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INVESTIGATING THE SALINITY TOLERANCE OF THE SWORDTAIL (Xiphophorus hellerii)

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INVESTIGATING THE SALINITY TOLERANCE OF THE SWORDTAIL

(*Xiphophorus hellerii*)

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THESIS ABSTRACT

The swordtail, *Xiphophorus hellerii*, is a freshwater fish species native to Mexico and Central America and is commonly used in the tropical aquarium trade. Swordtails have been shown to have limited survival in 6 ‰ salinity (Nanda et al., 2016), suggesting they may have a greater salinity tolerance than previously expected. Using a gradual acclimation method, we examined the salinity tolerance and swim performance of this species. Freshwater-reared female swordtails were housed in 29-gallon aquaria. A control group was held in freshwater throughout the experiment. For the experimental group, the salinity was increased by 2 ‰ every week until 30 ‰. Critical swimming speed (U_{crit}) was determined repeatedly for each individual fish (every second week) for both control and salinity acclimated (at 0, 4, 8, 12, 16, and 28 ‰) fish to assess the impact of increased salinity on swimming performance. The critical swimming speed was stable throughout the study for the control group. An initial increase in swim performance was seen at 4 ‰ salinity from the beginning of the experiment but then decreased to control levels by 8 ‰ and was not different from control rates for the remainder of the experiment. Gill Na^+/K^-—ATPase (NKA) activity was measured at the end of the experiment. There was a 64% increase in NKA activity for the experimental group over the control. Based on these results, swordtails can tolerate much higher salinity than originally thought, with minimal impact on their swimming performance and are able to increase their NKA activity to adapt to the higher salinity environment.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Most fish are classified as stenohaline meaning they can only tolerate a narrow range of salinity (McCormick et al., 2013). This means that most species are either entirely freshwater or marine and cannot migrate between the two environments. Fish capable of tolerating a range of salinities are termed euryhaline. The ability of euryhaline fish to survive in environments that differ in their salinity should greatly influence the geographic range over which a species is found. A classic question related to the distribution of fishes, especially those considered stenohaline freshwater, is how some species have dispersed over long geographic ranges when their only route from one freshwater river system to the next seems to require passage via the ocean. Myers (1949) stated how it might have been possible for some fish to swim through narrow sea barriers for short period of time. This would require at least a short-term tolerance of seawater, enough to allow them to swim from one river system to the next. My thesis will examine the swordtail, *Xiphophorus hellerii*, a species of Poecilid fish that is known to inhabit freshwater habitats over North and Middle America, the Caribbean, South America to southern Uruguay; Africa (congo Basin and the African rift lakes); Dar es Salaam and Madagascar (Parenti, 1981) and is considered a stenohaline freshwater fish. I will examine whether the swordtail has a tolerance of elevated salinity that might explain how it could migrate between river systems allowing for its dispersal and wide geographic range. To better introduce this topic and rationalize my experimental approach, I will provide background information of the swordtail, the difference between ionoregulation in freshwater and saltwater fish, migratory behavior of euryhaline fish, and the effects of swim performance on fish physiology.

1.2 *Xiphophorus hellerii*

Fish within the family, Poeciliidae can be found in freshwater or brackish water based on geographical distribution (Myers, 1949; Myers, 1976; Lucinda, 2003; Matamoros, 2014). The family consists of 299 species and can range in size from 13.9 mm up to 200 mm (Lucinda, 2003). Poeciliids are termed 'livebearers' because of their ability to give birth to live young (Avise, 2013). Poeciliids can be found in North and Middle America, the Caribbean, South America to southern Uruguay; Africa (congo Basin and the African rift lakes); Dar es Salaam and Madagascar (Parenti, 1981). Because many species have a varying range in salinity tolerance, research has been done with many of the fish within this family (Chiyokubo et al., 1998; Gonzalez et al., 2005; Kumaraguru vasgam et al., 2005; Nanda et al., 2016; Tumlinson, 2017). This family is divided into three subfamilies, Poeciliinae, Procatopodinae, and Aplocheilichthyinae (Ghedotti, 2000).

The family Poeciliidae consists of species that are euryhaline and stenohaline (Meffe and Snelson, 1989). Many species can be found near coastal waters such as the sailfin molly *(Poecilia latipinna*), the blackbanded limia (*Limia melanonotata*), and the mosquitofish (*Gambusia affinis*) (Nordie et al. 1992; Haney and Walsh, 2003; Purcell et al. 2008). Some species within this family, such as mosquitofish have shown that they can live in freshwater environments away from coastal areas yet retain some salinity tolerance (Tumlinson, 2017). Some stenohaline freshwater fish found within this family are species of Gambusia, and the swordtail, *Xiphophorus helleri*.

The swordtail, *Xiphophorus hellerii*, belongs to the subfamily, poeciliinae and are native to areas of northern Mexico, and into central and western areas of Guatemala and Honduras. They have been introduced in many other areas of the world including the United States, Sri Lanka,

Canada, and Africa (Tamaru et al., 2001). These fish are also sexually dimorphic. Male swordtails possess a modified anal fin called a gonopodium. The gonopodium aids in internal fertilization of the female. Males also have an elongation of their lower caudal fin rays which is how they assumed their common name, swordtail (Tamaru et al., 2001). Female swordtails also possess a feature that aids in identifying sex called the "gravid spot". This consists of a darker area on the body underneath the anal fin. The "gravid spot" usually changes in size and darkness based on whether the female is pregnant or not (Tamaru et al., 2001).

The swordtail, *Xiphophorus hellerii*, is one of the few species within this family that has an unknown salinity tolerance but is considered a stenohaline freshwater fish. Interestingly, the highest salinity this species has ever been tested to was up to 6 ‰ with a 74% survival rate. (Nanda et al. 2016). This survival rate is relatively high for a freshwater fish which may suggest it can tolerate higher salinity levels. Because this species is naturally found in freshwater river systems over a wide geographic area, where the only seemly reasonable route they could take to get from one river to the next is via the ocean, the question is can they tolerate salinity higher than 6 ‰. If I can provide evidence to support that they can handle a higher salinity range, I can use the swordtail as a model species to answer this question of whether other stenohaline freshwater fish are also capable of handling wider ranges of salinity than currently expected. Therefore, I plan to assess the salinity tolerance of *Xiphophorus hellerii*. In order to examine this, several physiological mechanisms which regulate ion and water balance in the fish will need to be assessed. To best explain this, it's important to first review what is known about how freshwater and marine fish ionregulate.

1.3 Ion regulation in Freshwater fish

Ion regulation is the control of the ionic composition of body fluids according to Moyes and Schulte, (2008). For freshwater fish, their big challenge is to acquire enough ions from ion-poor environments and dispose of excess water (Moyes and Schulte, 2008). Because these fish are taking in large amounts of water, they excrete large amount of urine and participate in active ion uptake across their gills and dietary absorption of salt (McCormick et al., 2013). Freshwater fish are considered ionregulators, meaning they control the levels of ions in their extracellular fluids and employ a combination of ion absorption and secretion strategies. All freshwater fish are also termed osmoregulators because they maintain internal osmotic pressure above that of the water (Moyes and Schulte 2008). Therefore, swordtails are ionregulators and osmoregulators due to being freshwater fish. The specific details as to how swordtails iononregulate have not yet been investigated, but it is likely similar to other freshwater fishes. In order to assess how salinity exposure effects their ionoregulatory mechanisms, we need to first understand the details of ion regulation of swordtails in freshwater, and then examine changes in those mechanisms in seawater.

Evans (1981) states that the major site of osmotic and ionic permeability is the branchial epithelium (gills). Ion transport in freshwater fish is carried out by ionocytes and pavement cells (PVCs) located in the gill epithelium. Pavement cells form an epithelial layer along the lamellae and interlamellar junctions. Tight junctions connect these two junctions and adjacent ionocytes (McCormick et al., 2013). Other cells such as chloride cells are also in the branchial epithelium and can be identified from pavement cells by using histochemical methods (**Figure 1.1a**). Chloride cells are also referred to as PNA^{+} cells whereas pavement cells are called PNA^{-} cells. These two types of cells aid in transport of different ions and water (Moyes and Schulte, 2008). Gills of freshwater fish take up Na^{+} and Ca^{2+}, and other ions from the water. PNA^{-} cells obtain

the Na^+ ions through a Na^+ channel. The PNA^- cells use a H^+ ATPase to acidify the water in the boundary layer. The Na^+ is exported to the blood via the basolateral Na^+/K^+ ATPase or a Na^+/HCO^{3-} exchanger. With PNA^+cells, $Cl^-$are transferred into the cells using an apical Cl^-/HCO^{3-}exchanger. The production of HCO^{3-} and H^+ is essential and allows ions to be used as counterions or to change pH (Moyes and Schulte, 2008) (**Figure 1.1a**). Since swordtails are considered stenohaline freshwater fish, I expect them to iono-regulate utilizing these mechanisms similar to other freshwater fish.

