FW is the predominant habitat for this species (Rao, 1971). Plaut (1999) found that the freshwater blenny (*Salaria fluviatilis*), a euryhaline species which inhabits FW, had significantly higher metabolic rates in FW compared to SW. Examination of more euryhaline species will help establish whether there are patterns in the relative metabolic rates in FW and SW that can be related to their normal or preferred halohabitats.

Most studies like those cited above examine the costs of osmoregulation in fish that have been fully acclimated to any given salinity. There is reason to suspect, however, that there are added costs associated with the process of acclimation. Branchial ionocyte remodeling occurs after exposure to SW as FW ion uptake mechanisms are lost and SW ion secretory mechanisms are gained (Inokuchi and Kaneko, 2012). It has been estimated that the theoretical energetic cost of new protein synthesis in fish ranges from 50 to 100 mmol ATP · g protein synthesized (Jobling, 1985). When expanded theoretically to oxygen consumption of a whole animal, protein synthesis accounts for approximately 20–40% (Smith and Houlihan, 1995). To date, only a few studies have examined changes in metabolic rate during the SW acclimation process, which includes an adaptive phase that occurs in the first 3–5 days and a chronic regulatory phase that follows where osmoregulatory traits reach homeostasis after 2 weeks (Soengas et al., 2007; McCormick et al., 2022). One study showed that FW-acclimated Mozambique tilapia had 20% greater oxygen consumption in SW compared to FW and isosmotic conditions 4 days after salinity changes (Morgan et al., 1997). Kammerer et al. (2010) found similar results that showed oxygen consumption was significantly greater after 24 h in FW-acclimated tilapia exposed to 25 ppt SW but went down 3 and 5 days after exposure. In contrast, Leray et al. (1981) found that FW-acclimated rainbow trout had a significant decrease in oxygen consumption 6 and 10 days after SW exposure. Finally, Kidder et al. (2006) found no effect of salinity on oxygen consumption in mummichog within the first 24 h of exposure to FW, isosmotic, and SW conditions. To our knowledge these are the only studies that have examined costs of osmoregulation during acclimation from FW to SW conditions. Thus, both the energetic costs of steady-state osmoregulation and the costs of acclimation to changes in salinity are still unclear.

The natural habitats of each species of the family Gasterosteidae are well described, with some species generally confined to marine and brackish (sea stickleback, *Spinachia spinachia* and blackspotted stickleback, *Gasterosteus wheatlandi*) or FW environments (brook stickleback, *Culaea inconstans*), while other species are capable of surviving broad salinity ranges from 0 ppt to 60 ppt (threespine stickleback, *Gasterosteus aculeatus*; ninespine stickleback, *Pungitus pungitius*; fourspine stickleback, *Apeltes quadracus*; Nelson, 1968). However, ionoregulation and osmoregulation have not been thoroughly investigated in most stickleback species other than the threespine stickleback (Grøtan et al., 2012; Divino et al., 2016; Li and Kültz, 2020). This lack of information on basic osmoregulatory physiology of stickleback is somewhat surprising given their use as an evolutionary model for FW invasions (Divino et al., 2016). The fourspine stickleback is a euryhaline species with populations generally in brackish water and SW, with a some resident populations occupying in FW, from Newfoundland to Virginia (Blouw and Hagen, 1984; Nelson, 1968). Fourspine sticklebacks were observed to have a near-isosmotic preference (7 ppt) in a horizontal gradient from 0 ppt to 35 ppt (Audet et al., 1985a). Further investigation showed that cortisol injections stimulated high salinity preference of 28 ppt while prolactin injections stimulated low salinity preference of 14 ppt in fourspine stickleback previously acclimated to 20 ppt (Audet et al., 1985b). Feeding in fourspine stickleback occurred at 38 ppt, which was higher than 24.5 and 28 ppt for observed for brook and ninespine stickleback, respectively (Nelson, 1968). These studies indicate the wide range of salinity tolerance and preference of fourspine stickleback.

The euryhaline capabilities of fourspine stickleback and their stability as a laboratory animal made it appealing as a model species to examine the metabolic costs of osmoregulation in teleosts. In the present study we measured routine metabolic rate in fourspine sticklebacks throughout a 2-week period following SW exposure, allowing us to assess both short-term and long-term metabolic rates. We also examined physiological responses, including survival, tissue water content, and gill NKA and CS activity to monitor the acute response (days 1 and 3) and acclimation process (days 7 and 14). A period of 2 weeks is generally recognized as sufficient for complete acclimation to altered salinity (McCormick et al., 2022). We expected that there would be higher metabolic rates during initial exposure to elevated salinity, but due to the high euryhalinity of this species there would be only small differences in animals fully acclimated to SW.

