The effects of sub-lethal salinity concentrations on the anti-predator responses of fathead minnows

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HIGHLIGHTS

- We exposed minnows to three levels of salinity and three levels of risk cues.
- Minnows exposed to increasing salinity showed a reduction in anti-predator responses.
- Threat-sensitive behavioural responses were absent in moderate and high salinity.
- Modified behavioural responses are evident well below physiological tolerance levels.

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ABSTRACT

Salinization, both natural and anthropogenic, of inland waters is a major facet of environmental change, and can have detrimental effects on aquatic systems. Fish facing increasing levels of salinity must do more than simply survive salinization, they must also undertake important behaviours such as predator avoidance. Here, we exposed fathead minnows (Pimephales promelas) to three levels of salinity crossed by three levels of predation risk cues. We found a reduction in pre-stimulus movement and a lowered intensity of anti-predator response for the highest salinity exposure (8000 ppm). We also found that the typical threat-sensitive anti-predator response (an important behaviour conferring fitness advantages) was absent in the two highest salinity exposure treatments. Our data demonstrate that salinization can have negative effects on critical behaviours well below physiological tolerance levels.

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1. Introduction

Environmental change can dramatically affect aquatic ecosystems. Climatic events such as drought can alter water temperature, dissolved oxygen, salinity, and the concentration of dissolved nutrients. These changes have the potential to stress organisms, and possibly entire aquatic ecosystems, beyond the point of recovery (Bond et al., 2008). Additionally, humans can exacerbate the effects of drought through practices such as water diversion and crop irrigation (Lake, 2011). However, aquatic systems tend to be resilient, and can often cope with environmental change if the rate of change is not excessive (Flower, 2001). Unfortunately, the rate of change associated with many human activities tends to be high, and the sheer number of ways in which humans can modify the environment is staggering. Anthropogenic environmental change also tends to be multi-faceted; aquatic ecosystems are often affected by more than one stressor at a time. In short, aquatic ecosystems face a number of challenges, and are affected by a variety of natural and anthropogenic changes.

Salinization, the increase of salinity in water bodies, is one such change facing many inland waters. Salinization can occur in two ways. Natural, or primary salinization, has no anthropogenic basis. It is typically caused by the accumulation and concentration of salts via ice-cover, weathering of rocks and soil, and the process of evaporation and subsequent concentration of salts. In contrast, secondary salinization is caused by human activities, and may be more acute. Secondary salinization can have many causes, including: clearing of natural vegetation for development, discharge of wastewater, irrigation, runoff, dams, and mining activities (Williams, 2001). Confounding both primary and secondary salinization, global climate change almost certainly plays a role, but is notoriously difficult to discern (Covich et al., 1997; Pratchett et al., 2011).

Increasing salinity can affect aquatic ecosystems in many ways. It can cause shifts in biotic communities, limit biodiversity, exclude less tolerant species, and cause acute or chronic effects at specific life stages (Weber-Scannell and Duffy, 2007). Additionally, salinity...
may increase the toxicity of other pollutants in the aquatic environment (Noyes et al., 2009). Salinization of inland waters represents a serious threat to ecosystems and humans alike. If left unchecked, increasing salinity could leave many inland water bodies unfit for animal and/or human uses (Williams, 1987).

The physiologic effects of increasing salinity have been well studied in aquatic organisms. However, many studies have focused on NaCl (for example, Kefford et al., 2004; Luz et al., 2008). This bias in previous research is a concern because other major ions, common to inland waters around the world (such as MgSO₄ dominated lakes in Saskatchewan), may have even more dramatic effects than NaCl. For instance, Mount et al. (1997) found that the 96-h LC50 for fathead minnows (Pimephales promelas) varied from <510 to 7960 parts per million (ppm) depending on the ion ratio and salts present in the experimental water, with the following relative ion toxicity: K⁺ > HCO₃⁻ > Mg²⁺ > Cl⁻ > SO₄²⁻. Similarly, for rainbow trout (Oncorhynchus mykiss) and larval chironomids (Chironomus tentans), Chapman et al. (2000) found the toxicity of mining effluents was not predictable from total dissolved solids (TDS) concentration alone, but instead depended on the specific combination and concentration of ions.