1.4 Ion regulation in Seawater

My main question is when swordtails are exposed to elevated salinities, will they be able to acclimate to their environment? To do so they would need to start ionregulating like other saltwater fish and begin to actively secrete ions from the body instead of absorbing them like a normal freshwater fish. Saltwater fish must secrete ions against a high electrochemical gradient and also work to obtain water against an osmotic gradient. Some saltwater fish are considered ionregulators, except they maintain their ion levels below that of their environment. Maintaining water and avoiding excessive ion uptake is crucial in saltwater fish (Moyes and Schulte, 2008). Fish drink saltwater to balance the osmotic water loss they endure while living in their environment. Once saltwater is ingested, that water is desalinated in the esophagus through passive and active NaCl transport (McCormick et al., 2013). The salt taken from the esophagus is then excreted through the gills due to high density capillaries (Ando et al., 2003).

In saltwater fish, their gills help control for ion balance by using chloride cells (PNA^+ cells) to aid in ion excretion. NaCl is excreted from the gills through passive transport of Na^+ with support to the secondary active transport of Cl^-(Evans et al., 2005). The Na^+/K^+ ATPase (NKA)

and Na^+-K^+-$2Cl^-$ cotransporters (NKCC) bring in $K^+$and Cl^- into the cell from the blood. Cl^-channels on the apical surface of the gill lead chloride to leave the body and into the external environment while K^+ channels allow $K^+$to enter the blood stream after being transported from the basolateral NKCC co-transporter (Moyes and Schulte, 2008) seen in **Figure 1.**1b. Na^+ ions leave the body through paracellular channels powered by the transepithelial membrane potential (Moyes and Schulte 2008) as seen in Figure 1b.

1.5 Na^+/K^+-ATPase (NKA) activity in fish gill epithelium

The Na^+/K^+-ATPase (NKA) is an active transport protein that is found in the plasma membrane of all cells specifically, the basolateral membrane of epithelial cells in the gills (McCormick et al. 2013). It is noted as a primary driving force for active transport in freshwater and marine fish (Marshall, 2002). The Na^+/K^+-ATPase protein consists of one α (catalytic) subunit and one β subunit. The α subunit is known to be the area where ATP and ion binding of ouabain to the K^+ site. The β subunit is glycosylated and facilitates trafficking of the complex to the plasma membrane (Marshall, 2002). This protein aids in the transport of three Na^+ out of the cell and two K^+ into the cell. The movement of these ions into and out of the cell creates low intracellular concentrations of Na^+ and high intracellular concentrations of K^+(McCormick et al. 2013). This pump also creates an electrical potential making the inside of the cell membrane electronegative and the outside of the membrane electropositive. The Na^+/K^+-ATPase also acts as a secondary transport to many other substances. This includes cell volume regulation, intracellular pH, osmoregulation and aids in many more functions (Suhail, 2010). When transferring to saltwater, the NKA activity is increased in fish due to the high number of ions the fish is coming in contact with over time (Marshall, 2002). Because this protein transport manages the movement of

sodium and chloride ions into and out of cells, it's important to make sure that cells are not overloaded with ions when fish are transitioning to an environment with high salinity. To monitor how swordtails are handling the transition to higher saline environments, I will be measuring the Na^+/K^+-ATPase (NKA) activity in the gills to monitor their homeostasis.

1.6 Migrating from one environment to another that differ in salinity

Because the family, Poeciliidae consists of stenohaline and euryhaline fish, their geographic distribution has been studied as to how different species tolerate different salinity ranges and how they may have been distributed around the world. Myers (1949) classifies the family, Poeciliidae, as secondary freshwater fish. Meaning, they are salt-tolerant but considered freshwater fishes. He mentions that the distributional pattern of these fish indicate that they were not distributed by sea per se but migrated through a narrow sea barrier to get from one area to another (Myers, 1949). Because poecilids are found in Central America and North America, it was thought that they traveled through a sea gap such as the Panamanian-Colombian sea gap during the Neogene period (Myers, 1976). This was supported by Matamoros et al., (2014) confirming that poecilids arrived in northern and/or middle America from Central America through a connection between these two areas during the Upper Cretaceous or Paleogene.

The internal conditions of an organism influence the fish's behavior during migration (McCormick et al., 2013). If poecilids were able to migrate from Central America, they would have had to change their physiology to meet the ion regulatory mechanisms of a saltwater fish to pass through a sea gap. For example, fish that are moving from a freshwater environment to a saltwater environment would have to change from absorbing ions and excreting large amount of water to excreting ions and maintaining water in a saltwater environment. If swordtails were to

take on an increase in salinity during migration from Central America, they would have had to change their ion regulation mechanism to that of a saltwater fish in a matter of days.

Stenohaline fish are said to tolerate only a small amount of salinity change yet, some freshwater fish populations may be more adapted to saltwater than others. Freshwater-linked estuarine migrants experience different salinity transitions that are usually low in salinity and patterns of migration are seen as due to trophic interactions (McCormick et al., 2013). For example, it has been seen that coastal largemouth bass populations were able to tolerate increased salinity habitats even though these fish are considered stenohaline freshwater (Lowe et al., 2009). Kultz (2015) found that the proteins required for euryhalinity are already found within stenohaline fish. They would just need to be activated in order to undergo the physiological transition. This brings into question whether some species of stenohaline fish can tolerate more salinity than what is thought. Therefore, we hypothesize that swordtails will tolerate salinity concentrations up to the isosmotic point (10 ‰). The isosmotic point is the osmolarity where the external environment matches the extr

acellular fluids (blood plasma) of the animal. For most fishes, whether they are freshwater, marine or estuarine the blood is regulated at ~300-350mOsmol/kg (~10 ‰ salinity). It is also important to note how physiology is impacted by exercise when an environmental stressor such as salinity is present.

1.7 Swim Performance

An ecologically relevant concept central to how likely a freshwater fish can tolerate elevated salinity is related to its' swimming ability. If salt levels in the blood increase due to the inability of a fish to regulate them while encountering a higher salinity environment, other physiological

processes may also be negatively impacted. Many fish allow their muscle to dehydrate during the initial phases of salinity acclimation. This helps to maintain proper blood osmolarity. The dehydrated muscle may not perform as well and therefore swimming ability may be compromised. A fish that loses swimming ability is at much greater risk of being eaten by predators. Physiologists can assess swim performance in a variety of ways, allowing them to give a general assessment of how fish are surviving in their everyday environment given many environmental factors that are present in them (Plaut, 2001). There are three types of swim performance tasks. Those include sustained, prolonged, and burst swimming. Sustained and prolonged swimming both require the organism of interest to swim for long durations (seen as > 200 minutes in Brett (1964) with prolonged swimming being a shorter period (20s – 200 minutes) over sustained (Beamish, 1978). Burst swimming requires the fish to swim at high speeds for short amounts of time usually less than 20 seconds (Beamish, 1978). Both prolonged and sustained swimming are aerobic activities whereas burst swimming is considered anerobic (Plaut, 2001).

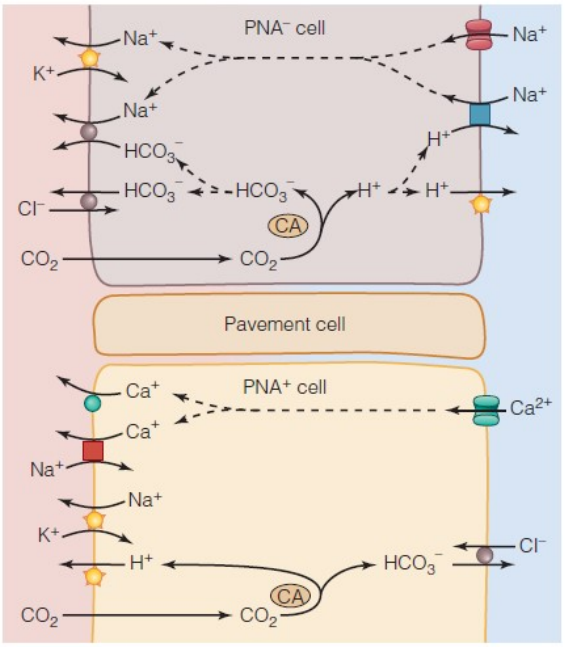
Determination of the critical swimming speed (U_{crit}) is a method that looks at the aerobic swimming capability of a fish (Plaut, 2001). Critical swimming speed was first conducted on young sockeye salmon by Brett (1964). To test critical swimming speed, a swim tunnel is used. Fish enter the swim chamber and swim against a increasing current of water (**Figure 1.2**). The critical swimming speed is measured at the time and velocity that the fish fatigues at over a certain period of time (**Figure 1.3**). The speed of the current that the fish swims at is increased every 30 seconds until the fish fatigues (Brett, 1964). Measuring the critical swimming speed is important to measure how certain environmental factors can affect the swimming ability of fish. Environmental factors such as temperature, salinity, feeding rate, body form, externally attached

tags, and transmitters all have an impact on the swimming speed of the fish (Plaut, 2001). Conducting a swim performance test and measuring the critical swimming speed of *Xiphophorus hellerii* would allow me to look at how salinity affects the performance of this fish and would give a better representation of how this species may be allocating energy during its transition into a high salt environment.

