

## ABSTRACT

ORR, SARAH ELIZABETH. Physiological Mechanisms of Major Ion Toxicity in Aquatic Insects. (Under the direction of Dr. David Buchwalter).

Human activities and changes in climate are profoundly influencing the ionic composition of freshwaters. Aquatic insects often dominate the ecology of these systems and ecologists report diversity losses associated with changes in salinity. Our central hypothesis is that the energetic cost of osmoregulation in saltier waters leads to detrimental toxicological consequences in sensitive aquatic insects.

First, we postulated that temperature modulates ion transport rates. Radiotracer ( $^{22}\text{Na}$ ,  $^{35}\text{SO}_4$ , and  $^{45}\text{Ca}$ ) experiments with the lab-reared mayfly, *Neocloeon triangulifer* and other field-collected insects showed that increasing temperature generally increased ion transport rates. For example, increasing temperature from 15°C to 25°C, increased Na uptake rates by two-fold ( $p < 0.0001$ ) in the caddisfly, *Hydropsyche sparna*. Then, we demonstrated that the toxicity of  $\text{SO}_4$  was influenced by temperature profoundly in a 96-hour toxicity test. Under the saltiest conditions (1500 mg/L  $\text{SO}_4$ ), *N. triangulifer* survival was 78% at 15°C, but only 44% at 25°C ( $p < 0.0036$ ). We concluded increases in salinity and/or temperature influence ion flux rates and ultimately, organismal performance in several species of aquatic insects.

Then, we asked if significant sensitivity differences occur among different larval life stages of *N. triangulifer* by conducting traditional 96-h toxicity tests with NaCl,  $\text{CaCl}_2$ , and  $\text{Ca/MgSO}_4$ . Using a general linear model, we observed that younger larvae were moderately more sensitive than older larvae in the three salts ( $p = 0.0065$ ). To assess the potential changes in ion flux between larval stages, we used radiotracers ( $^{22}\text{Na}$ ,  $^{35}\text{SO}_4$ , or  $^{45}\text{Ca}$ ) in 18-day old and 25-day old larvae and found no significant differences in ion uptake rates ( $p = 0.17$ ,  $p = 0.53$ , and  $p$

= 0.22, respectively). Our results indicate that ontogenetic differences should be considered in the future when using *N. triangulifer*.

Next, we used *N. triangulifer* to ask how ionic exposure history alters physiological processes and responses to subsequent major ion exposures. Using radiotracers, we observed that mayflies chronically reared in elevated sodium or sulfate had 2-fold ( $p < 0.0001$ ) and 8-fold ( $p < 0.0001$ ) lower ion uptake rates than naïve mayflies. These acclimatory ion transport changes provided protection in 96-hour toxicity tests for sodium, but not sulfate. Interestingly, calcium uptake was uniformly much lower and minimally influenced by exposure history, but was poorly tolerated in the toxicity bioassays. With qRT-PCR, we observed that the expression of many ion transporter genes in mayflies was influenced by elevated salinity in an ion-specific manner. To address the ion-specific physiological plasticity observed, we characterized the proteins on the gills of *N. triangulifer* through shotgun proteomic analysis, which revealed salinity-induced changes in protein expression. Ongoing analysis will elucidate the exact transporters involved in apical transport on the gill.

We then asked if osmoregulatory traits could explain the salinity niches and tolerances of various aquatic insects. We performed radiotracer experiments in different waters (dilute to salty) with various species of mayflies (*N. triangulifer*, *C. floridanus*, *D. coloradensis*, *M. modestum*, and *Isonychia sp.*), a mosquito (*A. albopictus*), and a caddisfly (*H. betteni*). Statistical analysis is ongoing, but has revealed interesting relationships among species' ion transport rates and permeability (e.g., *N. triangulifer* lost 29% of its  $^{22}\text{Na}$  label after 9 h DI water challenge while *H. betteni* lost only 11%). We also used tritium ( $^3\text{H}_2\text{O}$ ) to reveal a striking concept of readily exchangeable pools of body water that varied among species. This work characterizes the physiology that may provide clues for various salinity tolerances among aquatic insect taxa.

Our work provides supporting evidence that major ion toxicity is caused by energy depletion from excessive ion turnover. Better understanding the osmoregulatory physiology of freshwater insects is essential to improve regulatory efforts and protect freshwater ecosystems.

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Physiological Mechanisms of Major Ion Toxicity in Aquatic Insects

by  
Sarah Elizabeth Orr

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APPROVED BY:

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Dr. David Buchwalter  
Committee Chair

---

Dr. Chuck Hawkins  
External Committee Member

---

Dr. Thomas Augspurger

---

Dr. Russell Borski

---

Dr. Gerald LeBlanc

## **BIOGRAPHY**

Sarah Elizabeth Orr was born on April 26<sup>th</sup>, 1994 to Brent Orr and Shuri Roberts and raised on a farm in Dawsonville, Georgia. She quickly fell in love with the natural world and spent most of her time outdoors or with her numerous pets. Science was an obvious favorite in school, and Sarah graduated as salutatorian from Lakeview Academy in 2012. She joined the Honors Program at the University of North Georgia and excelled in the biology program with particular love for organismal biology courses. She graduated in 2016 and began her graduate studies at Mercer University studying renal toxicology with Dr. Christy Bridges. She graduated with her M.S. in Biomedical Sciences in 2018. Research science quickly became an obvious career path for Sarah and she decided to pursue a doctoral degree. In 2018, Sarah moved to Raleigh, NC to begin her PhD in toxicology in the lab of Dr. David Buchwalter. All of her interests seemed to collide into her doctoral project investigating osmoregulatory physiology of aquatic insects. In the future, she hopes to continue to do insect physiology research in academia.

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# CHAPTER ONE

## GENERAL INTRODUCTION

### *Freshwater Salinization*

The salinization of freshwater ecosystems has undoubtedly emerged as an ecological crisis (Canedo-Arguelles et al., 2013, 2016, 2018; Kaushal et al., 2005, 2018). Anthropogenic activities that have contributed to the increased major ion concentrations in freshwater bodies include resource extraction activities like hydraulic fracking (Entrekin et al., 2011) and mountain-top coal mining (Pond et al., 2008) that disturb the earth and mobilize salts. Similarly, the irrigation of arid landscapes can mobilize salts into nearby freshwater streams, rivers, and lakes (Allison et al., 1990; Halse et al., 2003; MacDonald et al., 2016). In cold urban areas, road salting can have dramatic effects on NaCl concentrations of adjacent freshwaters (Corsi et al., 2015; Karraker et al., 2008; Nava et al., 2020). Further, climate change has also led to freshwater salinization by rising sea levels and increased seawater intrusions (Barlow and Reichard, 2010; Kinzelbach et al., 2003; Post, 2005; Werner et al., 2013).

Affected areas have endured severe ecological consequences, such as the total loss of some sensitive, important species of aquatic insects (Cormier et al., 2013; Pond et al., 2008). Interestingly, some groups have recently proposed alternative road de-icing solutions, such as beet juice (Fay and Shi, 2012), but these products may actually pose a greater risk to sensitive aquatic species than salt alone (Gillis et al., 2021). Freshwater salinization has broadly led to a decrease in aquatic biodiversity and affected ecosystems have experienced a proliferation of salt-tolerant species (Hintz and Relyea, 2017; Kefford et al., 2016). The long term effects of salt contamination in freshwaters are unknown and swift mitigating actions are necessary to prevent further biodiversity loss.

The creation of water quality criteria in the United States is outdated (Stephan et al., 1985) and has historically relied on a handful of model organisms that do not accurately represent all aquatic taxa (Buchwalter et al., 2017). Generally, scientists have used species of *Chironomus* as an insect model, but these animals are typically much more tolerant of many environmental stressors than other taxa (Buchwalter et al., 2004; Hassell et al., 2006; Raby et al., 2018). To date, only one federal water quality criteria exists for salinity in the United States: chloride (US EPA, 1988). Clearly, this outdated standard does not appropriately protect aquatic life (Canedo-Arguelles et al., 2016; Cormier et al., 2013; Pond et al., 2008). It is imperative that we use a more ecologically defensible model for salinity pollution and quickly generate useful toxicity data to mitigate the harmful effects of freshwater salinization.

Salinity is challenging to study because of the ionic strength of major ions and the inability to manipulate major ion concentrations in isolation. Rather, an anion and cation must always be added to a water together (e.g., NaCl or MgSO<sub>4</sub>). Natural waters are primarily composed of the major cations: Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>+2</sup>, Mg<sup>+2</sup>, and the major anions: Cl<sup>-</sup>, HCO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>-2</sup>. It is reasonable to expect various compositions of major ions in different places due to the environmental scenario, which depends on weather, proximity to seawater, nearby human activities, and soil/sediment composition. For example, seawater intrusion and road deicing may elevate Na and Cl (Baek et al., 2014; Karraker et al., 2008; Venâncio et al., 2019; Zalizniak et al., 2006). Alternatively, mining operations may increase concentrations of Mg, SO<sub>4</sub>, Ca, and HCO<sub>3</sub> (Dam et al., 2010; Pond et al., 2008).

Scientists use multiple strategies to measure salinity. Major ion concentrations can be precisely measured using expensive chemical techniques, such as inductively coupled plasma mass spectrometry (ICP-MS), which would result in units of mg/L for each major ion measured.

Alternatively, conductivity is the measurement of electrical current in a solution and is representative of total dissolved solids, typically expressed in micro-Siemens per centimeter ( $\mu\text{S}/\text{cm}$ ). Conductivity probes are relatively inexpensive and are easy to use with immediate results, which may be quite helpful for ecological field work. While conductivity provides information on total dissolved solids (TDS) that can be generalized to a salt concentration, it fails to specify the unique ion matrices present in the solution (Horvath and 1932-, 1985; Vanysek, 2000). Thus, two streams with equal conductivities may have vastly different salt concentrations and/or ionic compositions. Although measuring conductivity is convenient and helpful in the field, we promote the use of mass spectrometry measurements to tease out ion-specific effects.

All major ions are essential for physiology, but have different functional roles. For example, it is well known that Na and K are critical for the osmotic stabilization of cells, which is established by the ubiquitous Na/K ATPase (Lodish, 2004). Na also plays a role in acid-base regulation (Cooper, 1994) and gas exchange of aquatic organisms (Evans et al., 2005). In addition, Ca is essential for many cell signaling pathways in nervous, muscular, and cardiovascular systems of the body (Flick, 1995), and for aquatic vertebrates specifically, forming and maintaining bone. Notably, organisms do not require the same amount of each major ion. Instead, the maintenance of optimal concentrations is achieved through physiological processes such as filtering blood through the renal system (for many vertebrates), hemolymph maintenance in the Malpighian tubules (for many invertebrates), and controlling expression of ion transporters at epithelial surfaces (e.g., gills or intestine) (Griffith, 2017).

Not surprisingly, ions with vastly different physiological roles will also have different toxicities. Mount et al. tested three common freshwater species (*C. dubia*, *D. magna*, and *P. promelas*) and found that the toxicity of major ions was generally  $\text{K} > \text{HCO}_3^- \sim \text{Mg} > \text{Cl} > \text{SO}_4$

(Mount et al., 1997). Moreover, a study by Kunz et al. found that waters with similar conductivities, but different compositions of major ions were differentially toxic to four test species (*L. siliquidea*, *C. triangulifer*, *D. dubia*, and *H. Azteca*) (Kunz et al., 2013). Most interestingly, some major ions (e.g., Cl, Ca, or K) may actually ameliorate salinity stress of other major ions (e.g., SO<sub>4</sub>) in aquatic organisms (Kennedy et al., 2005; Soucek, 2007). A study by Scheibener et al. found that increasing Na concentration improved survivorship of elevated SO<sub>4</sub> in the mayfly, *N. triangulifer*, which suggests interactions between ions of interest (Scheibener et al., 2017). Additional studies are needed to fully understand ionic interactions and their relevant toxicity on sensitive freshwater taxa.