In addition to overcoming the physiological stress imposed by salinity, aquatic animals must successfully forage, mate, and avoid predators while exposed to these stressors. The goal of our current work was to understand how an increase in salinity influences the ability of a prey fish (fathead minnows, P. promelas) to respond to predation risk, given that a combination of chemical and biological stressors have been shown to interact synergistically to influence the behaviour of aquatic animals (Relyea and Mills, 2001).

Fathead minnows are small bodied prey fish common throughout much of central North America and as such, are often subject to both primary and secondary salinization. Minnows have well characterized anti-predator behaviours and show a remarkable ability to distinguish among a variety of chemical cues indicating risk. For example, these fish can determine the size, density, and proximity of predators based on the odour signature of their predators (Ferrari et al., 2010). Minnows also respond to chemical alarm cues released by nearby conspecifics which have been attacked by a predator (Chivers and Smith, 1998). These cues serve as an early warning, alerting prey fish to the presence of a nearby predator.

Ferrari et al. (2005) showed that minnows exhibit threat-sensitive responses to chemical alarm cues. The minnows increase the intensity of their anti-predator response (an increase in shelter use and a reduction in activity) when exposed to increasing concentrations of alarm cues. Essentially, fathead minnows modulate their anti-predator response to match the perceived level of threat, based on the concentration of alarm cues present (Helfman, 1989). This confers a fitness advantage: time and resources are not wasted due to excessive or insufficient anti-predator behaviours (Lima and Dill, 1990).

Here, we tested whether salinity influenced the ability of fathead minnows to exhibit threat-sensitive anti-predator behaviour by exposing them to three sub-lethal concentrations of salinity and measuring their anti-predator response to three risk levels: no risk (water), low, or high risk (low or high concentration of alarm cues) on the anti-predator responses of fathead minnows (n = 180 fish, 20 per treatment). After an acclimation period to their respective salinity levels, the fish were exposed to one of three cues and their anti-predator response was recorded.

2.2. Experimental fish

Adult fathead minnows were captured from Feedlot Pond (salinity ~300 ppm), located on the University of Saskatchewan campus, in November 2009 and housed in the R.J.F. Smith Center for Aquatic Ecology in a 3500 L flow through tank (filled with dechlorinated tap water, salinity ~250 ppm). They were fed commercial fish flakes (Nutrafin Max Flake Food, Rolf C. Hagen Inc., Montreal, QC) ad libitum and held at room temperature with a 16:8 h light:dark photoperiod and at least 80% oxygen saturation. This experiment took place in the spring of 2010, prior to the breeding season of the minnows.

2.3. Stimulus collection

Alarm cues were prepared using 15 fathead minnows (mean ± SD: fork length 5.5 ± 1.3 cm; weight 1.9 ± 1.2 g) following the method described in Ferrari et al. (2005). The minnows were euthanized by cervical dislocation, in accordance with University of Saskatchewan Animal Care protocol #20100023. Skin fillets were removed from each side of the body and immediately placed in 100 mL of chilled distilled water. The skin solution was then homogenized and filtered through glass wool. This procedure resulted in 42.9 cm² of skin in 858.4 mL of distilled water to give a stock solution of 1 cm² of skin per 20 mL. This stock solution was then serially diluted to obtain high (1 cm²/40 L) and low (1 cm²/80 L) concentration alarm cue solutions. These solutions, along with distilled water, were frozen in 20 mL aliquots at −20 °C until use.