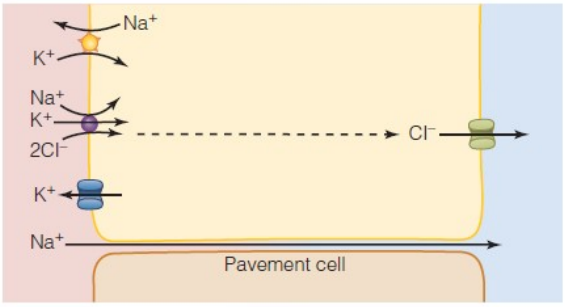
1.8 Thesis Objective

The current salinity tolerance of the swordtail is unknown. While this fish possesses a large geographic distribution and some low salinity tolerance, studying how these fish are impacted by exercise and increased salinity acclimation would allow us to understand why these fish have such a large geographic distribution. This study was designed to assess whether swordtails could survive in an environment with high salinity concentrations to assess whether bi-weekly aerobic prolonged swimming trials would affect their physiology.

This thesis addresses the questions: (1) Are swordtails able to tolerate higher salinity concentrations in their environment, (2) Will swordtails be able to switch their osmoregulatory mechanism like a freshwater fish to that of a saltwater fish; and (3) how does exercise impact energy allocation in swordtails in relation to salinity? It was predicted that swordtails would tolerate salinity concentrations up to the isomotic point (10 ‰) as a freshwater fish is not capable of excreting large amounts of ions like a saltwater fish does. It was also predicted that swim performance would decrease as fish were exposed to higher salinity concentrations. Overall, the results of this thesis provide insight into the salinity tolerance of swordtails and how that impacts swim performance. These things would allow fish to overcome the challenge of moving to new river systems via the ocean.



(a) Freshwater trout gill



(b) Marine fish gill

1.9 Figures

Fig. 1.1 Ion regulation mechanisms in stenohaline freshwater fish (a) and saltwater fish (b) (Moyes and Schulte, 2008).

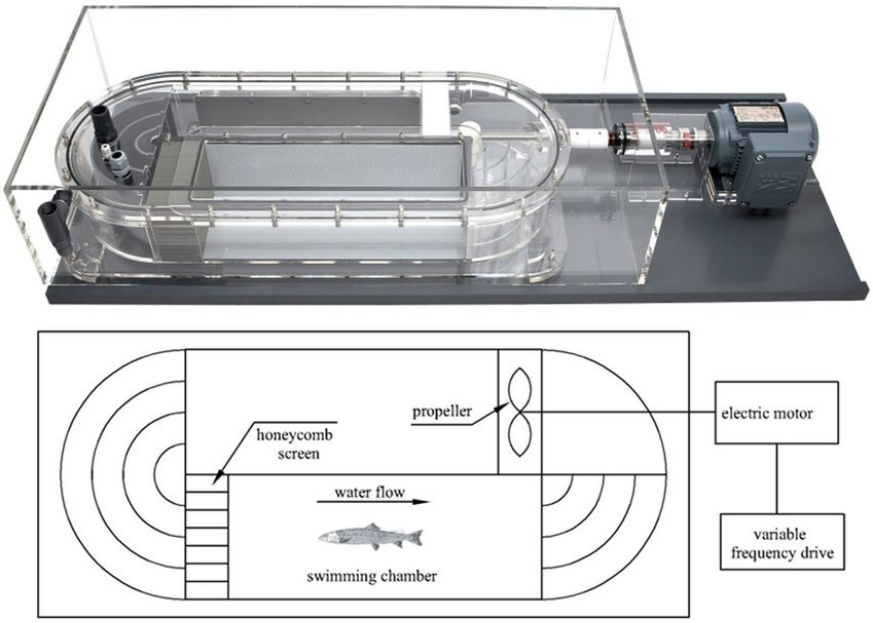


Fig. 1.2: Photograph of Brett-type swim tunnel to test critical swimming speed (Wang et al., 2018)

$$U_{crit} = U_i + [U_{ii}\left(\frac{T_i}{T_{ii}}\right)]$$

Fig. 1.3: Equation to calculate the critical swimming speed (U_{crit}) to assess swim performance (Plaut, 2001).

CHAPTER 2: ASSESSING THE SALINITY TOLERANCE AND SWIM PERFORMANCE OF SWORDTAILS (*Xiphophorus hellerii*)

2.1 Introduction

Most fish are classified as stenohaline, meaning they can only tolerate a narrow range of salinity (McCormick et al., 2013). This means that most species are either entirely freshwater or marine and cannot migrate between the two environments due to the ion regulation system they possess. Freshwater fish acquire ions from ion-poor environments and dispose of excess water, while marine fish secrete ions against a high electrochemical gradient and work to obtain water against an osmotic gradient (Moyes & Schulte, 2014). Euryhaline fish are capable of tolerating a range of salinities. These fish can switch their ion regulation system from actively acquiring ions in freshwater to actively secreting them in saltwater (Moyes and Schulte, 2014). The ability of euryhaline fish to survive in environments that differ in their salinity should greatly influence the geographic range over which a species is found.

A question related to the distribution of fishes, especially those considered stenohaline freshwater, is how some species have dispersed over long geographic ranges when their only route from one freshwater river system to the next seems to require passage via the ocean. Myers (1949) stated how it might have been possible for some fish to swim through narrow sea barriers for short periods of time. This would require at least a short-term tolerance of seawater, enough to allow them to swim from one river system to the next. For example, it has been seen that coastal largemouth bass populations were able to tolerate increased salinity habitats even though these fish are considered stenohaline freshwater (Lowe et al., 2009). Some families of fish consist of many euryhaline and stenohaline species that are found across large geographic regions. The family Poeciliidae consists of many species that can live in a range of environments

due to their euryhalinity (Meffe and Snelson, 1989). For example, the mosquitofish (*Gambusia affinis*) has been known to live in freshwater environments away from coastal areas yet, they still retain some salinity tolerance (Purcell et al. 2008; Tumlinson, 2017). Another example is with *Poecilia reticulata,* which can tolerate a wide range of salinity (Chervinski, 1984; Chiyokubo et al. 1998; Torres-Dowdall et al. 2013) but prefers to live in freshwater environments over a brackish water environment (Pethygoda et al. 2019). Although many species in the family Poeciliidae are euryhaline, the group also contains a number of stenohaline freshwater fish. The salinity tolerance of many of these freshwater species has not been thoroughly investigated, including the swordtail, *Xiphophorus hellerii*.

The swordtail has a wide geographic distribution spanning from freshwater rivers of northern Mexico, and into central and western areas of Guatemala and Honduras. (Parenti, 1981, Tamaru et al., 2001). Based on its geographic distribution it is considered a freshwater fish and presumed to have little to no salinity tolerance. However, the fact that its range is so large and the most likely route for range expansion would include passage from one river system to the next via an estuary or ocean passage, the question should be asked whether this species may have more salinity tolerance than expected. Interestingly, one study (Nanda et al. 2016) showed that swordtails had a 74% survival rate after exposure for 45 days to 6 ‰ saltwater. This survival rate seems high for a freshwater fish, which may suggest it can tolerate higher salinity levels.

In the wild, tolerating an increase in salinity on its own may not be enough to predict survival. To survive a migration from one river system to the next, fish would also need to be able to swim effectively enough to avoid predation. Swim performance is an excellent way to assess fitness in fish and how environmental stressors impact their swimming ability (Plaut, 2001). Their performance can be assessed by looking at their critical swimming speed, the time

and maximum speed a fish reaches before fatiguing out. Swim performance can be used to assess if ion and osmotic pressure can be impacted by swim performance over time. For example, Gonzalez and McDonald (1992) showed that there was a loss of Na^+ ions in relation to an increase in oxygen consumption during exercise training and Plaut (2000) found that killifish acclimated to a wide salinity range showed a decrease in activity in higher salinity concentrations. If ion regulation is impacted negatively by swim performance while trying to live in a high salinity environment, it could affect how long they survive.