### *Aquatic Insects*

Aquatic insects dominate freshwater ecosystems and over 8,000 species are found in North America alone (Merritt et al., 2008). Ecological analysis has revealed that between 70-95% of aquatic invertebrates are insects (Arscott et al., 2006). The most populous order is Diptera (43%), while Ephemeroptera only makes up 4% of aquatic insect taxa worldwide (Balian et al., 2008). Freshwater insects are considered secondarily aquatic; phylogenomic analysis of aquatic insects conclude that there is no clear common ancestor, but rather multiple invasions of water that occurred over millions of years (Misof et al., 2014; Wootton, 1988; Wootton and Clark, 1972). In today's world, very few species of aquatic insects can survive in saline waters, and this phenomenon has been attributed to both ecological and physiological reasons (Bradley, 2013; Bradley et al., 2009; Maddrell, 1998). However, the physiological processes of osmoregulation in sensitive aquatic insects remain understudied and poorly understood. Aquatic insects play a key role in freshwater ecosystem function as they serve as food sources for fish,



birds, and other animals and are involved in nutrient cycling (Covich et al., 1999; Huryn and Wallace, 2000; Jonsson and Malmqvist, 2000; Wallace and Webster, 1996).

Practically, aquatic insects also serve as ecological indicators via the USEPA Clean Water Act. Scientists heavily rely on the assessment of aquatic insect communities to infer information about water quality. Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies) are three aquatic insect orders known as “EPT taxa” that are used in ecological monitoring programs (Bonada et al., 2006; Cairns and Pratt, 1993). These orders are known for being more sensitive to pollution, including salinity stress (Cormier et al., 2013; Pond et al., 2008). Further, mayflies tend to have greater sodium uptake rate than other taxa (Scheibener et al., 2016) and are known to be the most sensitive group to salts (Hassell et al., 2006; Kefford et al., 2012). Unfortunately, their fragile, short adult life stage makes it highly unlikely for them to disperse great distances and find a more desirable habitat (Brittain, 1982). Despite the significance of aquatic insects to our environment, there is a shocking paucity of physiological understanding.

### *Osmoregulation*

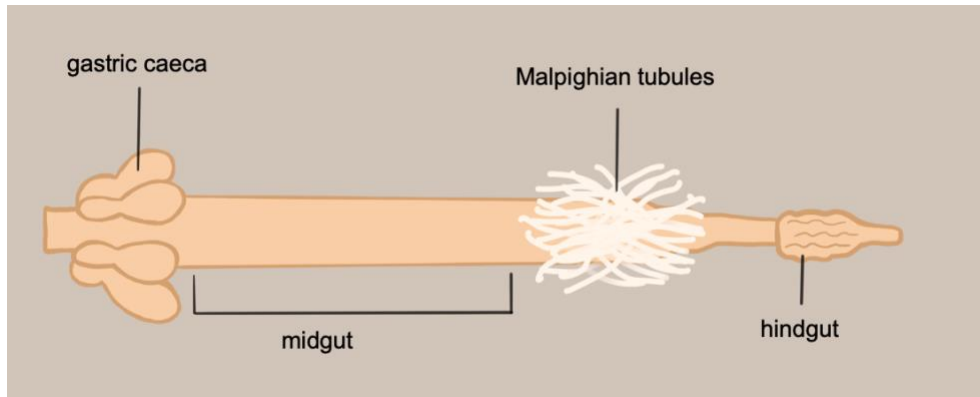
Freshwater insects are faced with the unique challenge of osmoregulation in aquatic environments hypotonic to their own hemolymph, and must prevent excess ion loss and water intake to maintain homeostasis (Silver and Donini, 2021). Most aquatic insects are strict osmoregulators (Komnick, 1977; Stobbert, 1974), even in the face of salinity challenge, which has been confirmed by experiments with *N. triangulifer* in our lab (Buchwalter et al., 2018; Scheibener et al., 2017a). Generally, aquatic insects cannot thrive in marine environments (Maddrell, 1998), however there are some exceptions. Some salt-tolerant insects, such as *Aedes*

*detritus*, can osmoconform or allow their hemolymph concentrations to fluctuate with the environment (Bradley and Philips, 1977; Patrick and Bradley, 2000a, 2000b).

Osmoregulation in dilute environments requires constant major ion uptake on epithelial tissues. Energy-demanding ionocytes found on the gills and abdomen of *N. triangulifer*, among other species of aquatic insects, are a focal point in our research. Ionocytes are rich in mitochondria and house a plethora of ion transporters essential for osmoregulation in their deeply folded plasma membrane (Komnick, 1977; Wichard et al., 1973). It remains unclear the exact location of ion transport, as most of the physiological research in aquatic insects is performed at the whole-body level, due to size restraints (Buchwalter et al., 2018). We hypothesize that the majority of ion uptake occurs at the epithelial surfaces of ionocytes found on the gills, but the exact transporters located on the gills remain unknown. Further, it is likely that at least some of the ion acquisition occurs through dietary means, since this route has been shown to be relevant for metal exposures in freshwater insects (Conley et al., 2011; Kim et al., 2012).

Acquired ions are regulated in the insect's body by the gut (gastric caeca, midgut, and hindgut) and the Malpighian tubules (Silver and Donini, 2021) (Figure 1.1). Malpighian tubules function as the insect's renal system and house a variety of major ion transporters to create an osmotic gradient, drive water out of hemolymph, and produce primary urine (Dow, 2009; Maddrell, 1980; Weng et al., 2003). After primary urine is produced, selective major ion reabsorption can occur in the Malpighian tubules and hindgut, to further prevent ion loss (Bradley, 1987). Aquaporins transport water and also play an important role in osmoregulation. However, salinity exposure does not change the expression of aquaporins in mosquito larvae (Misyura et al., 2020). Septate junctions, which control paracellular transport in invertebrates,

are also an important consideration in osmoregulation and have been shown to be altered by salinity stress (Jonusaite et al., 2016, 2017).



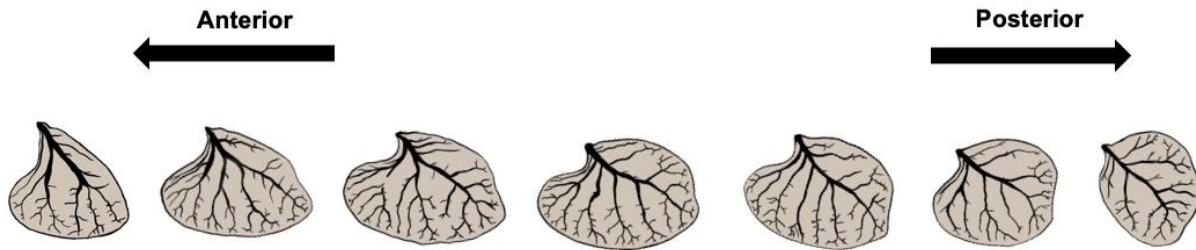
**Figure 1.1** Illustration of the gut of a *N. triangulifer* larva. Major organs involved in osmoregulation are highlighted: gastric caeca, midgut, Malpighian tubules, and hindgut. This illustration was inspired and redrawn from Silver and Donini, 2021.

Osmoregulation normally consumes a large portion of an animal's energy budget, but can be exacerbated by salinity stress (Verberk et al., 2020). *A. pusillus*, a salt-sensitive species of mayfly, was able to regulate hemolymph efficiently at several different concentrations of salt water, but still experienced mortality well before the isosmotic point (Dowse et al., 2017). Interestingly, individuals were able to maintain a constant hemolymph osmolality up to the brink of death, suggesting that there is an underlying physiological explanation for mortality that is not reliant on the ability of these organisms to osmoregulate. Our working hypothesis is that the energetic cost of osmoregulation in saltier conditions leads to a cascade of harmful physiological effects. The energy depletion hypothesis has been recently supported by data from our lab (Buchwalter et al., 2018; Chou et al., 2017; Scheibener et al., 2017b) and other labs (Johnson et al., 2015; Kefford, 2018; Struewing et al., 2014).

## *Neocloeon triangulifer as a model*

Ephemeroptera is one of the oldest living groups of insects, with their appearance in the evolutionary timeline dating back over 300 million years ago (Misof et al., 2014). The use of sensitive aquatic insects, such as mayflies, in laboratory settings has been difficult historically. Our collaborators at Stroud Water Research Center (SWRC) have helped us establish *N. triangulifer* as a model organism in our lab among a few others labs worldwide. This clonal line, originally collected from White Clay Creek, a pristine reference stream in Pennsylvania, is an environmentally-relevant model for sensitive aquatic insects and has led to greater physiological understanding of mayflies.

*N. triangulifer*, like other mayflies, are poikilothermic (i.e., cold-blooded) macroinvertebrates and are directly influenced by temperature of their environment (Sweeney and Vannote, 1984). Mayflies are also hemimetabolous; they lack a pupal stage and larvae morphologically resemble adults (Brittain, 1982; Sartori and Brittain, 2015). Larvae of *N. triangulifer* undergo an indeterminate number of molts (10 or more) as they grow and develop between 23-30 days at room temperature (22°C) (Kolpas et al., 2020). Larvae develop 14 gills on their abdomen that aid in gas exchange and ion regulation (Craig, 1990), and these gills vary dramatically in size and shape (Figure 1.2). It is currently unknown if each gill plays different roles in gas exchange or ion transport. Unlike any other extant group of insects, mayflies undergo two distinct adult stages (Kamsoi et al., 2021). First, they emerge as dark-winged, sexually-immature subimagos for a short time (hours) before molting once more into the clear-winged, sexually mature imago stage. Notoriously, mayflies do not have mouthparts as adults and undergo synchronized emergence to time sex in their short-lived terrestrial life.



**Figure 1.2** Illustration of the gills on each side of the abdomen on *N. triangulifer* larvae. The 7 gills vary dramatically in size, shape, and tracheal branching.

The use of *N. triangulifer* has ramped up in the recent years and this species has been used in toxicity testing (Soucek and Dickinson, 2015; Soucek et al., 2018), physiological experiments (Buchwalter et al., 2018; Scheibener et al., 2017a), and large -omics studies (Chou et al., 2017, 2020). The benefits of a clonal model organism are twofold: there is no mating required and all individuals are genetically identical, which provides a clean, experimental baseline. One downside to this model is their small size: less than 1 cm fully developed. Thus, we generally only work with large, late-stage larvae that may not fully represent the sensitivity and physiology of younger larvae. Additionally, a standardized diet for *N. triangulifer* has yet to be adopted. Some scientists use lab-grown diatoms (Raby et al., 2018; Soucek and Dickinson, 2015; Weaver et al., 2014), while others use natural periphyton (Jackson and Funk, 2019). Despite some discrepancies among rearing, *N. triangulifer* is clearly a powerful and promising model that represents sensitive aquatic taxa affected by environmental change.

### *Temperature Effects*

It is important to realize that controlled laboratory experiments do not accurately reflect the multitude of potential stressors in nature. Temperature is an important abiotic factor to

poikilothermic organisms, which can affect development time, size, and fecundity (Atkinson, 1994; Kolpas et al., 2020). Temperature fluctuates naturally across days and seasons (Vannote and Sweeney, 1980), but fluctuations have become more extreme in the Anthropocene (Sweeney et al., 1990). Other than the increase in global temperature (Hansen et al., 2006), warmer waters can also be a result of urbanization and removal of riparian vegetation (Madden et al., 2013; Quinn et al., 1994; Woodward et al., 2010).

In general, warmer temperatures speed ectothermic development up and produce smaller individuals, which has been coined the “temperature-size rule” (Atkinson, 1994). Most aquatic insects have a window of temperatures they can withstand; *N. triangulifer* fully develops in temperatures between 14 and 32°C (Kolpas et al., 2020). However, warmer temperatures can impose a greater energetic demand on metabolic processes (Verberk et al., 2020), which may make organisms more sensitive to other stressors (e.g., salinity). Increases in temperature can increase the toxicity of some environmental contaminants (Camp and Buchwalter, 2016; Holmstrup et al., 2010; Sokolova and Lannig, 2008), including salinity (Jackson and Funk, 2019). However, little is known about the physiological mechanisms behind temperature-salinity interactions.

### *Research Approach*

As freshwater salinization continues to pose a threat to sensitive aquatic insects, like mayflies, it is imperative that we better understand their physiology to mitigate devastating ecological effects. Here, the primary objective of my research was to better understand the physiological mechanisms of major ion toxicity in aquatic insects. This work is divided into 5 primary research

chapters that focus on different fundamental questions of the physiological effects of freshwater salinization.