2.4. Salinity preparation

Experimental water was prepared by reconstituting reverse osmosis water with sodium carbonate (Na₂CO₃; 1000, 4000, 8000 ppm treatments: 0.181 g, 0.722 g, 1.444 g), potassium chloride (KCl; 0.415 g, 1.661 g, 3.323 g), sodium bicarbonate (NaHCO₃; 1.261 g, 5.045 g, 10.091 g), magnesium sulfate (MgSO₄; 5.279 g, 21.117 g, 42.234 g), calcium sulfate dihydrate (CaSO₄·2H₂O; 0.512 g, 2.047 g, 4.093 g), calcium chloride dihydrate (CaCl₂·2H₂O; 0.074 g, 0.296 g, 0.592 g), and sodium sulfate (Na₂SO₄; 1.403 g, 5.613 g, 11.225 g). All chemicals were American Chemical Society (ACS) reagent grade or higher, and were chosen to mimic the ion ratio of Lake Lenore—a typical sulfate-dominated saline lake in Saskatchewan, Canada. See Fig. 1 for milligram equivalent per litre (mEq L⁻¹) ion composition. Due to the absence of published fathead minnow toxicity data for sulfate dominated water bodies, sub-lethal salinity concentrations were chosen based on the natural distribution of fathead minnows in these systems (maximum ~10000 ppm, Rawson and Moore, 1944). Although the test fish were collected from a pond with a salinity of ~300 ppm and maintained in the laboratory for several months at a salinity ~250 ppm (see above), we chose 1000 ppm as our lowest salinity concentration because preliminary experiments revealed that the behaviour (activity level, etc.) of the fish were not influenced at this salinity level.

Salinity levels were increased by removing 500 mL of water from each 9 L experimental tank, pooling the removed portions for each treatment level in a separate mixing container, adding salts while stirring with a hand mixer, and returning the removed portion to each tank. This approach ensured that the salts were completely dissolved in solution before they were added to

2. Methods

2.1. Experimental design

Using a completely randomized 3 × 3 design, we tested the effects of three salinity levels (1000 ppm, 4000 ppm, or 8000 ppm) and three predation risk cues (water, low, or high concentration of alarm cues) on the anti-predator responses of fathead minnows (n = 180 fish, 20 per treatment). After an acclimation period to their respective salinity levels, the fish were exposed to one of three cues and their anti-predator response was recorded.
experimental tanks, and helped minimize the precipitation problems associated with creating a single stock solution.

2.5. Test apparatus and acclimation period

A single minnow was placed in each experimental tank \( (n = 180) \). Experimental tanks consisted of 9 L plastic aquaria equipped with an airstone, a stimulus injection tube, a lid, and a shelter object \( (11 \times 11 \text{ cm ceramic tile with } 3 \text{ cm legs}) \). After 24 h, salinity was increased over 2 d to experimental levels using the procedure outlined in Section 2.4. Salinity was increased slowly to decrease stress to fish \( \text{(Kefford et al., 2004; Whiterod and Walker, 2006). Once experimental salinity levels were reached, the fish were given an additional 72 h to acclimate to the new environmental conditions.} \)

2.6. Testing procedure

Testing followed well established protocols \( \text{(Pollock et al., 2006). Following the acclimation period, fish were exposed to one of three risk cues and their anti-predator responses were measured for 8 min prior to and 8 min following the injection of cues into the test tank. During each of these observation periods, we recorded the time (s) that minnows spent moving (i.e. actively swimming and/or foraging) and the time (s) spent under shelter (at least 75% of body under shelter). Typical minnow anti-predator behaviours include a reduction in movement and an increase in shelter use from the pre-stimulus baseline; these behaviours render minnows less conspicuous to predators, and tend to increase survival \( \text{(Mathis and Smith, 1993)}. \text{ Tanks were observed between 10:00 and 16:00 in a random order to prevent bias due to order effects, and the same number of tanks from each treatment was observed each day. The observer sat two meters from the tanks and the room was darkened to reduce fright responses attributable to the presence of the observer. To ensure the injection tube was clear of any stagnant water, 60 mL of tank water was withdrawn and discarded just prior to the start of a trial. An additional 60 mL of tank water was then withdrawn and retained to later flush the stimulus into the tank. After the 8-min pre-stimulus injection period, 2.5 mL of the high or low concentration alarm cue solution or distilled water was injected into the tube and flushed into the tank with the retained tank water. Fish were then monitored for an additional 8 min. At the conclusion of the trial, water quality parameters (conductivity, pH, dissolved oxygen, and temperature) were recorded for each tank with a YSI probe \( \text{(Professional Plus, YSI Inc., Yellow Springs, OH). Additionally, 25 mL of tank water was withdrawn from each tank and combined by treatment group for analysis by an independent laboratory (Saskatchewan Research Council, Saskatoon, Canada) for verification of ion composition and TDS concentration (Fig. 1, Table 1).} \)