2. Methods and materials

2.1. Fish collection and maintenance

Adult fourspine sticklebacks (n = 198) were collected in the upper Quinnipiac River estuary (salinity ≤1 ppt), North Haven, CT, USA between August 12 to September 14, 2014; all fish were transported to laboratory facilities at the University of New Haven, West Haven, CT, USA. Fish were divided into four 150-L recirculating, aerated, filtered tanks. The fish were acclimated to 20 °C water temperature, 0.5 ppt salinity (equivalent to hard FW), and a natural photoperiod during the time of fish collection. Fish were fed an ad libitum daily diet of frozen bloodworms and brine shrimp for the duration of the experiment. Salinity and temperature were checked daily, and water quality parameters were checked weekly. Excess solid matter was siphoned daily and water changes occurred weekly. All experiments were performed under the guidelines for the use of laboratory animals approved by

University of New Haven Animal Care and Use Committee (protocol 2014–3)

2.2. Experimental design

Three experimental trials occurred September 29 to October 13, October 20 to November 3, and November 17 to December 1, 2014. Photoperiod was changed to 12:12 L:D on September 15, 2014, and water temperature was maintained at 20 °C for the duration of the experiment. For each trial, fish were placed individually into chambers and baseline metabolic measurements in FW were recorded as the day 0 measurements. The tank salinity was then increased to 35 ppt within a one-hour period after respirometry occurred; tanks were maintained at this salinity for the remainder of the trial. The FW control tank (0.5 ppt) also experienced a 50% water change within a one-hour period after respirometry. Fish were sampled at 1, 3, 7, and 14 days post-exposure. Fish were fed daily except for the 24-h period prior to each sampling. There was one tank for each SW exposure trial and one stock tank of FW control used throughout the experiment.

2.3. Respirometry

Ten identical glass respirometer chambers were constructed for use in the study. Each chamber was constructed from a 236-mL round glass container with a gasket-sealed lid. Each chamber included a 1.6 mm internal diameter tygon water inflow tube with a three-way stopcock that permitted control of inflow while minimizing disturbance of the fish. Water outflow was controlled via a tygon tube with a clamp. Approximately 50 mL of sterilized 1-cm diameter glass spheres were added to each chamber to reduce water volume and promote quiescence. The volume of each chamber and tubing, with spheres added, was individually measured, ranging from 184 to 191 mL, which produced an average respirometer volume to fish ratio of 589 ± 19. Each chamber was equipped with a dissolved oxygen sensor dot (O_2 2-mm non-autoclavable sensor dots, Loligo Inc.), which was secured to the inside of the chamber using silicone adhesive.

Prior to the start of a sampling time point, fish were fasted for 24 h. Chambers were filled with water from the experimental tank and were suspended in that tank to maintain temperature at 20 °C. Air bubbles were carefully removed from each chamber by repeated flushing with tank water. Ten fish were individually placed into respirometer chambers at 8 am. Respirometers were supplied with flow-through water using an external in-line pump, and fish rested for two hours to acclimate. This acclimation period was visually tested on three fourspine stickleback prior to the start of the experiment. Following an initial period of high activity and buccal pumping, the fish quickly became calm within one hour. Grøtan et al. (2012) observed similar behaviors in threespine stickleback within one hour of acclimation to static respirometer chambers.

At the start of the sampling period at 10 am, the chambers were sealed from the surrounding tank using the stopcocks and clamps, and the temperature-compensated dissolved oxygen (DO) level in each chamber was manually recorded using an optical oxygen meter (Witrox 1 and WitroxView software, Loligo Inc.). This marked the start of a three-hour respirometry period, and the fish remained undisturbed in the chambers during this time. During the respirometry period, the fish were physically separated from any human activity. At the end of the respirometry period, a final DO reading was manually taken for each sealed chamber. Oxygen levels in the chambers did not go below 3.5 $mg{\cdot}L^{-1}$. The change in DO reflected the amount of oxygen used by the fish for aerobic respiration and is a metric of routine metabolic rate (MO_2, mg $O_2{\cdot}g^{-1}{\cdot}h^{-1}$, Chabot et al., 2016).