Temperature largely dictates the physiology of poikilothermic animals, and has been shown to effect the toxicity of some environmental toxicants (Camp and Buchwalter, 2016; Holmstrup et al., 2010). Salinity can also have dramatic effects on the physiology of insects (Kefford, 2018; Scheibener et al., 2017a), but little is known about the interactive effects of these two abiotic factors. Recently, it was shown that colder temperatures resulted in decreased major ion toxicity to aquatic insects (Jackson and Funk, 2019). We hypothesized that warmer temperatures would increase major ion uptake rates and toxicity. To characterize temperature-salinity interactions, we used radiotracers ( $^{22}\text{Na}$ ,  $^{45}\text{Ca}$ , and  $^{35}\text{SO}_4$ ) to calculate ionic uptake rates at various temperature in *N. triangulifer*, *Isonychia sayi*, *Maccaffertium sp.*, and *Hydropsyche sparna*. We also explored the relationship between standard metabolic rate, ionic uptake rate, and temperature in *N. triangulifer*. Then, we assessed the interactive roles of temperature and elevated sulfate on major ion toxicity and development time. Chapter 2 details these experiments and reveals the interactive effects of temperature and salinity on aquatic insects.

It is well known that developing organisms have different windows of sensitivity throughout their life. Often, younger organisms are more susceptible to various toxicants than older individuals (Buchwalter et al., 2004; Gosselin and Qian, 1997; Nebeker et al., 1984). Because the majority of our experiments use *N. triangulifer* at late larval stages, we asked if sensitivity to salinity would differ across larval life stages. We performed a series of 96-h toxicity tests at different development stages and with three different salts ( $\text{CaCl}_2$ ,  $\text{NaCl}$ , and  $\text{Ca/MgSO}_4$ ). Further, we explored differences in ion uptake rates at two different larval life

stages. This work is summarized in chapter 3 and demonstrates a modest effect of age on major ion sensitivity in *N. triangulifer*.

Acclimation is an essential biological response to changing environments. The physiological plasticity of aquatic organisms to changing salinity regimes has been assessed in some organisms such as daphnids (Chen and Stillman, 2012; Coldsnow et al., 2017) and a species of mayfly (Dowse et al., 2017). However, the physiological mechanisms that influence increased tolerance are unknown. We hypothesized that mayflies would have lower ion uptake rates and changed gill protein expression with previous exposure to elevated salinities. We reared *N. triangulifer* larvae under control or elevated ionic conditions before assessing their ion uptake rates (Na, Ca, and SO<sub>4</sub>), relative mRNA transcript levels of several ion transporters, and acute survival in salinity challenge. We also analyzed histomorphological changes of the Malpighian tubules after salinity exposure. Further, we used a proteomic-approach to reveal the gill-specific ion transporters in *N. triangulifer*. The proteomic dataset provided evidence for differential protein expression among treatment groups (dilute, elevated NaCl, elevated CaCl<sub>2</sub>, or elevated Ca/MgSO<sub>4</sub>). We also imaged the ionocytes on the gills and abdomen using scanning electron microscopy. We found strong evidence for ion-specific acclimation and physiological plasticity, which is detailed in chapter 4. The proteomic-approach revealed salinity-induced osmoregulatory changes in the gill and is described in chapter 5.

The astounding evolution of insects has contributed to the most diverse and numerous group of animals on the planet. In particular, freshwater insect evolution has resulted in extreme variability among animals with numerous different strategies for gas exchange and osmoregulation. Some generalist aquatic insects species are highly tolerant to a wide range of salinities, while other specialists species have narrow ranges of salinity tolerance. We



hypothesized that by characterizing osmoregulatory traits (e.g., ion uptake rates), we could explain how animals adapt to specific salinity niches. To characterize these traits, we performed ion (Na, SO<sub>4</sub>, and Ca) and water uptake experiments on multiple taxa of aquatic insects, ranging from air-breathers to gill breathers. Many of the animals we studied are only found in freshwater streams (*D. coloradensis*), while others can be collected from salt-contaminated lakes (*C. floridanus*) or stagnant puddles (*A. albopictus*). We were particularly interested in characterizing species-specific and ion-specific differences in ion uptake rates. Further, we discuss the relatively novel idea of species-specific exchangeable pools of body water. These findings can be found in chapter 6. Taken together, this research makes significant strides in understanding osmoregulatory physiology of aquatic insects in a changing world.

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## **CHAPTER TWO**

### **IT'S ALL ABOUT THE FLUXES: TEMPERATURE INFLUENCES ION TRANSPORT AND TOXICITY IN AQUATIC INSECTS**

S. E. Orr and D. B. Buchwalter

North Carolina State University

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## Abstract

Many freshwater ecosystems are becoming saltier and/or warmer, but our understanding of how these factors interact and affect the physiology and life history outcomes of most aquatic species remain unknown. We hypothesize that temperature modulates ion transport rates. Since ion transport is energetically expensive, increases in salinity and/or temperature may influence ion flux rates and ultimately, organismal performance. Radiotracer ( $^{22}\text{Na}^+$ ,  $^{35}\text{SO}_4^{2-}$ , and  $^{45}\text{Ca}^{2+}$ ) experiments with lab-reared mayflies (*N. triangulifer*) and other field-collected insects showed that increasing temperature generally increased ion transport rates. For example, increasing temperature from 15°C to 25°C, increased  $^{22}\text{Na}^+$  uptake rates by two-fold ( $p < 0.0001$ ) and  $^{35}\text{SO}_4^{2-}$  uptake rates by four-fold ( $p < 0.0001$ ) in the caddisfly, *Hydropsyche sparna*. Smaller changes in  $^{22}\text{Na}^+$  and  $^{35}\text{SO}_4^{2-}$  uptake rates were observed in the mayflies, *Isonychia sayi* and *Maccaffertium* sp., suggesting species-specific differences in the thermal sensitivity of ion transport. Finally, we demonstrated that the toxicity of  $\text{SO}_4$  was influenced by temperature profoundly in a 96-hour bioassay. Under the saltiest conditions (1500 mg/L  $\text{SO}_4$ ), mayfly survival was 78% at 15°C, but only 44% at 25°C ( $p < 0.0036$ ). Conceivably, the energetic cost of osmoregulation in warmer, saltier environments may cause significant major ion toxicity in certain freshwater insects.

## **Introduction**

Abiotic factors such as temperature and salinity are fundamental determinants of aquatic species distributions (Carver et al., 2009; Kefford et al., 2012; Vannote and Sweeney, 1980). Few aquatic ecosystems maintain constant temperature and salinity (e.g. spring-fed headwater streams). Most freshwater ecosystems experience natural variations in temperature both daily and seasonally while major ion concentrations may naturally vary in a given location due to rainfall and evaporation (Canedo-Arguelles et al., 2013).

Layered upon these natural fluctuations are more extreme variances in temperature (Malmqvist et al., 2008; Webb et al., 2008) and salinity (Canedo-Arguelles et al., 2016; Kaushal et al., 2018; Pond et al., 2008) resulting from human activities. Warmer waters are a consequence of impervious surfaces in urban environments, removal of riparian vegetation, water drawdowns for human uses (e.g., agriculture, human consumption, and cooling for power plants and other industrial uses). Changing salinity regimes are often a result of road de-icing (Karraker et al., 2008), hydraulic fracturing (Entrekin et al., 2011), and mountain-top coal mining (Pond et al., 2008), among other land use activities.

Aquatic insects thrive in dilute, freshwater environments, yet are relatively rare in more saline and marine environments. Authors have offered both ecological (Maddrell, 1998) and physiological (Bradley, 2013) reasons for this phenomenon. However, our understanding of how salinity determines species distributions within freshwater ecosystems remains limited. Similarly, we understand that temperature imposes limits on where species thrive, but the mechanisms are relatively understudied in aquatic insects and remain unresolved (Chou et al., 2018; Kim et al., 2017; Sweeney, 1978; Sweeney et al., 1990, 2018; Verberk and Bilton, 2013).

There is evidence that both temperature and chronic salinity stress affect the energy budgets of aquatic insects. For example, recent work has demonstrated that chronic thermal challenge in aquatic insects is associated with changes in metabolomics linked to energy depletion (Chou et al., 2018), while acute thermal challenge has been linked to oxygen limitation (e.g. Verberk et al., 2013). Similarly, recent research on the toxic effects of salinity has provided evidence for the susceptibility of some freshwater insects (Cormier et al., 2013; Kefford et al., 2016; Scheibener et al., 2017; Soucek et al., 2018). Several authors have observed developmental delays and reduced growth rates in ion-challenged aquatic insects, suggesting a reallocation of energy to maintaining homeostasis in waters with elevated major ions (Buchwalter et al., 2018; Johnson et al., 2015; Sweeney et al., 2018).

In isolation, we are beginning to understand the mechanisms of how temperature and salinity affect aquatic insects, but our understanding of their interactions remains incomplete. Temperature is known to modulate the toxicity of certain environmental contaminants (Camp and Buchwalter, 2016; Holmstrup et al., 2010; Sokolova and Lannig, 2008). However, few studies have evaluated the physiological consequences of both stressful temperatures and salinities on aquatic insects. Among them, Jackson and Funk found that some streams were becoming saltier only in the winter months, but that colder temperatures were associated with a decrease in major ion toxicity to insects (Jackson and Funk, 2019). We hypothesize that physiological processes may exacerbate the effects of salinity under warmer conditions by increasing the energetic cost of osmoregulation.

Here, we ask if temperature is a significant modifier of ionic flux rates and toxicity in aquatic insects. We specifically evaluated the influence of temperature on the uptake rates of ( $^{22}\text{Na}$ ,  $^{35}\text{SO}_4$ , and  $^{45}\text{Ca}$ ) in both a lab-reared mayfly (*N. triangulifer*) and other field collected aquatic insects. We

further test the hypothesis that major ion toxicity is linked to ionic flux rates, and that toxicity may be modified by temperature via changes in these flux rates. Finally, we discuss the need to consider both temperature and major ion concentrations in the development of environmental standards in order to better protect aquatic life.

## Methods

### *Mayfly husbandry and aquatic insect field sampling*

Most experiments were done with the lab-reared parthenogenetic line of *Neocloeon triangulifer* (WCC-2 clone), which was originally gifted by Stroud Water Research Center (SWRC; Avondale, PA). *N. triangulifer* was maintained in laboratory settings (21-23°C and 14h:10h light:dark photoperiod) and fed natural periphyton from SWRC. Major ion concentrations (mg L<sup>-1</sup>) of artificial soft water (ASW) were: 55.8 NaHCO<sub>3</sub>, 3.5 KHCO<sub>3</sub>, 22 CaCl<sub>2</sub>, 18 CaSO<sub>4</sub>•2H<sub>2</sub>O, and 34 MgSO<sub>4</sub>•7H<sub>2</sub>O. ASW served as routine culture media, control treatments, and the base water to which sulfate was amended (see below).

Field-collected insects were sampled from the Eno River, NC (36.081, -79.140) with D-framed kick-nets in Spring 2019. Insects were sorted in pans and transported to lab in aerated coolers with stream water, ice packs, and mesh substrate. For experiments involving temperature treatments, insects of a given taxon were divided into separate temperature groups and held in incubators at their respective experimental temperatures for at least 24 hours before experiments began. Voucher specimens were preserved in 70% ethanol and identified using morphological features with a dichotomous key (Morse et al., 2017) .

### *Radioactivity measurement*

<sup>35</sup>S, <sup>22</sup>Na, and <sup>35</sup>Ca isotopes were obtained as Na<sub>2</sub><sup>35</sup>SO<sub>4</sub>, <sup>22</sup>NaCl, and <sup>45</sup>CaCl<sub>2</sub>, respectively (PerkinElmer, Billerica, MA, USA). Working stock solutions were made by diluting isotopes in ASW for a final exposure activities between 156 and 260 Bq mL<sup>-1</sup>. All experimental waters were sampled and counted on a Beckman LS6500 Multipurpose Scintillation Counter prior to establishing experimental exposures. Flux experiments were conducted either as dual-labeled

exposures, using both  $^{35}\text{S}$  and  $^{22}\text{Na}$  isotopes simultaneously or  $^{35}\text{Ca}$ -labeled waters. After an exposure period, for  $^{35}\text{S}$  and  $^{22}\text{Na}$  waters, insects were rinsed in two consecutive baths of ASW to displace any adsorbed radioactive ions from the exoskeleton. For  $^{35}\text{Ca}$  experiments, insects were sequentially rinsed in stable water, 0.05 M EDTA, 0.1 M L-ascorbic acid sodium salt, and stable water because Ca adsorption to the exoskeleton can be significant (Poteat and Buchwalter, 2014a).