2.7. Statistical analyses

Statistical analyses were performed with R version 2.13.2 \( \text{(R Development Core Team, 2011). All data were checked for conformance to test assumptions, and were transformed as noted below when those assumptions were not met.} \)

2.7.1. Fish characteristics and water quality

To ensure there were no confounding differences among treatment groups due to size differences, weight and length measurements of each fish were taken before tank assignment. Size and weight of test fish were compared between salinity and alarm cue treatments using a 3 \( \times 3 \) MANOVA. Water quality parameters \( \text{(conductivity, pH, dissolved oxygen, and temperature) were compared among the three salinity levels using a 1 \( \times 3 \) MANOVA. ANOVA’s were then performed to investigate which of the parameters differed significantly between levels. Significant differences were expected for conductivity and \( \text{pH} \), as conductivity increases with increasing salinity and \( \text{pH} \) tends to increase as well. Saline waters \( \text{(salinity >3000 ppm, Williams, 1964) trend to have higher pH values than freshwaters because 1) the salts in saline waters are involved in acid–base interactions in the water, and 2) increased alkalinity (due to increased concentrations of } \text{HCO}_3^- \text{ and } \text{CO}_3^{2-} \text{ ) raises the equilibrium } \text{pH of saline water (Morel and Hering, 1993; Hinga, 2002).}} \)

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
Treatment group & 1000 & 4000 & 8000 \\
\hline
Conductivity (\( \mu \text{s cm}^{-1} \)) & 1464 (1) & 4257 (11) & 7610 (29) \\
Dissolved oxygen (%) & 87.3 (2.3) & 87.4 (1.8) & 87.9 (2.2) \\
\text{pH (pH units)} & 8.3 (0.1) & 8.7 (0.1) & 8.6 (0.1) \\
Temperature (\(^\circ\text{C}\)) & 23.9 (1.1) & 23.7 (1.1) & 23.5 (1.1) \\
TDS (ppm) & 1067 & 4037 & 7783 \\
Error (%) & 7 & 1 & -3 \\
\hline
\end{tabular}
\caption{Mean (\( \pm \text{SD}\) water quality parameters. Percent error for TDS concentration is based on comparison with nominal theoretical values. The negative value for the 8000 ppm group denotes the slightly lower than theoretical value.}
\end{table}
2.7.2. Behavioural data

Data for pre-stimulus movement were heteroscedastic and were not normally distributed. Therefore, the data were square root transformed. A 1-way ANOVA was conducted to determine any effect of salinity on baseline activity among treatment groups. Due to differences in pre-stimulus movement, percent change in movement (post-stimulus–pre-stimulus/pre-stimulus) was used as a measure of response to alarm cues. Percent change in movement was not normally distributed. Because this measure is an unbounded percentage with both positive and negative numbers, data were rank transformed. A $3 \times 3$ ANOVA was then performed to determine any differences among treatment groups. To determine if salinity affected overall intensity of response, a 1-way ANOVA was performed on percent change in movement for fish exposed to alarm cues (excluding fish exposed to water), followed by Tukey’s HSD to evaluate any significant differences.