This study will examine the salinity tolerance of the swordtail. I will expose swordtails to a very gradual increase in salinity (2 ‰ per week) and monitor survival. I predicted that swordtails would not be able to survive past the isosmotic point of ~ 10 ‰. At the isomotic point, the fish would presumably need to switch their ionoregulatory strategy from active ion uptake (as used by freshwater fishes) to active ion secretion (seen in marine fishes). To this point, no study has investigated whether this species can accomplish this switch. During the experiment, I will also monitor swim performance. Even if a fish can survive an increase in salinity, it does not mean it will be able to swim effectively. If increased salinity has a negative impact on swim performance it could also mean an increased risk of predation, or loss of migratory capacity, which would reduce the change of individuals moving from one freshwater river system to the next. I predicted that swordtails would have a reduced swimming capacity due to the increase in salinity. Assessing the salinity tolerance of the swordtail will allow us to see how this stenohaline freshwater fish may be able to handle a high salinity concentration for a short period of time that may allow for geographic relocation and population spread of the species.

2.2 Materials and Methods

Acute Direct Salinity Transfer Experiment

A preliminary study was conducted to assess salinity acclimation and gill Na^+/K^+-ATPase (NKA) activity in swordtails, *Xiphophorus hellerii* following short term exposure to four salinity treatments (0‰, 10‰, 25‰, 34‰) . This study was conducted at Kansas State University in Dr. Tobler's lab. Thirty swordtails were housed in freshwater and fed tropical fish flake food daily for two weeks prior to the start of the experiment. On day one of the study, seven fish (control group, 0‰) were euthanized and the remaining 23 fish were moved to 10‰ salinity. After five days at 10‰ six fish were euthanized and the remaining fish were moved to 25‰. Five days later, six of those fish were euthanized and the remaining fish were moved to 34‰. These fish were euthanized following five days of exposure to 34‰. . Following euthanasia, gills were excised, and flash frozen in liquid nitrogen, then shipped to DePaul on dry ice, then stored at -80 ° C until analyzed for gill NKA.

Gill Na^+/K^+—ATPase Activity

Gill filaments were homogenized in an SEID buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, and 0.1% sodium deoxychlorate; pH 7.4 by hand with a ground glass homogenizer. This solution was then centrifuged at 5000 g for 1 minute at 4°C to remove insoluble materials from the supernatant. NKA activity was determined spectrophotometrically (Agilent Cary 60 UV-Vis, Agilent Technologies, Inc., Santa Clara, CA) at 25°C using an enzyme (NADH)-linked assay (Gibbs and Somero, 1990). Gill samples were measured for ATPase activity in the presences and absence of the NKA-specific inhibitor ouabain (1 mM). gill

homogenates were run in duplicates with and without ouabain to calculate the NKA activity. The gill homogenate protein was then determined spectrophotometrically using a Bradford assay (λ = 595 nm, SPECTRAmax PLUS 384, Molecular Devices Corp., Sunnyvale, CA) (Bradford, 1976). From this data we saw that gill NKA activity increased in each salinity treatment which suggested this species has the capacity to upregulate salt secretory mechanisms which may allow for long term acclimation to elevated salinity. This led to the development of a larger scale, longterm, gradual salinity acclimation experiment to determine if swordtails could survive and function at elevated salinities.

Chronic Gradual Salinity Transfer Experiment

To determine if swordtails are able to acclimate in the longterm to elevated salinity I designed an experiment that would assess the salinity tolerance of the swordtail over a gradual period of time with a slow increase in salinity. From there, I will assess their osmoregulation mechanisms and swim performance.

Animal Rearing Conditions

This experiment was conducted at an indoor research support facility (RSF) at DePaul University (DPU), Chicago, IL, USA from May – November of 2022. This experiment used 24 adult female (very few males were available from the supplier) swordtails (initial length 4.85 ± 0.31 cm) procured from a commercial distributor (Emark Tropical Imports Inc.) from Sri Lanka. All fish were acclimated in 29-gallon tanks at 76°F and under a (12L:12D) photoperiod for at least two weeks prior to experimentation. Swordtails were fed to satiation every day with tropical fish flake food (TetraMin). Water, temperature, and oxygen levels were maintained near saturation with air stones and supplied to each tank using aerators (Aquatop Breza, AP-20). Swordtails were randomly sorted into six groups of four fish and kept in (29-gallon) tanks. All

fish were allowed to acclimate to their new tank and salinity concentration for 24 hours prior to their first swimming trial. All procedures were performed according to an Animal Use Protocol approved by the DePaul University Animal Care and Use Committee (IACUC). Experimental procedures were designed to minimize handling stress throughout the study.

Nine aquaria (29-gallons) were used for the entirety of this experiment. A divider was placed inside to separate two fish on each side of the tank to aid with identification. Two sets of three tanks were used to house twelve fish for the control and experimental group. An additional three tanks were used to move the experimental fish over to a new salinity concentration every week. Every week, the three tanks that did not house fish were increased in salinity by 2 ‰. Fish were gently moved by net into those tanks the following week. This continued until the experimental fish reached a salinity of 18 ‰. Fish were then held at 18 ‰ for 11 weeks before acclimation to increasing salinity continued (also at a rate of 2 ‰ per week) until they reached 30 ‰. This delay was due to a need to modify the original IACUC protocol (which only allowed for a salinity increase to 18 ‰) and in part due to complications with the Covid pandemic.

Swim Tunnel and Calibration

A Brett style (Logilo, 90.15 L) swim tunnel was used to conduct swim trials for each fish. Oxygen was supplied throughout the swim tunnel with air stones and a heater (Marineland Precision Submersible, 75 Watt) was used to match the water temperature in the holding tank (25°C). A submersible water pump was used to provide circulation throughout from the exterior swim tunnel into the inner swim chamber. The flow of water was controlled by a submerged propeller attached to an electric motor. The velocity of the current was read in Hertz (Hz) and a flow probe (Hontzsch, Vane Wheel Sensor, HFA-Ex Hand Unit) was used to determine the velocity of the current in meters per second. The flow probe was calibrated in three different

areas of the swim tunnel at varying heights to account for differences in the water velocity. These areas included the center of the swim chamber, most inner area of the swim chamber, and most outer area of the swim chamber. Minimal differences were seen in calibrated water velocity in the areas described above (see Appendix 1).

Swim trials

To assess critical swimming speed, all fish underwent 20 minute preliminary swim trials in groups of four to acclimate and familiarize themselves with the swim chamber. Food was withheld from fish for 24 hours prior to each trial to exclude feeding/appetite as a confounding variable. Velocity was increased by the average BL of each group every 10 minutes. Preliminary swim trials were done three times before data collection began. All fish were placed back into their tanks after the preliminary trials were over. Critical swimming speed was assessed in the control and experimental group (alternating) every other week. 24 hours after fish were moved to a new salinity concentration their critical swimming speed was assessed. These tests were conducted separately for each individual. The salinity of the water in the swim tunnel was made to match the holding tank that each fish came from. To assess the size of each individual fish, they were photographed next to a ruler prior to being placed inside the swim tunnel. Fish were then acclimated to the swim chamber for 30 minutes at a low velocity of 0.03 M/s based on a previous study done by Oufiero et al. (2012). This speed was used to create enough flow within the swim tunnel to promote swimming and orient the swordtails into the current prior to the swim trial. Velocity was increased by 1 BL/s every 10 minutes until fish were fatigued. Once fatigued, the flow was immediately stopped and the time and final speed were recorded to calculate the critical swimming speed (**Figure 2.0**). Fish were placed back into their tank to recover for the remainder of the day. Swim performance was assessed for the experimental and

control group repeatedly until 16 ‰ was met. A final swim performance test was run at 28 ‰ before anesthetization at 30 ‰.

Anesthetization

Once the swim trials were completed and fish had been exposed to 30 ‰ each individual was euthanized in buffered tricaine methansulphate (MS-222) (300mg/L). Death was confirmed by severing the spinal cord. After euthanization, fish were weighed and measured. Gills were then excised and frozen in dry ice, then stored at -80 °C for later analysis of Na^+/K^+-ATPase activity. The head and tails of each fish were removed, and the body was placed in foil and on dry ice to determine whole-body sodium and chloride content.

Gill Na^+/K^+—ATPase Activity

Gill Na^+/K^+—ATPase activity was assessed using the same methods described in the acute direct salinity transfer experiment (see above).