After being rinsed, insects were blotted dry with a Kimwipe and weighed before being digested with 500  $\mu\text{L}$  Soluene 350 (Perkin Elmer) in a 20 mL glass scintillation vial. Digestates were mixed with 500  $\mu\text{L}$  of glacial acetic acid and 16 mL of scintillation cocktail (Perkin Elmer Ultima Gold uLLT) and counted for three minutes. Appropriate corrections for spill-over and quench were applied. Only measurements with lumex values  $<5\%$  and counting error rates  $<10\%$  were used in data analysis.

#### *Assessing temperature effects on ion uptake rates*

Flux experiments were performed in aerated 100 mL high-density polyethylene (HDPE) beakers with 20 mL of treatment solution. All beakers had an air line with gentle aeration, a Teflon square substrate, and a ParaFilm<sup>TM</sup> cover to avoid evaporative loss. Temperatures (15, 20, or 25°C) were controlled by incubators and monitored with a HOBO<sup>TM</sup> data logger device throughout the duration of all experiments. Some experiments only included two temperatures (15 and 25°C) due to limited availability of collected animals. All experiments had 6-8 replicates ( $n = 6-8$ ) and either 3 ( $^{45}\text{Ca}$  experiments) or 4 ( $^{35}\text{S}$  and  $^{22}\text{Na}$  experiments) time points, to calculate mass-specific unidirectional linear uptake rates, which were taken as the slopes of radioactivity acquisition vs time plots (Figure 2.1). The fourth time point of each experiment was only included if it changed the slope less than 5% in order to avoid underestimating uptake due to efflux of labeled ion.



### *Standard metabolic rates across temperatures*

Oxygen consumption rates (or standard metabolic rates, SMR) were analyzed using a fiber-optic based, intermittent flow respirometry system (Loligo Systems, Tjele, Denmark) using Autoresp™ 2.0 software. *N. triangulifer* larvae were acclimated for 24 hours before each experiment. They were individually placed in test chambers ( $1.28 \pm 0.1$  mL) and rested on a small piece of stainless steel mesh over a magnetic stir bar. Standard metabolic rates (SMRs) were taken as the mean of eight respirometry cycles (200 seconds for flush, hold, and measure phases) at the appropriate temperature (15°C, 20°C, or 25°C) subtracting blank chambers as background.

### *Life history outcomes across salinities*

To assess the effect of temperature on sulfate toxicity, we performed an acute 96-hour bioassay with 10 day old *N. triangulifer* larvae in 6-well plates. Before each experiment, waters were measured for conductivity ( $\mu\text{S cm}^{-1}$ ), pH, and dissolved oxygen (mg/L). All experimental waters were sampled (10 mL) and ion concentrations were verified by North Carolina State University Environmental and Agricultural Testing Service Lab (ICP-EATS). All waters were within 10% of nominal concentrations. Each well was rinsed well with deionized water, then filled with 8 mL of well aerated treatment water (~75% full). Treatment waters were amended with  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (Ca:Mg = 2:2.1 mass ratio) and had the following concentrations of  $\text{SO}_4$  (mg  $\text{L}^{-1}$ ): 23, 360, 515, 735, 1050, and 1500. Larvae were acclimated to various temperatures (15°C, 20°C, or 25°C) 24 hours prior to the beginning of the experiment. Temperatures were controlled by incubators with 14h:10h light:dark photoperiod and monitored with a HOBO temperature data logger throughout the duration of all experiments. Ten larvae were

seeded into each well and each treatment group had 5 replicates ( $n=5$ ). Wells were provided 100  $\mu\text{L}$  of food slurry (0.1 g periphyton per mL) prepared for each salinity treatment such that the addition of food did not affect the targeted treatment.

Each well was manually aerated with an airline daily (30 seconds /well) and larvae were visually assessed using a Leica DFC480 microscope. Mortalities were recorded and removed daily with a glass pipette. After 48 hours, 50% of the water was changed by removing 4 mL of liquid and all debris and replacing it with 4 mL of clean, well aerated water and 100  $\mu\text{L}$  of food slurry. After 96 hours, all wells had conductivity ( $\mu\text{S cm}^{-1}$ ) and pH measured to ensure consistent major ion concentration throughout the duration of the experiment. All wells were within 5% of initial conductivity measurements.

A chronic bioassay was also performed using 1.5 L glass jars. Fifteen <1 day old *N. triangulifer* larvae were seeded into each jar. Larvae were fed *ad libitum* with 1-3 mm coating of periphyton attached to 23x6.4x0.16 cm acrylic plates from SWRC. Three treatment waters (ASW, 665  $\text{mg L}^{-1}$   $\text{SO}_4$ , and 1300  $\text{mg L}^{-1}$   $\text{SO}_4$ ) were tested. These concentrations were chosen based on values found in literature discussing streams impacted by mountain top coal mining (Pond et al., 2008). We amended waters with  $\text{CaSO}_4$  and  $\text{MgSO}_4$  because these ions are found in mining-affected ecosystems, while Na remains relatively low (Cormier et al., 2013). Emerged subimagos were collected daily in a mesh lid and development time was recorded.

### *Data analysis*

GraphPad Prism (v6, GraphPad Software, La Jolla, CA, USA) was used for all data analysis. Errors bars represent mean  $\pm$  SEM for each plot. A p value of 0.05 was chosen *a priori*. For ion flux experiments, rates were determined by the slope of a linear regression across each

time course. Mass specific calculations were all based on wet weights. For respirometry experiments,  $\log_{10}$ -transformed data was graphed and  $Q_{10}$  estimates were obtained from the slopes. In all experiments, either a Student's t-test or a one-way ANOVA using Tukey's multiple comparison test was performed.

## Results

*Sulfate, sodium, and calcium uptake rates in N. triangulifer and field-collected aquatic insects across three temperatures (15°C, 20°C, and 25°C)*

In *N. triangulifer*, the effect of temperature on ion influx rates varied among ions. Sodium influx rates were strongly influenced by temperature, increasing 13% at 20°C and 49% at 25°C relative to the 56.8  $\mu\text{g g}^{-1} \text{hr}^{-1}$  sodium uptake rate observed at 15°C (Figure 2.2A). This nonlinear rate of increase was also observed for sulfate. At 15°C, sulfate uptake rate was 2.5  $\mu\text{g g}^{-1} \text{hr}^{-1}$ , but increased 23% at 20°C and 60% at 25°C (Figure 2.2B). Interestingly, calcium uptake rates changed very little (< 5%) across temperatures (Figure 2.2C).

Similar to *N. triangulifer* ion flux results, field-collected species also exhibited a general increase in ion uptake rates as temperatures increased. In the field-collected mayfly, *Isonychia sayi*, sulfate uptake rates increased 21% at 20°C and 28% at 25°C relative to 7.3  $\mu\text{g g}^{-1} \text{hr}^{-1}$  at 15°C (Figure 2.2D). Sodium uptake rates increased 32% and 37% at 20°C and 25°C, respectively, relative to 13.3  $\mu\text{g g}^{-1} \text{hr}^{-1}$  at 15°C ( $p < 0.05$ ) (Figure 2.2E). *I. sayi* calcium uptake rates were 0.3  $\mu\text{g g}^{-1} \text{hr}^{-1}$  at 15°C, but increased 99% at 20°C and 88% at 25°C ( $p < 0.05$ ) (Figure 2.2F). We observed similar patterns in *Maccaffertium* sp.; sulfate uptake rates increased 81% at 25°C relative to 7.8  $\mu\text{g g}^{-1} \text{hr}^{-1}$  at 15°C (Figure 2.2G), while sodium uptake rates increased 63% at 25°C relative to 9.8  $\mu\text{g g}^{-1} \text{hr}^{-1}$  at 15°C (Figure 2.2H). However, calcium uptake rates at 15°C were 0.4  $\mu\text{g g}^{-1} \text{hr}^{-1}$  and increased 75% at 25°C ( $p < 0.01$ ) (Figure 2.2I). Sulfate uptake rate in *Hydropsyche sparna* was 2.9  $\mu\text{g g}^{-1} \text{hr}^{-1}$  at 15°C and increased 2.4-fold at 25°C ( $p < 0.01$ ) (Figure 2.2J). Similarly, sodium uptake rates increased 4.3-fold at 25°C relative to the 1.0  $\mu\text{g g}^{-1} \text{hr}^{-1}$  observed at 15°C ( $p < 0.0001$ ) (Figure 2.2K). Calcium uptake rates also increased in *H. sparna* at 25°C, albeit not significantly (Figure 2.2L).

### *Standard metabolic rates correlate with ion uptake rates in N. triangulifer*

The non-linear effect of temperature on ion transport rates was also observed in the standard metabolic rates (SMRs). At 15°C, the average SMR was 539  $\mu\text{g O}_2/\text{g}/\text{hour}$  and increased 27 % to 686  $\mu\text{g O}_2/\text{g}/\text{hour}$  at 20°C and 2.2-fold to 1188  $\mu\text{g O}_2/\text{g}/\text{hour}$  at 25°C ( $p < 0.0001$ ) (Figure 2.3A). Interestingly, we observed almost perfect correlation ( $R^2=0.997$  for sulfate and  $R^2=0.998$  for sodium) of ion transport rates with SMRs for both sulfate and sodium (Figure 2.3B and 2.3C), suggesting that for a given ionic concentration, thermally driven changes in transport rates scale with metabolic rates in this species.

### *Temperature and salinity affect toxicity and development time*

Temperature strongly influenced sulfate toxicity in a 96-hour acute bioassay; 98%  $\pm$  2 of *N. triangulifer* larvae survived in water with 1050  $\text{mg L}^{-1}$  sulfate at 20°C, but in the same waters, only 84%  $\pm$  5.1 survived at 15°C and 70%  $\pm$  5.5 in 25°C (Figure 2.4A). At the highest salinity, 1300  $\text{mg L}^{-1}$  sulfate, 94%  $\pm$  4 of mayflies survived at 20°C, 78%  $\pm$  6.6 survived at 15°C, and 44%  $\pm$  5.1 survived at 25°C. In the chronic full-life cycle bioassay, control performance was too variable for in-depth toxicity analysis. However, the commonly observed phenomenon of developmental delay associated with salinity stress was observed at all test temperatures. For example, at 20°C, 665  $\text{mg L}^{-1}$  sulfate caused a 4.5% increase in development time compared to controls (ASW) ( $p < 0.0001$ ) and highly-elevated sulfate waters (1300  $\text{mg L}^{-1}$ ) caused a 27% increase in development time ( $p < 0.0001$ ). However, the magnitude of these delays was consistent across all three temperatures (Figure 2.4B).

## Discussion

Freshwater salinization is an emerging ecological problem worldwide (Canedo-Arguelles et al., 2016; Kaushal et al., 2018). Despite this growing problem, our understanding of how major ions affect the performance and distributions of most aquatic life remains unknown. This is particularly true for aquatic insects, which are used extensively to evaluate the ecological conditions of freshwater ecosystems (Pond et al., 2008). Here, we asked if temperature might be a modifier of ion transport (uptake) in aquatic insects, and by extension, a modifier of major ion toxicity as well.

To examine the effect of temperature on the uptake rates of major ions ( $\text{Ca}^{+2}$ ,  $\text{Na}^+$ ,  $\text{SO}_4^{-2}$ ) we used time course experiments over 36 hours for  $\text{Ca}^{+2}$  and 24 hours or less for both  $\text{Na}^+$  and  $\text{SO}_4^{-2}$ . This approach largely reduces the potential for adsorbed ions on the insect exoskeleton to confound the interpretation of results, particularly since there are no data that specifically examine the effect of temperature on adsorption. To evaluate the effect of temperature on adsorption to the exoskeleton, we compared y-intercept values between time course experiments and found some evidence that adsorption increased with temperature. Previous work with sulfate (Scheibener et al., 2017) and sodium (Scheibener et al., 2016) indicate very low to modest exoskeleton sorption potential of these ions respectively, whereas calcium is known to adsorb significantly (Poteat and Buchwalter, 2014a). Thus, if we assume that sorption is rapid (quasi-instantaneous), the interpretation of the increase in radioactivity over time can be interpreted as a unidirectional uptake rate. The potential confounding factor for time course experiments is the potential role of efflux or turnover at later time points that could result in under-estimating the true unidirectional uptake rates. Here, we compared the slopes of uptake rates with and without the last time-point to ensure that the rates we report are the best possible estimates of unidirectional uptake.