Background microbial respiration was measured for three hours using SW or FW from respective tanks following day 1 in the first trial and was subtracted from routine metabolic rate calculations. Probe calibration occurred before every sampling day, which included a 100% oxygen saturation sample and 0% oxygen solution prepared with sodium sulfite (1 g Na_2SO_3 in 100 mL deionized water). After each respirometry sampling event, equipment was cleaned and sterilized using 0.5% sodium hypochlorite solution and then air dried.

2.4. Sampling protocol

Immediately after the final DO reading was taken, fish were removed from the respirometers and rapidly euthanized in an overdose of anesthetic (500 $mg{\cdot}L^{-1}$, MS222). For each individual, total length and wet mass were recorded. The gill basket was dissected and placed in 100 μL SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and immediately stored at -80 °C for later determination of gill NKA and CS enzyme activity. Head and viscera were removed, and the trunks were blotted dry and weighed to the nearest 0.001 g. The trunks were placed into an oven at 60 °C for 24 h, then weighed again. The difference pre- and post-drying was used to determine the tissue water content.

In order to determine gill NKA and CS activity, gill tissue was homogenized in 150 μL of SEID (SEI buffer and 0.1% deoxycholic acid). Samples were centrifuged at 5000 ×*g* for 5 min and then run in 96-well microplates at 25 °C on a THERMOmax microplate reader using SOFTmax software (Molecular Devices Corp.). Gill NKA activity was determined with a kinetic assay as described in McCormick (1993) and expressed in units of μmoles ADP·mg $protein^{-1}{\cdot}h^{-1}$. Gill CS activity was determined using the same prepared samples with a kinetic assay described in McCormick et al. (1989a) and expressed in units of μmoles·mg $protein^{-1}{\cdot}h^{-1}$. Protein concentrations were determined using BCA Protein Assay (Pierce, Life Technologies).

2.5. Statistical analysis

Statistical differences were analyzed by GraphPad Prism v9.2.0. Respirometer to fish mass ratio (RFR) was calculated as volume of the respirometer in mL divided by mass of fish in g. Mortality was compared using the Chi-squared test between salinity and between time points. The three replicate trials within each salinity were first compared to one another using a one-way analysis of covariance (ANCOVA) for each physiological response variable, where the independent variable was time and trial was considered a covariate. There were no significant differences among any variables between trials ($p > 0.05$), thus all three trials were treated as replicates and were grouped together for further analysis (Tables 1 and 2). Two-way ANCOVA was used to look for effects of independent variables of time and salinity and their interaction with mass as a covariate. Tukey's HSD post hoc test was used to test differences in salinity treatment at days 1, 3, 7, and 14. In all cases, results were considered significantly different when $p \leq 0.05$.

3. Results

There was significant mortality of 18% in the SW-exposed group, primarily within 3 days after SW exposure (Fig. 1). SW day 1 had significantly higher mortality than SW day 2 ($p < 0.01$). There was a significant difference in mortality between SW and FW on day 1 ($p < 0.01$). No mortality was observed in the FW group.

There was a significant time ($F = 20.00$, $p < 0.01$), salinity ($F = 55.68$, $p < 0.01$), and interaction ($F = 15.54$, $p = 0.01$) effect on gill NKA activity. Enzyme activity levels increased by 55% and 45% from day 1 to 3 and 3 to 7, respectively, with peak levels measured at day 7 and 14 in

Table 1
Mean (± SEM) mass and total length for seawater (SW) exposed and freshwater (FW) control groups of fourspine stickleback.

Group	Initial sample size	Mass, g	Total length, mm
SW-exposed	150	0.380 ± 0.016	38.4 ± 0.49
FW control	48	0.370 ± 0.079	38.1 ± 2.43

Table 2
Sample sizes (N) and respirometry volume-to fish body mass ratio (RFR) for experimental and control groups of fourspine stickleback for each trial and salinity.

		Trial 1		Trial 2		Trial 3	
		N	RFR	N	RFR	N	RFR
FW	Day 0	10	737 ± 55	7	683 ± 75	7	665 ± 148
	Day 1	4	702 ± 87	4	490 ± 112	4	645 ± 150
	Day 3	4	490 ± 71	4	701 ± 175	3	556 ± 122
	Day 7	4	809 ± 176	4	451 ± 83	4	572 ± 74
	Day 14	4	611 ± 89	4	631 ± 112	3	509 ± 51
SW	Day 1	9	719 ± 74	7	474 ± 44	7	615 ± 96
	Day 3	8	862 ± 98	5	463 ± 58	7	443 ± 36
	Day 7	7	624 ± 107	5	1589 ± 92	9	469 ± 61
	Day 14	7	623 ± 107	5	364 ± 60	10	383 ± 35

RFR was calculated as volume of the respirometer in mL divided by mass of fish in g. FW = freshwater, SW = seawater.