Statistical Analysis

All values are represented as means ± standard error of the mean (s.e.m.) (N=12). Two-sample t-tests with a bonferroni correction was conducted at experimental treatments 4, 8, 12, 16, and 28 ‰ with the corresponding control group at that salinity to assess a relationship between critical swimming speed (U_{crit}). A repeated measures two-way ANOVA was used to assess difference in swim performance between the experimental groups at 0, 4, 8, 12, 16, 28 ‰. A repeated measures one-way ANOVA was used to assess how swim performance may have changed over time in the control group at week 1, 3, 5, 7, 9, and 27. Gill NKA activity for the fish that underwent the acute direct salinity transfer was compared using a one-way ANOVA. Differences in gill NKA activity for the chronic gradual salinity transfer was determined using a

two-sample t-test. For length, paired t-tests were run in the experimental and control groups. A two-sample t-test was determined to assess if there was a difference in growth between the control and experimental group. A two-sample t-test was used to determine if a difference was present in body weight. Significance was determined at $P < 0.05$. All statistical tests were performed using R Studio version 2022.12.0 (RStudio, Inc., Boston, MA, USA).

2.3 Results

Acute Direct Salinity Transfer Experiment

Gill Na^+/K^+-ATPase Activity

NKA activity was an average of 1.40 μmol ATP/mg protein/hr for the control group. Fish exposed to 10, 25 and 34 ‰ salinity had a 30.2 %, 56.5 % and 200 % higher gill NKA activity than control fish (0 ‰). Despite this step-wise increase in gill NKA with increasing salinity, only the 35 ‰ group was found to be significantly higher ($p < 0.05$) than the control group and experimental treatment group at 10 ‰ and 25 ‰ (**Figure 2.1**).

Chronic Gradual Salinity Transfer Experiment

Fish Health and Survival

All fish were in good health at the beginning of the experimental procedure. There were no deaths over the first 8 weeks of the salinity acclimation procedure. A 66% survival rate was seen in the experimental group and a 75% survivorship from the control group over the course of the entire 28 weeks of the experiment (**Figure 2.2**). The control group had a steady increase in mortalities after week 14. For the experimental group, most mortalities were seen after week 22 with the exception of one at week 8. Overall, fish in both the control and experimental groups

showed positive growth rates over the course of the experiment. A significant growth increase of 56.2% was seen in the control group over the experimental group (**Figure 2.3**). The control group has a significant length increase ($P < 0.05$) of 39.7% over the course of the experiment starting with an initial weight of 4.85 ± 0.37 cm and a final weight of 6.78 ± 0.58 cm. The experimental group also had a significant length increase ($P < 0.05$) of 25.2% over the course of the experiment with an initial weight of 4.92 ± 0.30 and a final weight of 6.16 ± 0.39 cm (**Figure 2.4**). For this study, weight was only determined at the end of the experiment. Final weight of the control group was 2.3 ± 0.5 grams in relation to the experimental group's weight at 1.62 ± 0.83 grams which was a 42.0% increase and not significantly different ($P > 0.05$).

Prolonged Swimming (1.0 BL/s)

Critical Swimming Speed

Critical swimming speed (U_{crit}) for all fish were determined at 0 ppt prior to the start of salinity treatments. The mean critical swimming speed was 59.3 M/s and is included as a pre-experimental data point on **Figure 2.5**. The critical swimming speed of control fish did not differ throughout the experiment. Fish exposed to increasing salinity showed a slight decrease (although not significant) in U_{crit} at ~ 8 ‰ before following a similar trend as the control fish for the remainder of the experiment (**Figure 2.5**). The overall U_{crit} was not significantly different between experimental and control groups for the duration of the experiment. ($P > 0.05$). When comparing U_{crit} in fish acclimated to increasing salinity, there was a significant decrease at 8, 12, 16 and 28 ‰ when compared to fish at 0 ppt ($P < 0.05$). Critical swimming speed between the control treatment groups did not differ significantly throughout the experiment ($P > 0.05$). There was no significant difference between the control and experimental group at each time point.

Gill Na^+/K^+-ATPase Activity of Fish in Chronic Gradual Salinity Transfer

Gill NKA activity was significantly higher (a 67.4% increase) in swordtails acclimated to increasing salinity during the 28 week swimming trial ($P < 0.05$). (**Figure 2.6**).

2.4 Discussion

Overview

Assessing salinity tolerance in different species of fish allows us to learn more about the habitat, diet, and physiological mechanisms they possess to survive in different environments. Myers (1949) stated that secondary freshwater fish could retain a high salt-tolerance even if they were never to reach the sea. He also mentions how secondary freshwater fish of Central America have colonized nearby islands and suggests their distribution is through narrow sea barriers. This theory is further supported by the results of this thesis which show the swordtail's ability to tolerate a high salinity concentration over time. Swordtails are classified as secondary freshwater fish and appear to have retained this ability even though they live in a freshwater environment. It could be possible that other freshwater fish such as the swordtail also retain some salinity tolerance to enable to them to increase their geographic distribution by moving across an estuary or ocean-passage.

This study assessed the salinity tolerance of the swordtail, by looking at their survival, gill Na^+/K^--ATPase activity and their swim performance. From this data, it is clear that swordtails are much more salt tolerant than originally thought. This information may be able to provide insight into other freshwater fish's physiological capabilities and assess whether some species may possess the ability to tolerate higher salinities to increase their geographic

distribution. The following discussion will outline the main results of this study: (1) Swordtails were able to switch their ion regulation system from active ion uptake to active ion secretion to aid in saltwater acclimation, (2) swordtail growth and length increased significantly in both groups but more in the control, and (3) overall swim performance was not impacted by salinity acclimation in swordtails.

Survivorship of Swordtails

Swordtail survivorship for the experimental and control groups were not impacted severely. For the control group, deaths occurred gradually throughout the experiment whereas with the experimental group, most of the deaths were at the higher salinity concentrations after the final swim performance test had been conducted. This suggests that it may be too energetically taxing to swim at high speeds while trying to maintain a balance of ions within the body at that time. Control group survivorship was at 75% while the experimental was at 66% with a difference of only one death between each group further supporting the notion that salinity is not significantly impacting these fish.

Osmoregulation in Swordtails

Freshwater fish use active ion uptake to absorb ions from their environment while marine fish actively secrete excess ions from their body to maintain proper salt and water homeostasis. Euryhaline fish can go back and forth between these two ionoregulatory strategies and can therefore survive in different salinity environments (McCormick et al., 2013). A previous study (Nanda et al. 2016) had shown that swordtails may have a higher than expected salinity tolerance but only assessed their capacity up to 6 ‰. In order to potentially migrate across an ocean passage or even though an estuary fish would likely experience more than 6 ‰. The isosmotic

point is where the salinity level is equivalent to the blood salinity of an organism (~10 ‰). In theory, stenohaline freshwater fish should not be able to tolerate a salinity above this isosmotic point due to physiological restraints (McCormick et al. 2013). If swordtails are able to surpass the isosmotic point, they would need to begin excreting Na^+ and Cl^- ions from their gills and begin drinking saltwater and excreting less urine. If they are able to do this, they would be considered more euryhaline than thought.

From the acute direct salinity transfer collected for this study, NKA activity was significantly higher in the treatment group at 34 ‰ compared to the other treatment groups by over 200%. Gill NKA activity in all other salinity treatments was increased. Gill NKA activity for this current experiment was significantly higher in the experimental group over the control by 67.4%. This increase in activity further supports that swordtail were able to move pass the isosmotic point (~10 ‰) and switch their ion regulation mechanisms from active ion uptake to active ion secretion. These results provide further context into the swordtail's gill regulation mechanisms as well as more insight into their ability to handle the gradual salinity change needed to move from one river system to another.