We show that there are significant species-specific and ion-specific differences in the magnitude of uptake rates and the extent to which temperature modifies them. For example, in all four species we examined, calcium uptake rates were consistently 1 to 2 orders of magnitude slower than those for sulfate and sodium. One reason for the generally slow rate of calcium uptake in insects relative to crustaceans is that the insect exoskeleton is proteinaceous rather than calciferous. Calcium was most dramatically changed by temperature in the caddisfly *Hydropsyche*, and was unchanged in *N. triangulifer*. In contrast, sodium transport varied by 2 orders of magnitude across species, and was consistently elevated by increasing temperature. Sulfate transport rates varied least among species, but were consistently elevated by increasing temperatures. Previous work by Scheibener et al. showed elevated sodium transport rates of mayflies relative to other taxa (Scheibener et al., 2017) and Poteat and Buchwalter demonstrated that body size and phylogeny both contributed to differences in calcium transport among aquatic insects (Poteat and Buchwalter, 2014b).

We are not aware of any studies that examined the effect of temperature on ion transport rates in aquatic insects. In other freshwater taxa however, there is evidence for the temperature sensitivity of ion transport. For example, Dunson and Weymouth showed that soft shell turtles (*Trionyx spinifer*) significantly decreased sodium uptake in cold waters (4°C) vs warm waters (21°C) (Dunson and Weymouth, 1965). Isaia studied the gills of a freshwater fish, *Carassius auratus*, and found that a ten degree increase (10°C to 20°C) nearly doubled sodium permeability (Isaia, 1972). There is also evidence for temperature-dependent osmoregulation in marine organisms such as sea bass (Masroor et al., 2019), threespine stickleback (Gibbons et al., 2018), and yellowleg shrimp (Vargas-Albores et al., 1998). While the data on this topic are not abundant, it is apparent that temperature is a major determinant of ion transport rates.

One potential reason for the increase in ion transport rates with increasing temperature could be related to changes in the permeability of respiratory epithelia. Isaia and Motais reported that freshwater-adapted eels, *Anguilla anguilla*, had a higher osmotic to diffusional permeability ratio (Motais and Isaia, 1972). Robertson and Hazel demonstrated that osmotic water uptake was greater in teleost fishes in warmer waters (Robertson and Hazel, 1999), and Buchwalter et al. showed similar increases in water permeability with acute thermal challenge in aquatic insects (Buchwalter et al., 2003). It remains unclear if changes in epithelial permeability that result in increased water influx are also associated with increased diffusive ion losses through paracellular channels. However, we can surmise that thermally induced increases in epithelial permeability could impose an increased demand for active ionic uptake to maintain hemolymph osmolality.

Alternatively, warmer temperature could increase ion transport rates simply by accelerating physiological processes in general. The nonlinear relationship of ion uptake across temperatures observed in *N. triangulifer* and other field-collected aquatic insects might also be explained by temperature-dependent metabolic rates. In *N. triangulifer*, we observed almost perfect correlation of sodium and sulfate transport rates with metabolic rates across temperatures. As oxygen consumption rates generally increase with temperature in a logarithmic fashion (Portner, 2001), the degree to which ion transport rates are affected by temperature change may be determined by how those exposure temperatures relate to the species thermal tolerances (i.e. larger differences as organisms approach their thermal limits). Much more work is needed to better understand the relationships between metabolic rates and ion transport, but it is tempting to think that temperature effects on ion transport are more modest within a given species' Thermal Acclimation Zone (Sweeney et al., 2018), and are exacerbated as the organism approaches thermally stressful conditions. Thus, both epithelial permeability and temperature-dependent metabolism remain



possible, but not mutually exclusive explanations for temperature driven changes in ion transport rates.

Although there is relatively little information about the interactions of salinity and temperature in aquatic insects, our finding that major ion toxicity was temperature dependent in a mayfly generally fits with existing information. Jackson and Funk reported that cooler temperatures were shown to ameliorate NaCl toxicity in mayflies (Jackson John K. and Funk David H., 2019). Similarly, there is growing evidence that increasing temperature often leads to increased toxicity of contaminants (Sokolova and Lannig, 2008). In aquatic organisms, the temperature-dependent toxicity of metals and insecticides has been demonstrated (Brecken-Folse et al., 1994; Cairns, 1986; Camp and Buchwalter, 2016; Holmstrup et al., 2010; Macaulay et al., 2019). One study with *G. rosesli*, a freshwater amphipod, showed increased major ion toxicity with increasing temperatures (Sornom et al., 2010). Our results demonstrate mayfly tolerance is modulated in response to warmer temperatures: exposure to a borderline-stressful temperature of 25°C reduced survivorship to 44% in our 96-hour bioassay relative to a more ideal temperature of 20°C where 94% survived. However, we acknowledge that survivorship at 15°C (78%) was slightly reduced under salinity stress relative to 20°C. One explanation is that 20°C is closer to an optimal temperature for this species (D. Funk, personal communication) and that under these conditions larvae can better respond to stressful salinities (e.g., more appropriate efflux rates to maintain homeostasis). Alternatively, in an R-strategist, such as *N. triangulifer*, a few spurious deaths could account for these differences.

The toxicity of increasing major ion concentration has been well described for sensitive species of aquatic insects in the laboratory (Buchwalter et al., 2018; Hassell et al., 2006; Kefford, 2018; Soucek and Dickinson, 2015), mesocosm experiments (Canedo-Arguelles et al., 2012; Clements

and Kotalik, 2016), and in the field (Cormier et al., 2013; Pond et al., 2008). Previous observations show that salinity is stressful at concentrations lower than the osmolality of the hemolymph (Dowse et al., 2017; Kefford, 2018) and insects do not appear to be dysregulated with respect to hemolymph osmolality or whole body element concentrations (Buchwalter et al., 2018; Scheibener et al., 2016). Salinity stress is also associated with developmental delay (Johnson et al., 2015) and all of these observations point to the energetic costs of strict osmoregulation by aquatic insects. If ion transport is energetically expensive, than anything that increases flux rates (increases in ionic concentrations and/or increases in temperature) divert nutritional resources from other critical functions (e.g. growth and reproduction).

Regulatory entities have been slow to respond to the consequences of major ions on freshwater biodiversity. In the US for example, few scientifically defensible Water Quality Criteria exist for major ions. The EPA currently regulates 60 pollutants, including chloride (US EPA, 1988) and hardness (US EPA, 1986), but no other major ions. Regardless of whether future standards to protect aquatic life are based on lab-based studies of individual ions, or field based methods based on community responses to conductivity (Cormier et al., 2013), it is apparent that temperature is a major modulator of major ion toxicity that should not be ignored if we are to better protect freshwater biodiversity from salinity in a changing world.

In summary, our results suggest that warmer temperatures increase metabolic rate, major ion uptake rate, and ultimately energy consumption across several genera of aquatic insects. Although we have gained knowledge about the interactions of temperature and salinity in aquatic insects, the exact mechanism of toxicity is unclear. Understanding the underlying physiology of critical organisms in freshwater ecosystems is important and may help with regulatory purposes and interpreting biomonitoring data.

## **Author Statement**

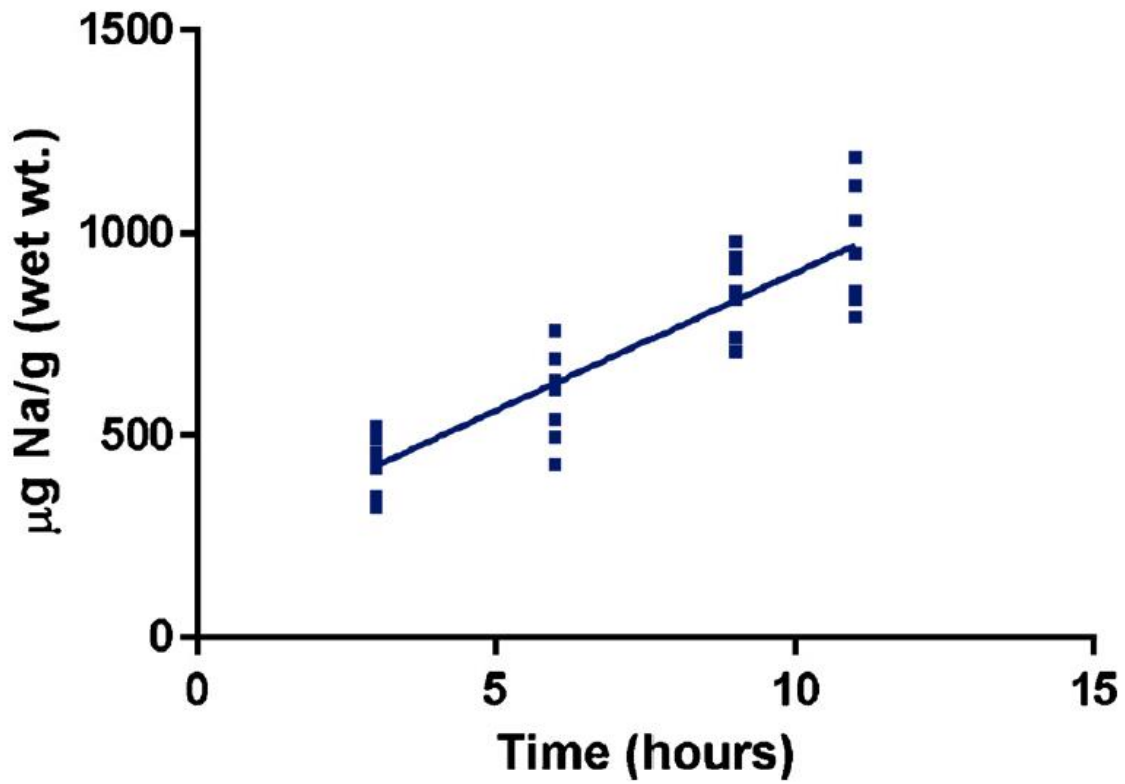
David Buchwalter conceived of the work and provided research oversight and editorial assistance of the manuscript. Sarah Orr conducted the research and wrote the manuscript.