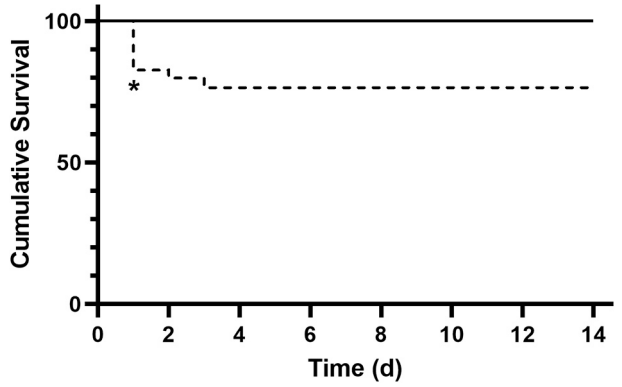

Fig. 1. Cumulative survival of fourspine stickleback in freshwater (FW, solid line) and 35 ppt seawater (SW, dashed line) groups. Exposure to each salinity occurred within one hour after day 0 sampling, where a FW-to-FW water change occurred as a control. An asterisk indicates significant difference between FW and SW exposure at that time ($p \leq 0.05$).

SW (SW day 1 to 3: $p < 0.01$; day 1 to 7: $p < 0.01$; day 1 to 14: $p < 0.01$; day 3 to 7: $p < 0.01$; day 3 to 14: $p < 0.01$; Fig. 2). Peak levels were 2.6-fold greater than the initial FW levels (FW day 7 to SW day 7: $p < 0.01$; FW day 14 to SW day 14: $p < 0.01$). The FW control group did not exhibit significant changes in gill NKA enzyme activity during the experiment ($p = 0.33$). Mass of the fish did not have a significant effect on gill NKA activity ($p = 0.16$), and there were no interactions with time or salinity.

Gill CS activity levels showed a significant time ($F = 3.04$, $p = 0.03$), salinity ($F = 13.66$, $p < 0.01$), and interaction effect ($F = 3.71$, $p = 0.01$). Values peaked in the SW-exposed group on day 7, which was significantly greater than days 1 and 3 ($p < 0.01$), and remained elevated on day 14 (Fig. 3). Gill CS activity levels on day 7 in the SW-exposed group were two times greater than day 0 enzymatic activity. Comparatively, gill CS activity in the FW control group did not change as time progressed, and no significance difference was found among those points ($p = 0.94$). Fish in SW on day 7 had significantly greater gill CS activity than fish in FW (FW day 7 to SW day 7: $p < 0.01$). Mass of the fish did not have a significant effect on gill CS activity ($p = 0.58$), and there were no interactions with time or salinity.

Tissue water content showed a significant time ($F = 6.19$, $p < 0.01$), salinity ($F = 12.99$, $p < 0.01$), and interaction ($F = 2.89$, $p = 0.04$) effect between the SW-exposed and FW control groups (Fig. 4). The SW-exposed group had a significant increase in water content between day 1 from the other sampling days (day 1 to 3: $p < 0.01$; day 1 to 7: $p < 0.01$; day 1 to 14: $p < 0.01$); the levels were maintained from day 7 to the end of the trial. In contrast, the FW control group showed no change in water content as the trial progressed. Fish in SW had a 4% decrease in tissue water content on day one compared fish in FW on day 1 ($p < 0.01$). On days 3, 7, and 14, differences in tissue water content between FW and SW ranged from 0.63 to 1.54%, which was not significantly different between experimental and control groups (FW day 3 to SW day 3: $p = 0.99$; FW day 7 to SW day 7: $p = 0.99$; FW day 14 to SW day 14: $p = 0.75$). Mass of the fish did not have a significant effect on tissue water content ($p = 0.71$), and there were no interactions of mass with time or salinity.