The Effect of Saltwater on Gill Na^+/K^+-ATPase (NKA) Activity in Swordtails

Gill NKA activity has been studied thoroughly and is a primary driver for active transport of ions in freshwater and marine fish (Marshall, 2002, McCormick et al., 2013). Many studies have looked at the NKA activity specifically with other species of poecilids such as *Poecilia latipinna* (Yang & Lee, 2008), *Gambusia affinis* (Tsai et al. 2017), and *Poecilia vivipara* (Amaral et al., 2001) but no studies have looked at gill NKA activity in the swordtail. In the present study, gill NKA activity was significantly higher in the experimental group over the control. There was a 67.4% increase in gill NKA activity for the experimental group compared to

the control. This data supports the idea that swordtails are able to switch their ionoregulatory strategy to secrete ions from their body to tolerate up to 30 ‰. Studies such as Lisboa et al. (2015), Zhang et al. (2019), and Yang et al. (2009) have demonstrated an increase in NKA activity when euryhaline fish such as *Mugil liza*, *Dicentrarchus punctatus*, and *Poecilia latipinna* have been moved to higher salinity concentrations. This increase in NKA activity is standard for euryhaline fish. From this data we can infer that swordtail are more euryhaline than thought. Even though they are able to tolerate a higher ion concentration in their environment, they did show physical signs of degradation over time due to the gradual salinity acclimation such as a decline in growth rate, length, and it was observed that their slime coat seemed compromised at higher salinity concentrations (~26 ‰). During the beginning of the experiment, normal activity significantly decreased around 8 ‰ during the salinity acclimation. Fish became sluggish and lethargic at this time. Once fish were moved to 10 ‰ the following week, normal behavior and feeding resumed. This may have been a time when the fish were struggling to upregulate their salt secretory mechanisms as they neared the isosmotic point. Other studies have described this stage of acclimation to elevated salinity as a 'crisis period' which is very common amongst other fish adjusting to higher salinity concentrations. The 'crisis period' is when fish undergo a rapid increase in Na^+ and Cl^- ions in their environment which causes them to adjust their ion regulatory system. If fish are able to survive this period, their ionoregulatory system will have adjusted to deal with the increase in ions in their environment (Eddy, 1981). He et al. (2009) observed juvenile Chinese sturgeon undergoing a 'crisis period' upon being moved to brackish water. This was also seen in rainbow trout transferring from freshwater to saltwater (Bath and Eddy, 1979). This study supports the idea that swordtails can survive higher salinity concentrations by increasing their gill NKA activity.

The Effect of Saltwater Acclimation on Exercise in Swordtails

Environmental factors such as salinity, pH, temperature and contaminants can affect the swimming capabilities of fish (Plaut, 2001). Assessing a fish's swimming speed is a useful technique to see how environmental factors impact their swimming capability and energy used during that time. If swimming performance is negatively impacted, fish may become more susceptible to predation, have habitat restrictions, and more difficultly foraging for food. My thesis is the first study to look at how salinity impacts critical swimming speed in swordtails. Fish in the experimental group showed a decline in swimming speed around 8 ‰ during experimentation. This could be due to a possible 'crisis period' that the swordtails were undergoing with the salinity acclimation. Critical swimming speed was similar to the control group the rest of the experiment with no significant difference between the two groups. This data suggests that critical swimming speed is not impacted by a gradual increase in salinity. While the swordtails were able to survive up to 30 ‰ over a gradual acclimation, this gradual transfer may not be realistic to a salinity transfer in their natural environment. This current experiment increased in salinity by 2 ‰ every week. Further studies should be done to assesses how a fish's physiology would be impacted if the time and salinity concentration were altered to mimic those moving through an estuary or ocean passage. With the family Poeciliidae being classified as secondary freshwater fishes, this further supports the idea that swordtails could maintain the exercise needed to travel to one river system to another via the ocean.

Critical swimming speed between experimental treatment groups was significantly different in all salinity treatments after 4 ppt in relation to the experimental group at 0 ‰. This could have been due to a training effect or the adjustment to the different salinity concentrations. It should also be noted though that when critical swimming speed was recorded at higher salinity

concentrations (~26 ‰), swordtail mortality did increase. This could be due to the fact that they have to allocate energy towards regulating their ion concentration in their body. McKenna et al. (2007) found that continuous rigorous exercise can cause NKA inactivation which would reduce the fish's ability to regulate their ion concentrations at high salinity levels. This could have been happening with the swordtails which could have resulted in their death at those high concentrations. An energy tradeoff between swimming speed and ion regulation may be present in this circumstance.

2.5 Conclusion

This study has explored the salinity tolerance of swordtails and assessed their swim performance and osmoregulatory mechanisms in relation to the gradual acclimation to different salinity concentrations. My first prediction was not supported, indicating that swordtails are able to surpass the isosmotic point and change their ionoregulatory mechanism to secrete ions from their environment. Preliminary gill NKA activity was increased in all treatment groups and significantly different in the treatment group at 34 ‰. Gill NKA activity was significantly higher in the experimental group over the control allowing for the acclimation to higher salinity levels. My second prediction was also not supported. Swimming capacity was not reduced due to the increase in salinity. Fish that underwent the gradual salinity acclimation had no significant decrease in their critical swimming speed compared to the control group. There was a significant difference in critical swimming speed between treatment groups within the experimental group. A slight drop was seen in the experimental group's critical swimming speed around 8 ppt due to a possible 'crisis period' observed. Overall, the findings above support the idea that swordtails can move to new freshwater river systems via the ocean to increase their geographic range.

Further studies on the physiological effect during the gradual salinity acclimation could provide more insight into how swordtails regulate their bodies during this process. For example, looking at the whole-body sodium and chloride content would be beneficial to assess the total concentration of those ions in the body. For this study, female swordtails were only used due to a lack of males received in the shipment. It would be interesting to see if males are just as tolerable as female swordtails for this experiment. Female swordtail also gave birth to fry at 18 ‰ but not at higher salinity concentrations. This suggests that fitness is not impacted by moderate salinity ranges but may be impacted at higher salinity concentrations. Overall, this study entails vital information about the effects that salinity has on the osmoregulatory mechanisms of the swordtail and how it impacts their swimming capability.

2.6 Figures

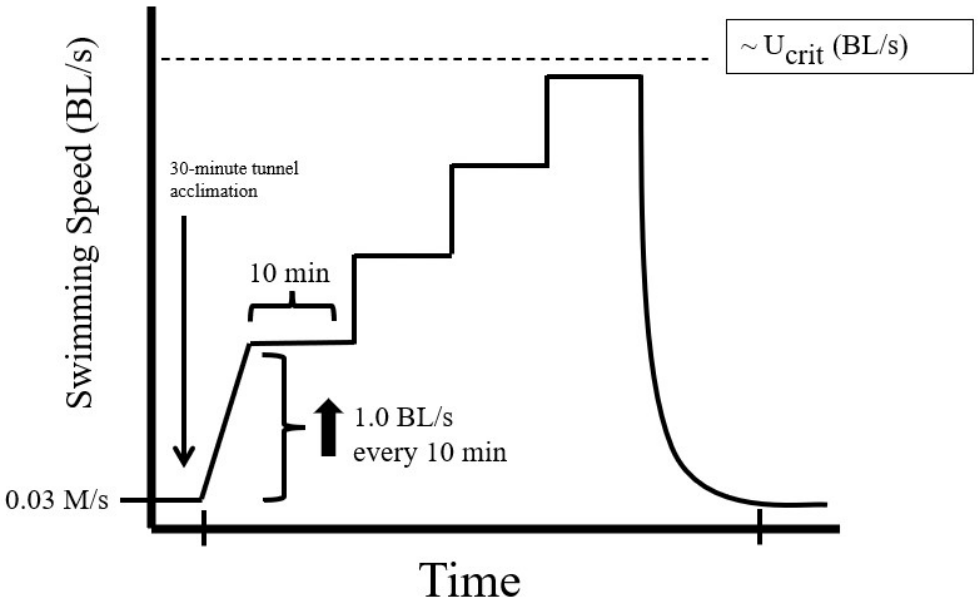


Figure 2.0: Swim performance protocol for swordtails to assess the critical swimming speed.

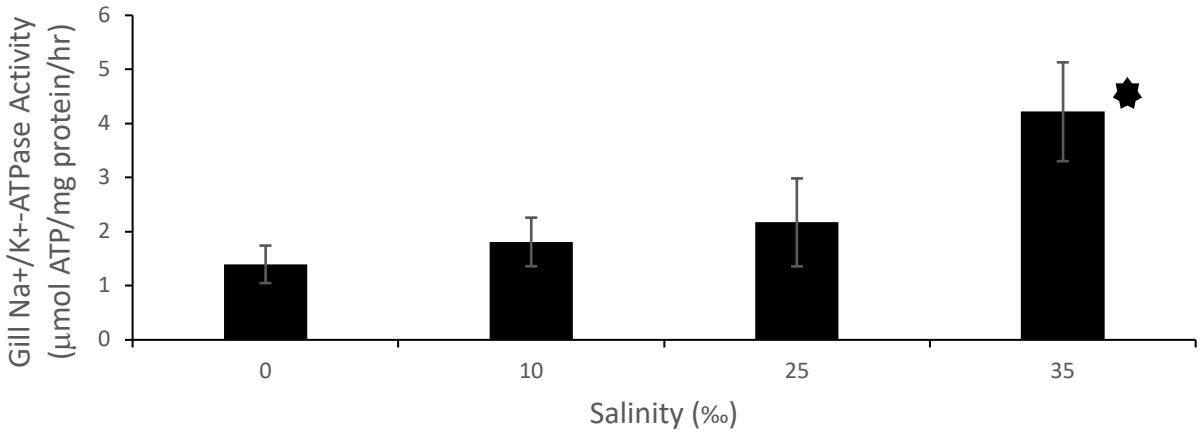


Figure 2.1: Gill Na^+/K^--ATPase (NKA) activity for the acute direct salinity transfer of *X. hellerii*. Statistical comparisons made between each treatment group (one-way ANOVA; $P < 0.05$): * denoted significantly different from other treatment groups and the control group.