To determine if salinity affected the ability of minnows to respond to risk in a threat-sensitive manner, separate ANOVA’s were performed on percent change in movement among alarm cue treatments (within each salinity group), and any significant differences were evaluated with Tukey’s HSD.

Examination of the means for pre-stimulus shelter use revealed that minnows spent a large percentage of time under shelter (mean ± SE: 1000 ppm 78 ± 5%; 4000 ppm 72 ± 5%; 8000 ppm 84 ± 4%). Shelter use is a measure of risk avoidance. Therefore, sheltering behaviour should be rare in the absence of risk. Because minnows in this experiment spent so much time under shelter before being exposed to alarm cues, shelter use was deemed an inappropriate measure, and subsequently removed from further analyses (see Discussion for details).

3. Results

3.1. Fish characteristics and water quality

Standard length (mean ± SD: 5.2 ± 0.9 cm) and weight (2.4 ± 1.2 g) did not differ among experimental groups (Pillai’s Trace, salinity: $F_{(2,167)} = 0.02$, $p = 0.6$; cue: $F_{(2,167)} = 0.03$, $p = 0.2$; interaction: $F_{(4,167)} = 0.05$, $p = 0.3$). A significant difference in water quality parameters was found among salinity treatment groups (Pillai’s Trace: $F_{(2,173)} = 1.7$, $p < 0.001$, Table 1). Both conductivity ($F_{(2,173)} = 17876$, $p < 0.001$) and pH ($F_{(2,173)} = 287$, $p < 0.001$) showed significant differences among groups. These differences were present among salinity groups—no significant differences were found among alarm cue treatments with the same salinity group (conductivity: $F_{(2,173)} = 0.02$, $p = 0.98$; pH: $F_{(2,173)} = 0.04$, $p = 0.96$). Similarly, no difference was found among treatment groups for dissolved oxygen ($F_{(2,173)} = 0.4$, $p = 0.6$) or temperature ($F_{(2,173)} = 0.2$, $p = 0.8$). All TDS values were within 7% of theoretical Lake Lenore values.

3.2. Behavioural measures

Pre-stimulus data for time spent moving showed that minnows were significantly affected by salinity treatment ($F_{(2,173)} = 22$, $p < 0.001$; Fig. 2), with fish in the 8000 ppm treatment displaying a lower baseline activity level than those in lower salinity treatments. Percent change in movement was significantly affected by both cue and salinity levels (salinity × cue: $F_{(8,167)} = 32$, $p < 0.001$; Fig. 3).

Salinity concentration affected the overall intensity of anti-predator responses ($F_{(2,114)} = 21$, $p < 0.001$), with minnows in the 8000 ppm treatment group displaying lower intensity anti-predator responses than minnows in other treatments (Tukey comparisons: $p < 0.001$).

Minnows in the 1000 ppm treatment displayed typical threat-sensitive anti-predator responses ($F_{(2,55)} = 64$, $p < 0.001$), that is, displayed stronger anti-predator responses to increasing concentrations of alarm cues (Tukey comparisons: all $p < 0.005$). Minnows exposed to higher salinity levels, however, failed to show this pattern, and did not respond differently to the high and low concentration of alarm cues (4000 ppm: $F_{(2,56)} = 34$, $p < 0.001$, high vs. low: $p = 0.8$; 8000 ppm: $F_{(2,56)} = 11$, $p < 0.001$, high vs. low: $p = 0.9$).

4. Discussion

Our study clearly demonstrates that sub-lethal levels of salinity have the potential to affect the behavioural responses of fathead minnows, in terms of baseline activity level, overall intensity of anti-predator response, and threat-sensitivity. We found that fish maintained in 8000 ppm salinity water were less active than fish exposed to lower concentrations. This indicates that such levels provide a significant physiological and homeostatic cost to the fish. Lower activity levels relating to such intoxication have been linked to lower food-anticipatory activity, food intake, and growth (Luz et al., 2008).