Following an increase in environmental salinity, oxygen consumption showed a significant salinity treatment effect ($F = 5.25$, $p = 0.02$) but did not show any significant change over time ($F = 1.98$, $p = 0.13$) or interaction effect ($F = 0.19$, $p = 0.91$). SW oxygen consumption was consistently lower than FW oxygen consumption throughout the experiment (Fig. 5). Oxygen consumption was highest in both FW and SW on day 3; SW fish returned to initial levels by day 14 while FW fish remained elevated. Fish in SW had a 22% lower oxygen consumption compared to FW fish on day 14. Mass of the fish had a significant effect on metabolic oxygen consumption ($p = 0.043$), but there were no interactions with time or salinity.

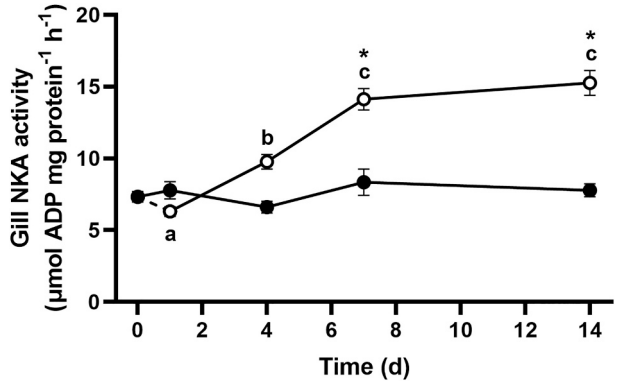

Fig. 2. Time-course changes in gill NKA activity in fourspine stickleback exposed to freshwater (FW, black circles) or 35 ppt seawater (SW, white circles). Dashed line indicates that fish previously in FW have been exposed to SW, which occurred within one hour after day 0 sampling. Values are means (± SEM). An asterisk indicates an effect of SW exposure at that time ($p \leq 0.05$). Different letters indicated significant differences within the SW time course ($p \leq 0.05$) as determined by a two-way ANOVA followed by Tukey's HSD post hoc test.

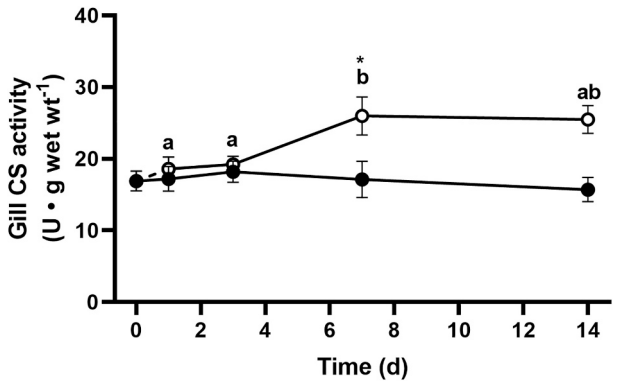

Fig. 3. Time-course changes in gill CS activity in fourspine stickleback exposed to freshwater (FW, black circles) or 35 ppt seawater (SW, white circles). Dashed line indicates that fish previously in FW have been exposed to SW, which occurred within one hour after day 0 sampling. Values are means (± SEM). An asterisk indicates an effect of SW exposure compared to FW controls at that time ($p \leq 0.05$). Different letters indicated significant differences within the SW time course ($p \leq 0.05$) as determined by a two-way ANOVA followed by Tukey's HSD post hoc test.

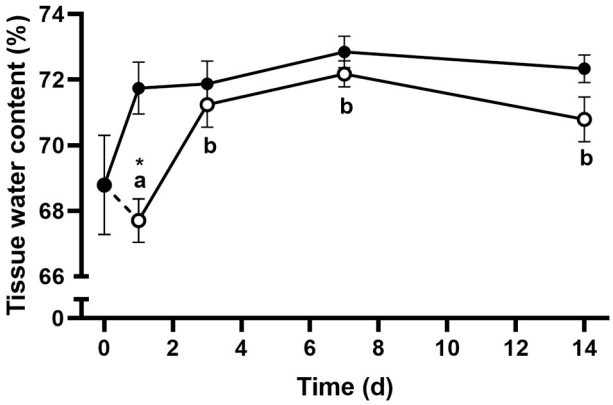

Fig. 4. Time-course changes in tissue water content in fourspine stickleback exposed to freshwater (FW, black circles) or 35 ppt seawater (SW, white circles). Dashed line indicates that fish previously in FW have been exposed to SW, which occurred within one hour after day 0 sampling. Values are means (± SEM). An asterisk indicates difference between FW and SW exposure at that time ($p \leq 0.05$). Different letters indicated significant differences within the SW time course ($p \leq 0.05$) as determined by a two-way ANOVA followed by Tukey's HSD post hoc test.