## **Declaration of Competing Interest**

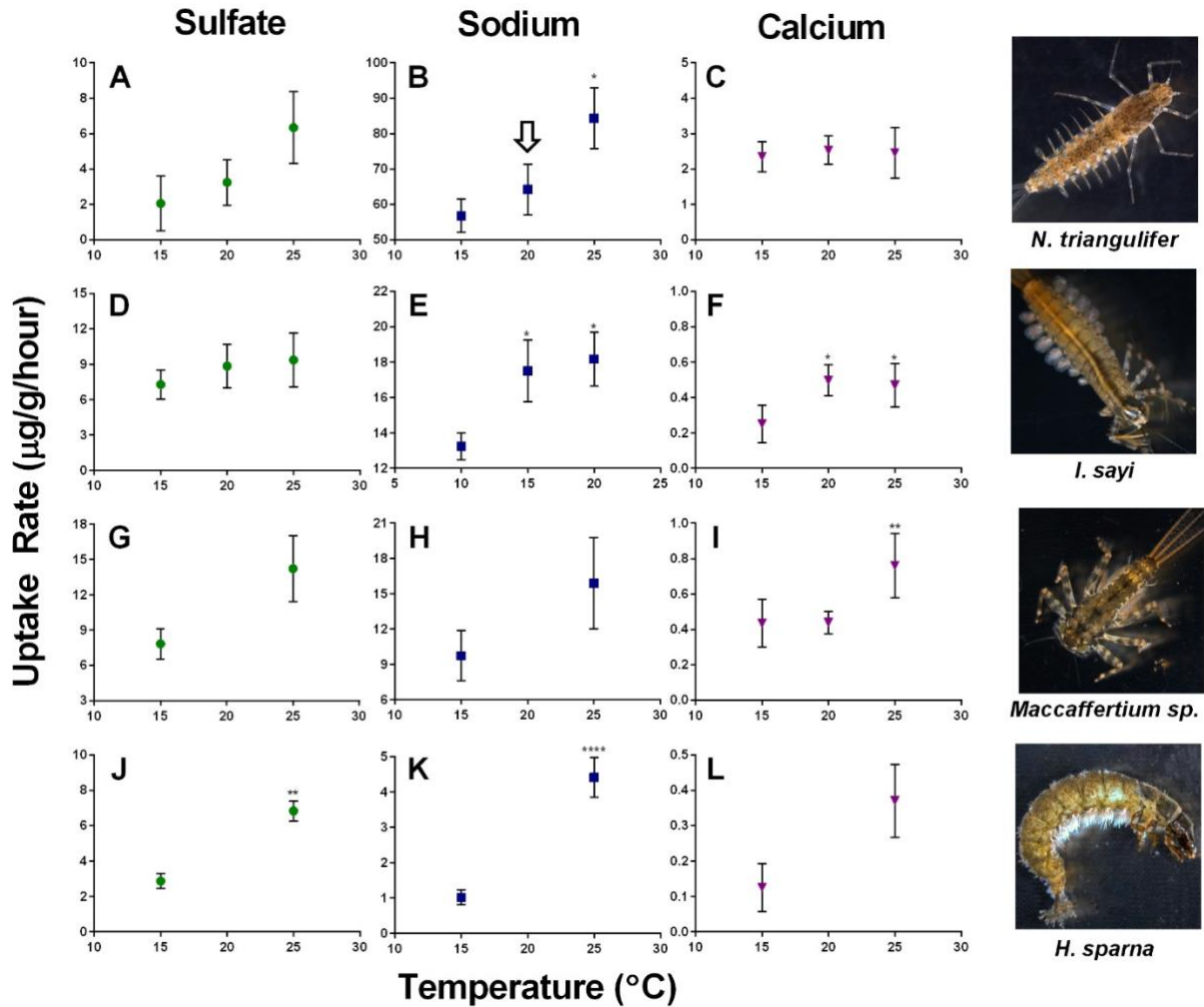
The authors declare that they have no known competing financial interests of personal relationships that could have appeared to influence the work reported in the paper.

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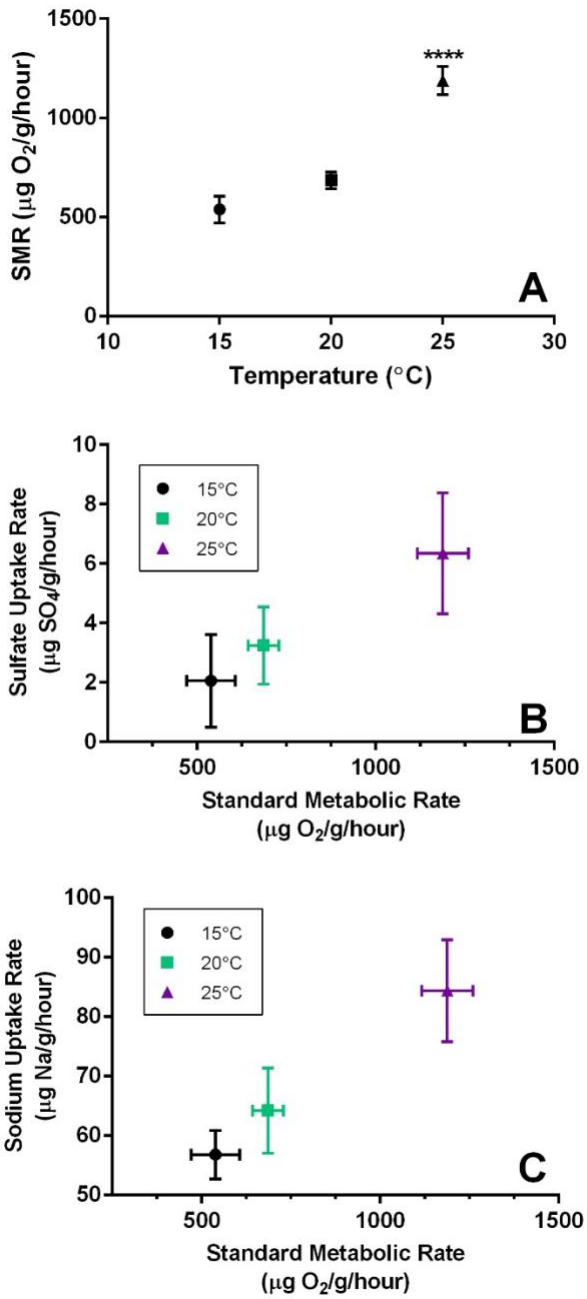
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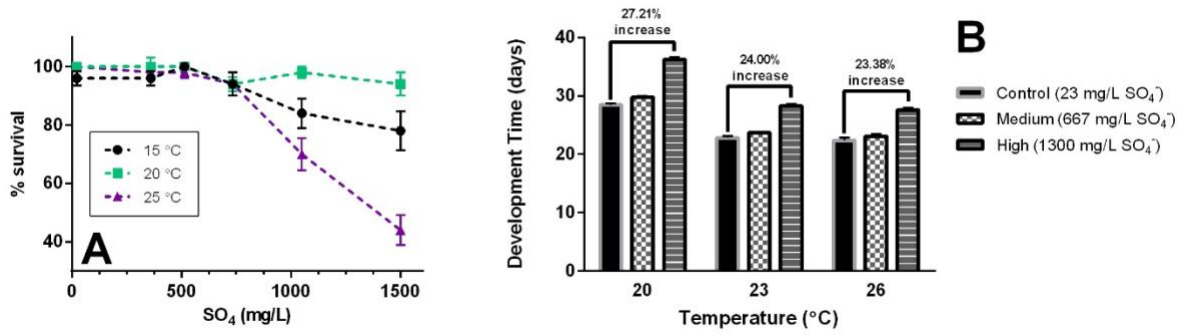
**Figure 2.1** Example of the approach taken to assess ion uptake rates in aquatic insect larvae. In this case, each point represents the acquisition of  $^{22}\text{Na}$  in an individual *N. triangulifer* larva at  $20^{\circ}\text{C}$ . Linear regression analysis is based on the mean values for each time point. These particular data are presented in figure 2.2B and indicated by an arrow.



**Figure 2.2** Uptake rates (mean  $\pm$  SE) of sulfate, sodium, and calcium across two or three temperatures in *N. triangulifer*, *I. sayi*, *Maccaffertium sp.*, and *H. sparna*, depending on number of animals available. Each point represents the slope of a time-course of 8 individuals per time point ( $n=8$ ). In general, uptake rates of ions increased with warmer temperatures across the four species.



**Figure 2.3** (a) *N. triangulifer* standard metabolic rates (SMR) across three temperatures. Each point represents the mean of 8 individuals. (b) Sulfate uptake rate plotted against SMR ( $R^2=0.997$ ). (c) Sodium uptake rate plotted against SMR ( $R^2=0.998$ ).



**Figure 2.4** (a) The percent survival of *N. triangulifer* across 6 concentrations of sulfate and three temperatures (15, 20, and 25°C). Each point represents the mean of 5 wells with 10 mayflies/well. (b) Development time of *N. triangulifer* across 3 concentrations of sulfate and three temperatures (20, 23, and 26°C). Each bar represents the mean of 5 jars with 15 mayflies/jar. Brackets indicate the percent increase from control to high sulfate-exposed mayflies.

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## CHAPTER THREE

### **WEAK DIFFERENCES IN SENSITIVITY TO MAJOR IONS BY DIFFERENT LARVAL STAGES OF THE MAYFLY *NEOCLOEON TRIANGULIFER***

S. E. Orr, J. K. Cochran, I. G. Wallace, R. W. Gray, G. E. Overmyer, and D. B. Buchwalter

Department of Biological Sciences, North Carolina State University, 850 Main Campus Drive,  
Raleigh, North Carolina 27606 USA

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## Abstract

Freshwater salinization is a global ecological concern because of the alarming biodiversity declines associated with increases in major ion concentrations. Loss of mayfly diversity appears to be a common ecological response to anthropogenic salinization worldwide. Remarkably few regulatory standards exist to protect aquatic life from major ions, and antiquated approaches for setting such standards rely on traditional laboratory toxicity tests, which do not address sensitivities of mayflies at different larval stages. The lab-reared mayfly *Neocloeon triangulifer* (McDunnough, 1931) has emerged as one of the very few useful aquatic insect models for studying the effects of environmental stressors, including salinity, in the laboratory. Here, we asked if different larval life stages are differentially sensitive to ion concentrations by conducting traditional 96-h toxicity tests with NaCl, CaCl<sub>2</sub>, and Ca/MgSO<sub>4</sub>. We used a general linear model to determine if survivorship differed among larval stages as well as ion type and concentration. We also calculated median lethal concentrations (LC<sub>50</sub>) for each larval stage. Larval sensitivity to NaCl decreased slightly with age (2–6, 9–13, and 17–21 d, with LC<sub>50</sub> values of 401, 441, and 570 mg/L, respectively, when expressed as Na concentrations). Similarly, larval sensitivity to Ca/MgSO<sub>4</sub> differed slightly among age groups (LC<sub>50</sub> = 5748, 1503, and 1439 mg/L, respectively, when expressed as SO<sub>4</sub> concentrations). Reliable confidence intervals on LC<sub>50</sub> values for CaCl<sub>2</sub> could not be calculated because of high survivorship. However, our general linear model revealed that age played a moderate role in survival ( $p = 0.0065$ ) across all salts of interest. To assess the potential changes in ion flux between larval stages, we used radiotracers (<sup>22</sup>Na, <sup>35</sup>SO<sub>4</sub>, or <sup>45</sup>Ca) in 18- and 25-d-old larvae and found no strong differences in ion uptake rates. We also qualitatively examined morphological differences between larval life stages, including the appearance of gills and number of ionocytes. Our results indicate that younger *N.*

*triangulifer* larvae may be more sensitive to major ions than mature larvae. These results should be considered when experimentally using larger, late-stage *N. triangulifer* larvae to study the physiological effects and acute toxicity of salinity.

## Introduction

Many freshwater ecosystems are becoming saltier worldwide (Canedo-Arguelles et al. 2016, Kaushal et al. 2018). Anthropogenic activities such as resource extraction, irrigation runoff, and road deicing are the main contributors to salt pollution in freshwaters (Pond et al. 2008, Entrekin et al. 2011, Kefford et al. 2016). However, drought and seawater intrusions also play a role in altering major ion concentrations in freshwater ecosystems (Kinzelbach et al. 2003, Barlow and Reichard 2010, Mosley 2017). Ecologists have observed sensitive aquatic organisms disappearing in affected areas (Pond et al. 2008, Entrekin et al. 2011), but mitigation is unlikely without governmental water quality standards for salinity.

The only current federal water quality criteria for salinity in the United States is for a single ion: chloride (USEPA 1988), which does not sufficiently protect aquatic life (Pond et al. 2008). Because water-quality criteria still rely on outdated approaches, such as an amalgamation of single-species tests (Stephan et al. 1985), it is important to broaden the array of suitable test organisms to address specific ecological scenarios (Buchwalter et al. 2017). For example, the use of laboratory experiments on established model species, such as *Ceriodaphnia dubia* (Richard, 1894) (Armstead et al. 2016), have been inappropriately applied to assess the safety of total dissolved solids (TDS) pollution in systems with declining mayfly populations (Pond et al. 2008). Chironomids are also widely used as model species, but species in this family are typically tolerant of many environmental stressors (Buchwalter et al. 2004, Hassell et al. 2006, Raby et al. 2018). No single species is a perfect representation of the extreme biodiversity found among freshwater organisms (Dijkstra et al. 2014), but a more ecologically defensible toxicity model species for total dissolved solids pollution in streams is needed (Sibley et al. 2020).