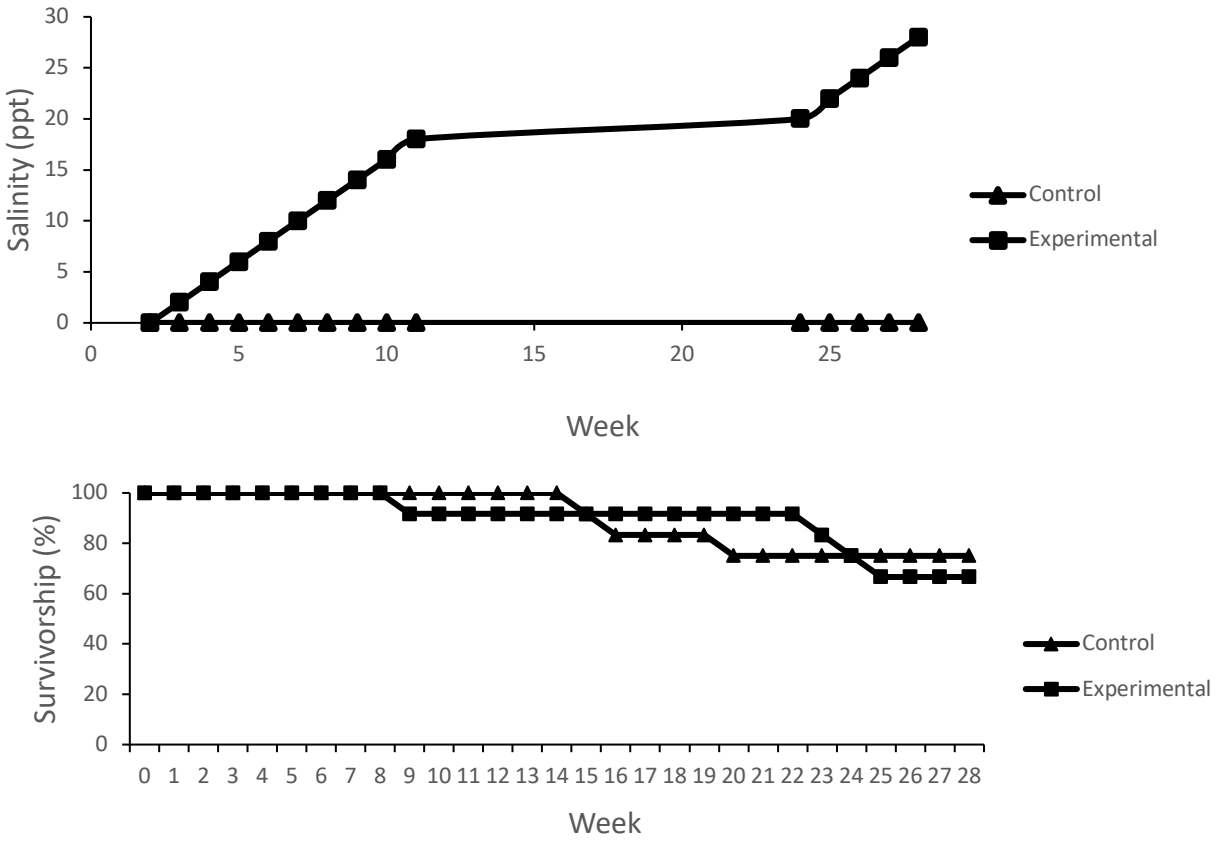


Figure 2.2: Survivorship of *X. hellerii* during the chronic gradual salinity experiment for the experimental and control groups each week. The control group is indicated with a triangle and the experimental group is indicated with a square.

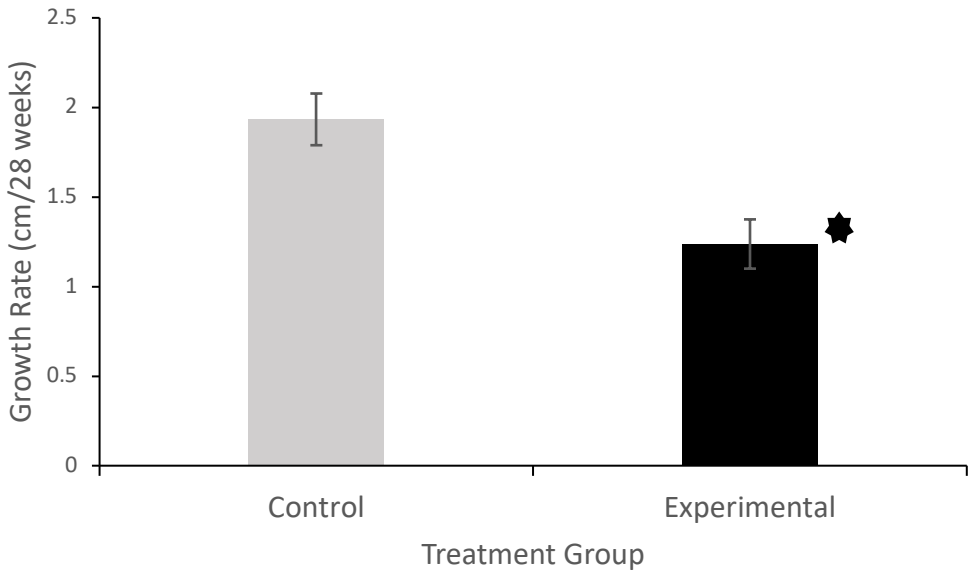


Figure 2.3: Growth rate for the experimental and control group for fish undergoing the chronic gradual salinity transfer. Statistical comparisons between each group's growth rate (Two-sample t-test; $P < 0.05$): * denotes significantly different length from corresponding pre-experimental values in the same group.

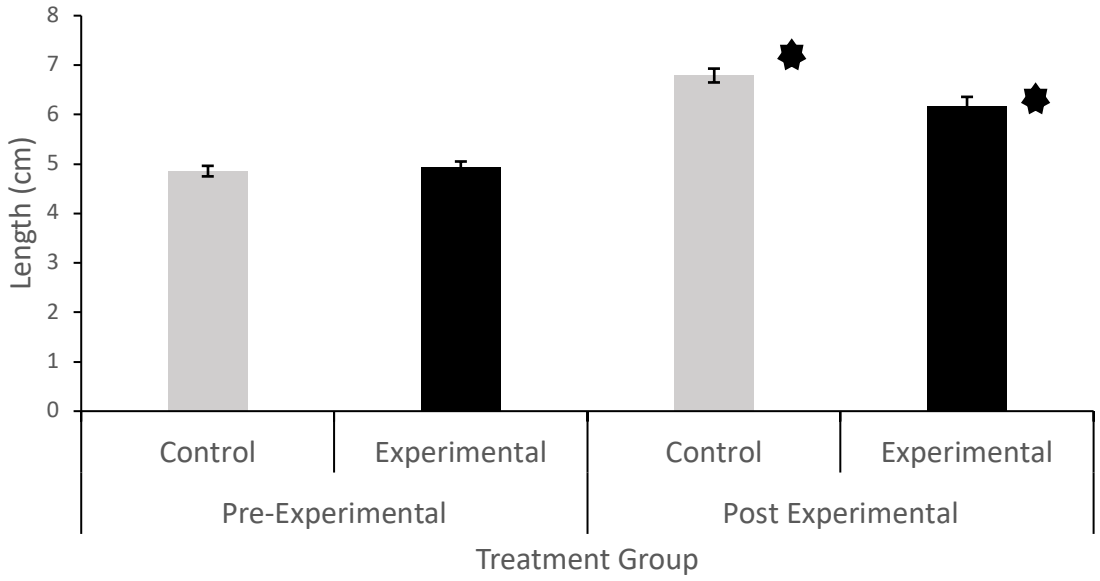


Figure 2.4: : Pre- and post-experimental lengths for the experimental (black bars) and control fish (gray bars) that survived the chronic gradual salinity transfer. Statistical comparisons between each group's length before and after (Paired t-test; $P < 0.05$): * denotes significantly different length from corresponding pre-experimental values in the same group.