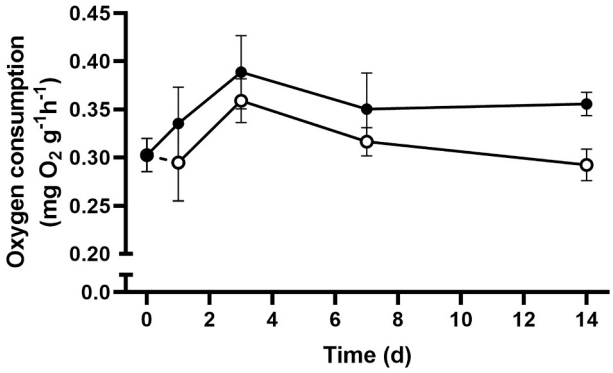

Fig. 5. Time-course changes in oxygen consumption in fourspine stickleback exposed to freshwater (FW, black circles) or 35 ppt seawater (SW, white circles). Dashed line indicates that fish previously in FW have been exposed to SW, which occurred within one hour after day 0 sampling. Values are means (± SEM). There was a significant effect of salinity but not time or their interaction on oxygen consumption (two-way ANCOVA), Post-hoc tests did not detect salinity differences at any time point.

4. Discussion

To date, only a few studies have examined the metabolic rates of osmoregulation over a time course, and there have been no studies of oxygen consumption and routine metabolic rate in fourspine sticklebacks, a common euryhaline species in the eastern United States. In this study, the exposure of FW-acclimated fourspine stickleback to SW resulted in: 1) mortality in the first 3 days, 2) transient loss of water content, 3) increase in gill NKA activity within 3 days and that remained elevated, 4) transient rise in gill CS activity at day 7, and 5) lower routine metabolic rate in SW compared to FW throughout the experiment. These results provide evidence that there are physiological changes in response to SW acclimation, but that both the metabolic demands of SW acclimation and long-term osmoregulation in SW are small compared to osmoregulation in FW and are unlikely to be limiting to fourspine stickleback. These findings have important implications for understanding the metabolic costs of osmoregulation in sticklebacks and other teleost species.

There are multiple methods for observing changes in the maintenance of homeostasis at the whole body level as a response changes in the environment (Febry and Lutz, 1987; McCormick et al., 1989a; Boeuf and Payan, 2001; Sampaio and Bianchini, 2002; Dalziel et al., 2011; Patterson et al., 2012). One common approach to determining salinity tolerance is direct testing, where environmental salinity is altered instantaneously, and mortality is an endpoint of inherent physiological capacity (Schultz and McCormick, 2013). In the present study, cumulative mortality of 23% occurred in the first 3 days after SW exposure, indicating a crisis phase of acclimation. This result corresponds to other studies that observed mortalities of landlocked threespine stickleback within the first 3 days after direct exposure to population-specific upper halotolerance (DeFaveri and Merilä, 2014, Divino et al., 2016). Plasma osmolality is a reliable method for observing changes in ion regulation in large species of teleosts, typically showing peak ion levels within the first 1–3 days after exposure to full-strength SW (Schultz and McCormick, 2013). However, smaller fish, such as fourspine sticklebacks, do not have enough blood for this method of sampling, making it difficult to measure osmolality directly. Instead, the tissue water content was used as a proxy to determine the capacity for osmoregulation. This study showed that fourspine stickleback exposed to full strength SW experienced a transient dehydration event, where the tissue water content was significantly lower than the baseline FW levels on day 1 but recovered by day 3. This time course is similar to that seen for mortality, suggesting that dehydration contributed to the mortality after SW exposure and that dehydration may have been higher for those individuals that did not survive. Several studies have shown a similar connection between halotolerance, mortality, and dehydration in euryhaline fishes (Maceina and Shireman, 1979; Laiz-Carrión et al., 2005; Kang et al., 2008; Küçük et al., 2013).