Because there are knowledge gaps in the toxicological responses to salinity in sensitive aquatic insects, our lab, among others, has been working to develop a lab-reared model organism, *Neocloeon triangulifer* (McDunnough, 1931). This small baetid mayfly performs well under laboratory conditions and is a parthenogenetic species with an ~25-d larval lifespan at room temperature (21–23°C; Sweeney and Vannote 1984). This model species has already proven useful to learn about major ion sensitivity (Johnson et al. 2015, Soucek and Dickinson 2015, Buchwalter et al. 2018, Jackson and Funk 2019, Orr and Buchwalter 2020), ion transport rates (Poteat and Buchwalter 2014, Scheibener et al. 2017, Orr and Buchwalter 2020, Orr et al. 2021), and gene expression patterns in response to abiotic stressors (Kim et al. 2017, Chou et al. 2020, Orr et al. 2021). However, the degree to which different larval life stages (Figs 3.1A–C, 2A–C) affect sensitivity and physiological endpoints, such as ion uptake rates, remains unclear.

Importantly, many aquatic insect species exhibit greater sensitivity and mortality as juveniles than as adults (Nebeker et al. 1984, Gosselin and Qian 1997, Buchwalter et al. 2004, Mebane et al. 2008). For example, the freshwater shrimp *Halocaridina rubra* (Holthuis, 1963) demonstrates molecular developmental changes in salinity tolerance and energy acquisition across life stages (Havird and Santos 2016). Few studies, however, have examined age differences in sensitivity of aquatic insects, likely because of the difficulty of culturing aquatic insects in the laboratory. Some mesocosm studies have examined the impact of metals across multiple differently-aged aquatic insect taxa (Kiffney and Clements 1996, Clark and Clements 2006, Clements et al. 2013). Other studies have found increased sensitivity in early mayfly instars to orthophosphate and fine sediment (*Serratella ignita* Poda, 1761; Everall et al. 2018) and zinc (*Baetis tricaudatus* Dodds, 1923; Cadmus et al. 2020).

In this study, we aimed to assess the sensitivity of *N. triangulifer* to salinity at different stages of development. We hypothesized that *N. triangulifer* larvae would be more sensitive at early-stage development because of previous findings in other aquatic taxa. Because earlier work demonstrates a direct relationship between ion flux rates and toxicity (Orr and Buchwalter 2020), we also investigated differences of ion uptake rates between larvae of different age groups. We hypothesized that younger larvae may have increased ion flux because of their small surface area to volume ratio. Finally, we discuss the need to establish and standardize an appropriate mayfly model to protect freshwater ecosystems from salinization.

## Methods

To assess differences in the sensitivity of *N. triangulifer* to salinization at different stages of development, we performed a series of 96-h toxicity tests with 3 different salts (NaCl, CaCl<sub>2</sub>, and MgSO<sub>4</sub>/CaSO<sub>4</sub>) across multiple larval stages to reflect the predominate total dissolved solids issues in different systems. Then, we recorded survival after 96-h for each test ( $n = 3-6$ ), performed a general linear model analysis to assess effects of life stage, ion treatment, and ion concentration on survival, and calculated median lethal concentrations (LC<sub>50</sub>) values for each salt and larval stage. For SO<sub>4</sub> results, we used previous chronic data to calculate acute-to-chronic ratios (ACR), which represent the relationship between modes of action of acute and chronic toxicity. Additionally, we characterized ion uptake rates in time course experiments using radiotracers for Na, Ca, and SO<sub>4</sub> in 2 larval stages to help explain our toxicity observations ( $n = 6$ ; 12, 18, 24 h for Ca and 3, 6, 9 h for Na and SO<sub>4</sub>). Then, we conducted a student's *t*-test to assess differences between calculated uptake rates.

### *Mayfly culture*

We reared mayflies in the laboratory to obtain populations at different stages of larval development based on age. *Neocloeon triangulifer* larvae were originally obtained from the White Clay Creek in Pennsylvania, USA, (Clone WCC-2) by our collaborators at the Stroud Water Research Center (Avondale, Pennsylvania) (Sweeney and Vannote 1984). We reared mayfly larvae in room temperature (21–23°C) control water (artificial soft water [ASW]) or very soft water [VSW; Table 3.1), which were made with recipes obtained from the United States Environmental Protection Agency (D. Mount, Environmental Protection Agency, Duluth, Minnesota, 2017, personal communication). All water was made using pure distilled water (18.0

megohm) with laboratory-grade salts (Thermo Fisher Scientific, Waltham, Massachusetts). Rearing took place on the bench top in 200-mL glass Pyrex® dishes (South Greencastle, Pennsylvania) with a 14:10-h light:dark photoperiod. We lightly aerated rearing dishes to provide highly oxygenated water and covered them in parafilm to prevent evaporation. We fed mayflies natural periphyton ad libitum on acrylic plates ( $6.5 \times 23 \times 0.15$  cm).

### *Ionocyte staining*

We stained live *N. triangulifer* larvae to visualize ionocytes. After isolating a single larva onto a glass microscope slide, we applied several drops of 2% AgNO<sub>3</sub> solution. After resting in direct light for ~2 m, we imaged mayflies on a MZ 16F stereoscope (Leica, Wetzlar, Germany) or BX41-P light microscope (Olympus Life Science, Center Valley, Pennsylvania). For visualization of late-stage larvae, we followed the same method but removed gills and imaged them separately.

### *Toxicity tests*

We made experimental waters for toxicity tests with a base of ASW for Na and Ca or VSW for SO<sub>4</sub>. For convenience, we describe our waters by the 3 major ions (Na<sup>+</sup>, Ca<sup>2+</sup>, and SO<sub>4</sub><sup>-</sup>), but we acknowledge that the companion ions associated with these salt additions may also contribute to toxicity. Control waters contained low levels of major ions: 15 mg/L Na (ASW), 12 mg/L Ca (ASW), and 23 mg/L SO<sub>4</sub> (VSW) (Table 3.1). We amended Na and Ca waters with NaCl and CaCl<sub>2</sub>, respectively. We made sulfate waters with a blend of both CaSO<sub>4</sub> and MgSO<sub>4</sub> because of relatively low solubility of CaSO<sub>4</sub> and to reduce the likelihood of cation effects. We chose a series of concentrations for each major ion of interest based on previous environmental

and laboratory assessments: NaCl: 15 (ASW), 205, 280, 387, 535, or 743 mg/L Na; Ca/MgSO<sub>4</sub>: 23 (VSW), 378, 630, 1050, 1750, or 2500 mg/L SO<sub>4</sub>; CaCl<sub>2</sub>: 12 (ASW), 127, 212, 352, 588, or 980 mg/L Ca. All waters were filtered through nylon 0.45- $\mu$ m syringe filters (Thermo Fisher Scientific) into sterile 15-mL test tubes (Olympus Life Science). Several major ion concentrations were verified by North Carolina State University's Environmental and Agriculture Testing Services Lab with inductively coupled plasma mass spectrometry and were within 10% of nominal values, except 743 mg/L Na, which was 21% higher than expected (Table 3.1). Because of the expensive nature of this technique, only some of the relevant major ions were measured. We used an Orion 5-Star™ Benchtop pH meter (Thermo Fisher Scientific) to measure the pH of all waters, which were in the expected range of 6.91 to 7.89 (Table 3.1).

To assess survival across different concentrations of major ions, we performed acute 96-h toxicity tests in sterile 6-well plates. The toxicity test experiments were conducted with different ages of *N. triangulifer* (2–6, 9–13, 17–21, and 23–27-d old, with the 23–27 d group for CaCl<sub>2</sub> experiments only). Four larval life stages were tested for CaCl<sub>2</sub>, but not other salts of interest, because of numbers of available animals. Our toxicity test protocol has been previously described in detail (Orr and Buchwalter 2020, Orr et al. 2021), but here we give a brief summary. We filled wells ~75% full (8 mL) with experimental solution. For each age group, we seeded 10 *N. triangulifer* larvae into each well ( $n = 3$  wells/treatment) with a glass pipette and a Leica MZ 16 F stereoscope. Then, we added 200  $\mu$ L of diatom slurry prepared in each exposure concentration as food. This procedure maintained the desired salinity treatment regimes. We kept the plates spatially randomized in an incubator at 21°C. We monitored and aerated the plates (60 s/well) daily. We performed a 50% water change after 48 h to ensure no excess debris and waste accumulated. We measured survivorship in each well after 96 h.

We used a general linear model (Prism, version 9.0.1; GraphPad Software, La Jolla, California) to assess how strongly survivorship varied with ion type, ion concentration, and age group (2–6, 9–13, or 17–21 d). Survivorship data were arcsine transformed prior to analysis. Ion concentrations were expressed in mM. We verified normality of model residuals based on visual examination of a Q-Q plot and a Shapiro–Wilk test ( $p = 0.10$ ). The model was first fit with all possible interaction terms as:  $\text{survival} = f(\text{ion} + \text{concentration} + \text{age} + \text{conc:age} + \text{conc:ion} + \text{age:ion} + \text{age:conc:ion})$ . Then, because the parameter estimates for all interaction terms had  $p > 0.05$ , we dropped them from the model. Because our data were unbalanced, we intentionally chose Type II sums of squares, which is appropriate for models without interactions and only main effects (Hector et al. 2010).

We calculated  $LC_{50}$  values as concentrations of  $Na^+$  for NaCl tests or  $SO_4^-$  for Ca/Mg $SO_4$  tests and estimated 95% confidence intervals for each ion treatment with Toxicity Relationship Analysis Program (TRAP) software (version 1.30a; United States Environmental Protection Agency, Mid-Continent Ecology Division, Duluth, Minnesota), which incorporates classic probit analysis ( $n = 3–6$  for each treatment within each larval age group) (Erickson 2010). In the Ca toxicity experiment, we were unable to calculate  $LC_{50}$  values because of high survivorship.

In addition, we calculated the approximate ACR for  $SO_4$  based on previous chronic  $SO_4$  data and our  $LC_{50}$  values. First, we used previous chronic data in *N. triangulifer* (Buchwalter et al. 2018) to calculate the chronic value (ChV) by taking the geometric mean of the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC). Then, using our  $LC_{50}$  data for  $SO_4$ , we calculated ACR using the equation  $ACR = LC_{50}/ChV$  for each larval life stage. These ACR values are only approximate because the  $LC_{50}$  and ChV values used to

calculate the ACR values were determined in separate, independent tests instead of in the more traditional concurrent, paired acute and chronic toxicity tests.

### *Ion flux experiments*

To assess the effect of larval stage on ion flux rates, we conducted ion flux experiments on 2 ages of mayfly larvae. We reared mayfly hatchlings from the same cohort in control water until they reached 18 or 25 d old. We chose these age groups based on our ability to experimentally work with the smallest (18 d old) and largest (25 d old) size possible. We used a Leica MZ 16 F stereoscope with Leica camera and Leica Application Suite X software (version 4.13) to measure lengths of *N. triangulifer* larvae. We made radioactive waters in ASW with  $^{45}\text{CaCl}_2$  or dual-labeled with  $^{22}\text{NaCl}$  and  $\text{Na}_2^{35}\text{SO}_4$  (PerkinElmer®, Billerica, Massachusetts) with exposure activities ranging from 156 to 260 Bq/mL. We measured experimental waters with an LS6500 multipurpose scintillation counter (Beckman Coulter, Brea, California). Experiments were performed in clean, acid-washed, 100-mL high-density polyethylene beakers with 20 mL of experimental water that were gently aerated and sealed with ParaFilm™ M Wrapping Film (Thermo Fisher Scientific) to prevent evaporation. Each experiment had 3 mayflies in each of 6 replicate chambers spatially randomized for each of the 3 time points (12, 18, 24 h for Ca and 3, 6, 9 h for Na and  $\text{SO}_4$ ). Relatively short time points are required to capture unidirectional uptake rates, as described previously (Orr and Buchwalter 2020). Longer time points for Ca were required because Ca uptake is physiologically much slower than Na or  $\text{SO}_4$ .