Our results also indicate that exposure to salinity can decrease the intensity of response to a general risk cue, such as alarm cues. This can be explained in one of two ways. First, salinity may interact with the olfactory receptors of the fish, or the alarm cue itself, causing a decrease in the detection of cues in the water. A lower detection would lead to a lower anti-predator response. Alternatively, the lower anti-predator response may be the result of a change in the cost-benefit trade-offs of the fish. The increasing salinity likely causes an increase in metabolic costs (Pistole et al.,...
mediated behaviours, such as learned predator recognition (Leduc et al., 2004a). Such alterations, in turn, cause deficiencies in alarm cue-processing (Leduc et al., 2004b) and have potential survival consequences (Leduc et al., 2010). Therefore, these specific costs should also be evaluated for salinity with fathead minnows in order to gain a better understanding of the mechanisms limiting their ability to properly respond to risk.

While it is true that both predators and prey may be affected by increasing salinity, predators tend to be larger-bodied, and there is evidence that larger body size may decrease the intensity of adverse effects associated with increasing salinity (Mojazi Amiri et al., 2009). Additionally, species-specific salinity tolerance may play a role in predator-prey interactions. For instance, Ingersoll et al. (1992) found that NaCl dominated water was acutely toxic to adult fathead minnows between 8000 and 10000 ppm, while Jacobsen et al. (2007), summarizing previous research, reported NaCl toxicity of 12000–18000 ppm for northern pike (Esox lucius), a common minnow predator. Though no studies have been conducted to explicitly evaluate potential predator-prey interactions when both fishes are affected by salinity changes, differential species tolerances may lead to differential survival.

Typical anti-predator responses of fathead minnows include a reduction in movement and an increase in shelter use. Our study did not include change in shelter use in the analyses because of high levels of pre-stimulus use. This pre-stimulus sheltering behaviour may be explained by the size of the shelter relative to the size of the tank. Shelter use experiments are typically undertaken in tanks with a volume of at least 37 L (for example, Pollock et al., 2006). Though shelters used in this study were roughly the same size as those used in 37 L tanks, the experimental tanks used here had a 9 L volume. Therefore, the shelters occupied a much greater area of the tank, and this may help to explain the high pre-stimulus use. In a larger tank, there is more forage area available than in a 9 L tank. Therefore, it may be that the fish found the shelter a preferable environment to rest of the tank—a location from which they could easily monitor the rest of the forage area without having to leave.

Despite the vast number of physiological studies, very few have evaluated the effects of sub-lethal salinity concentrations on freshwater fish behaviour. The distinction between physiological tolerance and behavioural modification threshold is important because some studies have found changes in behaviour well below lethal levels. For instance, Whiterod and Walker (2006) found an LC50 for common carp (Cyprinus carpio) of ~13000 ppm with behaviour modification at concentrations as low as 7500 ppm. Similarly, at sub-lethal concentrations, Alcaraz et al. (2008) found that mosquitofish (Gambusia holbrooki) competed less successfully with Mediterranean killifish (Aplocheilus fasciatus) for food, and Luz et al. (2008) found decreased locomotion and feeding behaviour in goldfish (Carassius auratus).

Salinization, specifically secondary salinization, has been called the greatest cause of degradation in some aquatic ecosystems (James et al., 2003). This statement can be even more pertinent when secondary salinization compounds primary salinization. Driver and Peden (1977) report that salinity concentrations of permanent saline water bodies in the Great Plains of North America can vary seasonally up to 20%. Therefore, most established populations, particularly those which have successfully experienced such salinity fluctuations in the past, should be able to accommodate mild natural salinization. However, when this natural variation is combined with unpredictable rates of secondary salinization, salinity concentrations may become unbearable, compromising the ability of organisms to undertake important behaviours. Future experiments should consider the effects of such stressors on reproductive behaviours and any generational effects. Additionally, acclimation to increased salinity levels should be investigated. It remains unknown whether minnows would eventually overcome the negative effects of salinity, and if so, the time period involved.

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