Time-course studies investigate and describe how physiological variables respond over a period after environmental conditions change (Lin et al., 2006). This study showed that gill NKA activity increased by 2.6-fold within 7 days after SW exposure and remained elevated through the experiment, indicating a regulatory phase of acclimation. Numerous studies have found gill NKA activity increases 1–7 days after SW exposure in other euryhaline species (Morgan et al., 1997; Wang et al., 2009; Urbina et al., 2013; Velotta et al., 2014; Velotta et al., 2015; Divino et al., 2016). Recent molecular studies have found that the two major subunits of the NKA enzyme are significantly upregulated in euryhaline teleosts within hours to days of being exposed to SW (Scott and Schulte, 2005; Evans and Somero, 2008; Havird et al., 2013; Norman et al., 2014; Taugbøl et al., 2014; Rind et al., 2017). The relatively slow increase in gill NKA activity seen in fourspine stickleback in the present study is similar to that seen in other intertidal species such as tilapia (Tipsmark et al., 2008). Marshall (2013) has suggested that this delay in physiological and morphological protein changes of the gill may be a mechanism of tolerance, as a way save energy in the case where environmental conditions may return to a lower salinity.

Citrate synthase, the first rate-limiting enzyme of the citric acid cycle in mitochondria, has been widely used to estimate the aerobic capacity of tissues (Pelletier et al., 1994; Gagnon and Holdway, 1999; Friedman et al., 2012). In mammals, nearly 90% of cellular oxygen consumption occurs in the mitochondria, mainly for the production of ATP that is used for most energy demands of the cell, including NKA activity and protein production (Rolfe and Brown, 1997). However, based on theoretical calculations of protein bond synthesis and transport processes, it has been estimated that approximately 20–40% of oxygen consumption goes toward protein synthesis (Houlihan et al., 1988). In this study, gill CS showed increased activity in the SW-exposed group during the acclimation phase, which indicates that there was a greater capacity for metabolic aerobic respiration in the gill. This time course is similar to that seen for gill NKA, which also significantly increased by day 7 and remained elevated. The higher levels of gill CS may in fact allow for the greater oxidative demands of protein, morphological, and cellular differentiation of the gill (Lyndon and Houlihan, 1998) as well as elevated gill NKA activity required for increased ion transport activity of gill ionocytes in SW (Marshall, 2013).

As noted in the introduction, there has been a wide range in measured metabolic rate differences in long-term acclimated animals in FW and SW. We found a significant effect of salinity on metabolic rate in fourspine stickleback, with levels that were 22% lower in SW after 2 weeks of acclimation. Previous studies have found no significant difference in routine metabolic rate between salinity treatments for several highly euryhaline species, including sheepshead minnow (Haney and Nordlie, 1997), European sea bass (*Dicentrarchus labrax*, Chatelier et al., 2005) and threespine sticklebacks (Grøtan et al., 2012). But several species' metabolic rates due to salinity have been relatively high in SW, including Mozambique tilapia (Job, 1969, Morgan et al., 1997), rainbow trout (Rao, 1971), shiner perch (*Cymatogaster aggregata*, Christensen et al., 2018), amphibious mangrove rivulus (*Kryptolebias marmoratus*, Sutton et al., 2018), common snook (*Centropomus undecimalis*, Gracia-López et al., 2006) and white seabream (*Diplodus capensis*, Kemp, 2009). In one study, SW-acclimated Arabian killifish (*Aphanius dispar*) had 28% greater oxygen consumption rates in SW compared to the highest values observed in our SW trial (Skadhauge and Lotan, 1974). More specifically to the Gasterosteidae family, Armitage and Olund (1962) found similar oxygen consumption levels of FW-acclimated brook stickleback as this study found in the SW fourspine stickleback; however, as the salinity increased, oxygen consumption peaked in isosmotic conditions and levels decreased as salinity exposure increased to 14 and 17.5 ppt, presumably due to physiological capacity failures. Alternatively, a low-salinity (4 –5.5 ppt) coastal population of ninespine stickleback held in FW at 19 °C had oxygen consumption values approximately two-fold lower than those found in our study (Bruneaux et al., 2014). Many factors affect metabolic oxygen consumption in teleosts, including natural salinity, natural temperature, body mass, life stage, and critical oxygen tension (Haney and Nordlie, 1997; Clarke and Johnston, 1999; Killen et al., 2010; Ern et al., 2014).

The present study applied direct exposure to SW as a means to examine metabolic rates and physiological changes in response to increased salinity. This approach allowed us to observe acute and acclimation responses to SW exposure compared to FW controls over an extended time course. It is possible, however, that there will be differences in the long-term metabolic rates of animals directly transferred to SW (as in the present study) compared to those that are gradually acclimated. For instance, stress associated with direct transfer may have long term impacts (Mommsen et al., 1999). However, our results suggest that after the acute phase (first 1–3 days) of direct exposure is completed, strongly euryhaline species such as fourspine stickleback are fully capable of ion regulation in SW, and thus long-term differences in metabolic rate due to gradual versus direct transfer are unlikely. This may not be the case for less euryhaline species that may struggle to fully acclimate at elevated salinities.