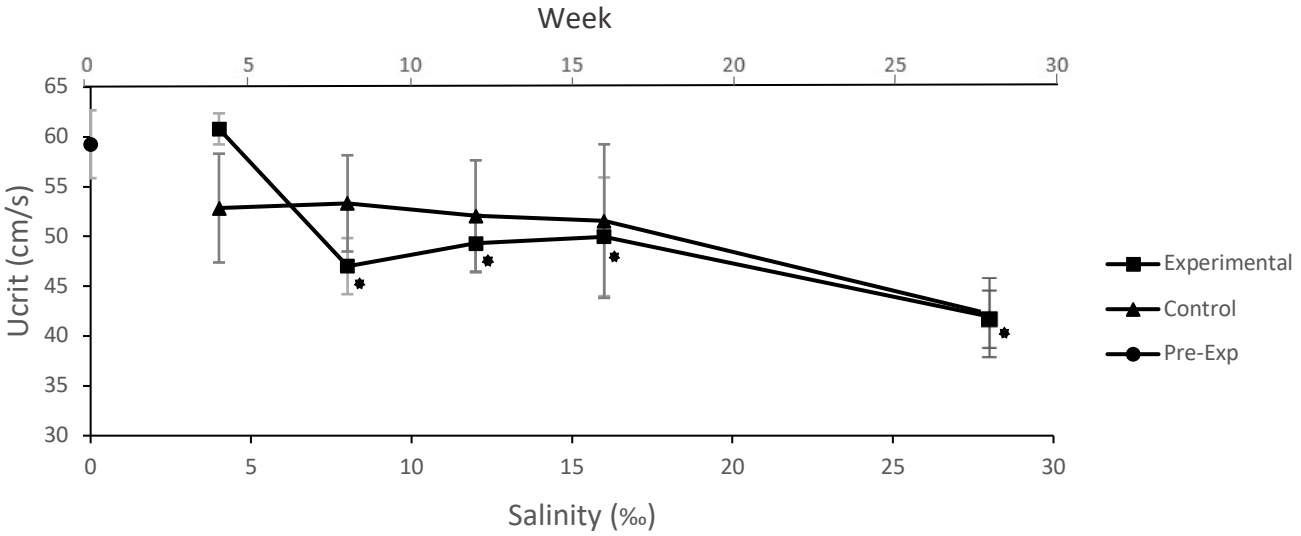


Figure 2.5: Critical swimming speed **(**U_{crit} **)** of the experimental (filled square) and control group (filled triangle) during the chronic gradual salinity transfer. Statistical comparisons made between both groups at each salinity point (Two-sample t-test with Bonferroni correction; $P< 0.05$). * denotes significantly different length from pre-experimental values.

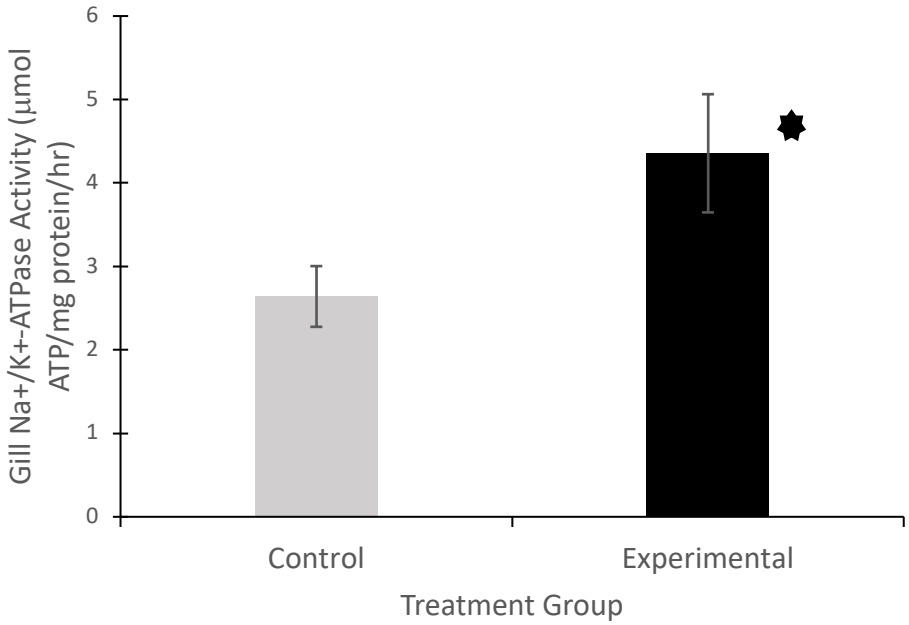


Figure 2.6: Gill NKA activity of control and experimental fish following the chronic gradual salinity transfer. Asterisk above indicates significant difference between control and experimental groups (Two-sample t-test, $P < 0.05$).

2.7 Appendix

Swim Tunnel Calibration

	Top			Middle			Bottom		
	Inside	Center	Outside	Inside	Center	Outside	Inside	Center	Outside
Interval	top inside	m/s	m/s	m/s	m/s	m/s	m/s	m/s	m/s
0	0	0	0	0	0	0	0	0	0
150	0.07	0.06	0.07	0.06	0.07	0.07	0.06	0.06	0.06
250	0.13	0.15	0.15	0.14	0.15	0.15	0.15	0.14	0.15
350	0.21	0.22	0.24	0.22	0.25	0.25	0.22	0.24	0.24
450	0.28	0.31	0.32	0.31	0.34	0.34	0.31	0.34	0.32
550	0.35	0.39	0.4	0.39	0.42	0.43	0.39	0.42	0.42
650	0.42	0.47	0.47	0.46	0.52	0.53	0.47	0.52	0.5
750	0.5	0.56	0.56	0.56	0.6	0.61	0.56	0.61	0.6
850	0.59	0.64	0.66	0.63	0.7	0.73	0.66	0.71	0.7
950	0.66	0.74	0.74	0.7	0.8	0.81	0.74	0.81	0.8
1050	0.73	0.81	0.82	0.78	0.89	0.91	0.82	0.89	0.88
1150	0.81	0.89	0.91	0.87	0.99	0.99	0.91	0.98	0.96

Table 2.1: Values for swim tunnel calibration for three separate locations within the swim tunnel

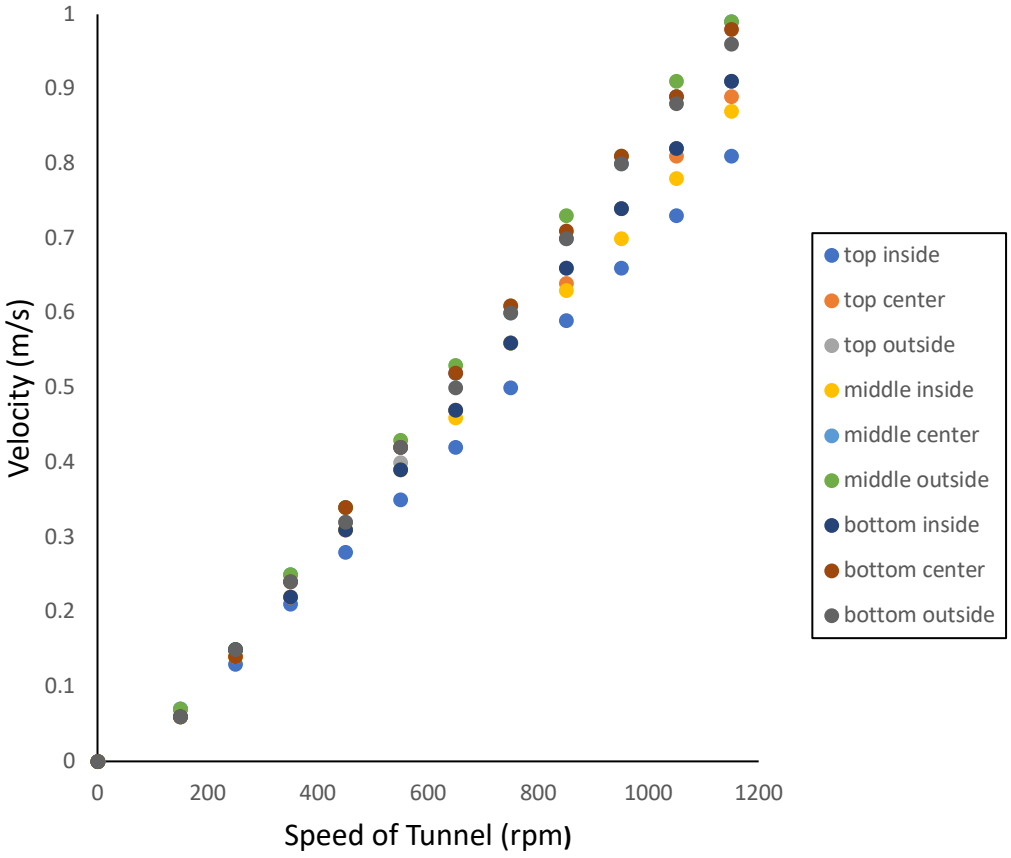


Figure 2.7: Swim tunnel calibration before swimming trials

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