As described in the introduction, only a few studies have examined the effect of salinity on metabolic rates over a short time course (Kidder et al., 2006; Morgan et al., 1997; Kammerer et al., 2010; Leray et al., 1981). We have demonstrated that FW-acclimated fourspine sticklebacks have lower routine metabolic rates after 1 day in SW and these lower rates were maintained throughout the 14 day study. The lower metabolic rates during SW acclimation were contrary to our expectations, and indicate that the metabolic costs of acclimation to SW are small in fourspine stickleback. This lower metabolic rate during initial SW exposure may be due in part to the high euryhalinity of this species that has been shaped by the large salinity changes in their estuarine habitat and has resulted in high physiological plasticity in response to salinity (Marshall, 2013). Furthermore, the lower costs in SW at 2 weeks suggests that the metabolic costs of osmoregulation are lower in SW than FW for this species. These results also support the idea proposed by Ern et al. (2014) that oxygen consumption is lowest in a species' natural habitat where osmoregulation costs will be lowest. Fourspine stickleback generally occupy marine and brackish environments, with a few known freshwater populations throughout its range (Lee et al., 1980; Blouw and Hagen, 1984). Additional studies, including maximum metabolic rate and aerobic scope, may help further elucidate the effects of salinity on performance and fitness in fourspine stickleback.

Transient changes in oxygen consumption were observed in this study, with individual variation occurring within each time point, suggesting genetic and/or physiological variation in individual inherent capacity to respond to SW exposure. We also observed individual variation in salinity tolerance since 18% of individuals died after exposure to seawater. These findings suggest that there may be substantial standing variation in euryhalinity that could be acted on by natural selection. However, on day 14, we saw a decrease in variation in SW metabolic rates compared to the rest of the time course, which may indicate the ability of fourspine stickleback to physiological acclimate to SW.

The lower metabolic rate in SW at the whole animal level does not mean there are no changes in metabolic rate of the gill or other osmoregulatory organs after salinity change. Higher levels of gill NKA and CS activity after seawater exposure in fourspine stickleback and many other teleosts indicate that the metabolic demands may have increased. The intestine and kidney also have prominent roles in osmoregulation in both FW and SW. The intestine plays a role in water absorption in SW, as ions are actively taken up from the lumen and water passively follows (Grosell, 2010). Kidneys in FW produce a very dilute urine that is excreted at a high flow rate as it reabsorbs ions, while in SW, kidneys produce a urine that is isosmotic to the plasma at a low flow rate as it reabsorbs water (Takvam et al., 2021). Thus, the kidney may reduce metabolic demands in SW at the same time that the gill metabolic demands are increasing. It should also be noted that the relatively small size of these organs relative to body size (< 5%) indicates that only very large changes at the organ level would influence whole animal oxygen consumption (McCormick et al., 1989a). In addition, metabolic rates may be partitioned among other aspects of the energy budget, such as reduced growth and reproduction (Febry and Lutz, 1987, Gibbons et al., 2018).

5. Conclusions

Understanding the energetic costs of SW acclimation furthers our understanding of osmoregulation in fishes in general and provides insight into the evolution of euryhaline fishes (Schultz and McCormick, 2013). With natural selection placing pressures on high-cost activities, it was previously thought that euryhalinity was too expensive to be maintained unless there was a clear benefit to having the trait (Kültz, 2015). Yet, euryhalinity has evolved repeatedly in most major families of teleosts (Schultz and McCormick, 2013), which may indicate that there are lower costs imposed by osmoregulation than expected, allowing the convergent adaptation of euryhalinity to occur more widely (Morgan and Iwama, 1999; Evans et al., 2005). It has also been shown that there is not a high cost associated with retaining ancestral osmotic abilities (Grøtan et al., 2012). Rather, stenohaline fishes do not have the physiological mechanisms (ion uptake in FW, ion secretion in SW) to withstand living in environments other than their own. We hypothesize that as fishes evolved the physiological capacity to withstand euryhaline environments, osmotic costs may play a role in the selection process of maintaining euryhalinity. Our results indicate that in highly euryhaline species the actual costs of osmoregulation appear to be small and are likely not limiting to euryhaline species such as fourspine stickleback.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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