At the designated experimental time points, we removed mayflies from the radioactive water and prepared them for radioactivity analysis. First, we rinsed removed mayflies in 2 consecutive baths of clean water to remove any adsorbed ions from exoskeletons. For the Ca

experiments, mayflies were additionally rinsed with freshly made 0.05 M ethylenediaminetetraacetic acid and 0.1 M L-ascorbic acid Na salt to remove adsorbed Ca on the exoskeleton (Poteat and Buchwalter 2014). We then blotted mayflies dry with a tissue, weighed them, and digested them in a 20-mL glass vial with 500  $\mu$ L of Soluene<sup>®</sup> 350 (PerkinElmer) for 48 h in a dark, 28°C incubator. After digestion, we neutralized samples with 500  $\mu$ L of glacial acetic acid. To quantify radioactivity, we added 12 mL of scintillation cocktail (PerkinElmer Ultima Gold<sup>™</sup> uLLT) before measuring radioactivity of samples (counts/min) for 3 min each with the Beckman LS6500 multipurpose scintillation counter. We corrected all measurements for quench, which is the interference of sample and cocktail characteristics on the radioactivity quantification. Only measurements with counting error values <10% and lumex (non-radioactive luminescence) values <5% were included for analysis, which has historically been our lab's conservative data quality threshold for quantifying radioactivity.

We calculated ion uptake rates by normalizing the amount of radiolabeled ion uptake to the wet mass of tissue to account for differences in mass among the replicates. Next, we performed a simple linear regression of mass-normalized ion uptake on time in the Prism software and used the positive slope of the regression as the ion uptake rate. We assessed all models for normality of residuals through visual examination of Q-Q plots. Then, we used Student's *t*-tests to determine if uptake rates varied between larval stage (18 or 25 d) for each ion.



## Results

### *Toxicity tests*

Mayfly survival generally decreased with increasing major ion concentration for all 3 salts of interest (Fig. 3.3A–C). For NaCl, life stages had LC<sub>50</sub> values of 401 mg Na/L (2–6 d), 442 mg Na/L (9–13 d), and 570 mg Na/L (17–21 d) (Table S1, Fig. 3.4A). Mayfly survival decreased at the highest Ca concentration across all 4 larval life stages (Table S3, Fig. 3.3C). Because of high survival, we were unable to calculate LC<sub>50</sub> values. Mayfly survival also generally decreased with increasing SO<sub>4</sub> concentration (Table S2, Fig. 3.3B). The 3 different larval life stages had LC<sub>50</sub> values of 749 mg SO<sub>4</sub>/L (2–6 d), 1503 mg SO<sub>4</sub>/L (9–13 d), and 1439 mg SO<sub>4</sub>/L (17–21 d) (Table S2, Fig. 3.4B). Based on previous chronic SO<sub>4</sub> work in *N. triangulifer*, we were able to use the NOEC (444 mg SO<sub>4</sub>/L) and the LOEC (667 mg SO<sub>4</sub>/L) to calculate the ChV (544 mg SO<sub>4</sub>/L) based on mean days to emergence (Buchwalter et al. 2018). Then, using the current LC<sub>50</sub> data from each of the 3 life stages, we calculated approximate ACR (LC<sub>50</sub>/ChV) as 1.4, 2.8, and 2.6, respectively.

Based on the general linear model results (Table 3.2, model  $R^2 = 0.71$ ), all 3 variables appeared to influence mayfly survival (ion:  $p < 0.0001$ ; age:  $p = 0.0065$ ; concentration:  $p < 0.0001$ ). However, the effects of ion and concentration were much stronger than the effects of age.

### *Ion flux experiments*

*Neocloeon triangulifer* larvae had similar ion uptake rates at 18 and 25 d old. Larvae were  $1.57 \pm 0.04$  and  $3.26 \pm 0.12$  mg wet mass ( $n = 54$ ,  $p < 0.0001$ ) at 18 and 25 d old, respectively. For 18- and 25-d-old larvae, Na uptake rates were  $49.1 \pm 5.8$  and  $39.3 \pm 3.2$   $\mu\text{g g}^{-1}$

$\text{h}^{-1}$ ,  $\text{SO}_4$  uptake rates were  $5.7 \pm 3.0$  and  $8.1 \pm 1.8 \mu\text{g g}^{-1} \text{h}^{-1}$ , and Ca uptake rates were  $5.3 \pm 0.1$  and  $4.5 \pm 0.6 \mu\text{g g}^{-1} \text{h}^{-1}$ , respectively (Fig. 3.5). Student's  $t$ -tests revealed that larval stage did not substantially affect ion uptake rates for Na ( $p = 0.17$ ),  $\text{SO}_4$  ( $p = 0.53$ ), or Ca ( $p = 0.22$ ).

## Discussion

In this study, we aimed to uncover differences of major ion sensitivity between larval stages of the mayfly *N. triangulifer*. We found that for all 3 salts (NaCl, Ca/MgSO<sub>4</sub>, and CaCl<sub>2</sub>) older larvae were slightly more tolerant than younger larvae. Further, we found weak differences in ion transport rates of Na, SO<sub>4</sub>, and Ca in 2 differently-aged groups of larvae. These results have implications for establishing water-quality criteria for salinity in freshwater ecosystems and point to the need to establish a model organism (*N. triangulifer*) to improve our understanding of aquatic insect osmoregulation and sensitivity.

### *Freshwater salinization*

Freshwater salinization is an emerging issue that merits scientific attention and regulatory action to mitigate ongoing harmful ecological impacts (Pond et al. 2008, Stepanian et al. 2020). Previous work has demonstrated the importance of assessing ion-specific effects on physiology and sensitivity rather than effects of total salinity. For example, one study found that waters with similar conductivities but different major ion compositions had different toxicities on aquatic organisms (Kunz et al. 2013). Another study showed that major ions had different mechanisms of toxicity in *N. triangulifer* (Orr et al. 2021). This information is environmentally relevant because different scenarios increase the concentration of different major ions. For example, we would expect increased Na, Mg, and Cl ions in freshwaters that are polluted by road deicing in urban areas with colder climates. Alternatively, mountaintop coal mining operations in West Virginia, USA, often cause increases in SO<sub>4</sub> and Ca ions in nearby freshwater systems (Pond et al. 2008, Cormier et al. 2013, Jackson and Funk 2019).

### *Aquatic insects*

Aquatic insects are disproportionately affected by increases in major ion concentrations (Pond et al. 2008, Griffith 2017) compared with other aquatic organisms, but their sensitivity is not well understood. Chemical benchmarks for altered major ion concentrations have been developed from field surveys of aquatic invertebrate communities (Cormier and Suter 2013a, b) but are not yet legally enforceable. Because these approaches cannot determine mechanisms of toxicity nor identify which part of the life cycles of sensitive aquatic insects are affected, it is important that we develop a better understanding of how TDS affects aquatic organisms. Sensitive aquatic organisms, such as mayflies, are challenging to study because of their intolerance of laboratory conditions. However, the development of *N. triangulifer* as a lab-reared model organism has opened many doors to ecotoxicology research in sensitive aquatic insects (Sweeney and Vannote 1984, Sweeney et al. 1993). Scientists can rear this species through its entire life cycle in laboratory conditions and produce valuable chronic toxicity data for salts and other stressors (Soucek and Dickinson 2015, Buchwalter et al. 2018, Jackson and Funk 2019, Chou et al. 2020). Here, we used this developing model organism, *N. triangulifer*, and produced valuable toxicity data for 3 different water chemistries. Our results are an important contribution to the growing body of data on salinity stress in aquatic life, taking into consideration both larval life stage and different major ions.

### *Acute and chronic toxicity to major ions*

We examined 3 different salts of interest because previous findings have demonstrated clear physiological and toxicological differences between major ions (Kunz et al. 2013, Scheibener et al. 2017, Orr and Buchwalter 2020, Orr et al. 2021). Larvae tolerated  $\text{Cl}^-$

concentrations as high as 933 mg/L in the CaCl<sub>2</sub> treatments, which leads us to speculate that Na is driving NaCl toxicity. We presume that Ca is the driver of CaCl<sub>2</sub> toxicity because the larvae tend to maintain a very low uptake rate of Ca, and we have observed histological changes in Malpighian tubules (Orr et al. 2021). Our concentration choices were made based on previous laboratory results and environmental values, and our acute toxicity data is relatively consistent with the literature. Recently, our group calculated LC<sub>50</sub> values for Na and Ca with ~10- to 17-d-old *N. triangulifer* larvae in similar water chemistries: 1169 mg Na/L and 901 mg Ca/L, respectively (Orr et al. 2021). Other groups have calculated LC<sub>50</sub> values for SO<sub>4</sub> (1227 mg/L; Soucek and Dickinson 2015) and NaCl (2755 mg/L, equivalent to 1084 mg N/L; Jackson and Funk 2019). Some potential explanations for differing results between studies include different natural periphyton quality based on seasons, different cohorts, or a combination of both. Our results emphasize the importance of studying salts of major ions individually rather than using total salinity or conductivity as a surrogate in salinity research. It is important to note, however, that animals are exposed to several major ions (among other stressors) in nature and that further work should be done with different combinations of relevant salts to improve our understanding of ionic interactions.

Historically, the United States has relied upon ACRs in ecological risk assessment to estimate chronic toxicity of chemicals for aquatic species that lack chronic data (Stephan et al. 1985, Raimondo et al. 2007). Normally, ACRs are calculated as a ratio of the median LC<sub>50</sub> and the ChV, which can only be done for species that have both acute and chronic data (Raimondo et al. 2007). Thus, a larger ACR value (>10) would indicate different modes of action between acute and chronic exposures. Here, our calculated approximate ACR values were low, suggesting major ions elicit similar mechanisms of toxicity for both acute and chronic exposures in this

species. Using similar endpoints, one study found ACRs ranging from 2.3 to 8.5 for  $\text{SO}_4$  in this species (Soucek and Dickinson 2015). The differences may be explained by variances in nutritional provisions, ionic compositions of the water, or a combination of both. Soucek and Dickinson (2015) used laboratory-cultured diatoms and  $\text{NaSO}_4$  rather than natural periphyton and a blend of  $\text{CaSO}_4$  and  $\text{MgSO}_4$ . At least some of the variability in the ACR values might also be a result of determining the  $\text{LC}_{50}$  values in a separate, independent study from that used to determine the ChV values. Soucek and Dickinson (2015) also calculated ACRs for  $\text{Cl}^-$  (2.1–6.4) and nitrate (2.5–5.1) in *N. triangulifer*, using  $\text{NaCl}$  and  $\text{NaNO}_3$ , respectively (Soucek and Dickinson 2015). Additional chronic toxicity tests are needed to fully characterize the toxicity of the salts of different major ions and then develop predictive toxicity models.

#### *Ion uptake rates*

Previous work has demonstrated the highly concentration-dependent and ion-specific nature of ion uptake rates and, further, that increased ion flux rates are associated with increased toxicity (Orr and Buchwalter 2020, Orr et al. 2021). We hypothesized that smaller animals may have greater ion flux rates because of their increased surface area-to-volume ratio. Here, we observed that basal ion uptake rates in control water remain unchanged between 18- vs 25-d-old larvae from the same cohort. This result aligns with the marginal differences in  $\text{LC}_{50}$  values observed between differently-aged larvae in this study.

#### *Life-stage sensitivity*

Aquatic insects develop through various life stages and may be more sensitive to metals and other toxicants during transitional stages that face steep energetic demands. Research on

other aquatic insects has demonstrated greater sensitivity to toxicants in the transitional stages, including the last molting stage and metamorphosis (Palmquist et al. 2008, Schmidt et al. 2013, Wesner et al. 2017, Wesner 2019). Similarly, we have often observed mortality in large, late-stage *N. triangulifer* larvae soon before emergence in chronic salinity experiments (SEO, DBB, personal observations, unpublished), which may suggest that energy budget, rather than size, determines sensitivity. A previous study demonstrated that pre-exposure of *N. triangulifer* to elevated, but subtoxic, SO<sub>4</sub> concentrations stimulated an acclimatory response (reduction in SO<sub>4</sub> uptake rates) but also increased subsequent toxicity (Orr et al. 2021). Molting drastically disrupts breathing and metabolic rate in this species (Camp et al. 2014), and greater mortality has been observed at the penultimate larval stage of a chronic NaCl exposure, emphasizing the energetic demand of metamorphosis combined with stressful salinities (Soucek and Dickinson 2015). Future studies should focus on fully understanding the energy requirements and increased sensitivity of the transition from the penultimate larva to subimago in *N. triangulifer*.

#### *Neocloeon triangulifer* as a model species

Nutrition is an important consideration when using *N. triangulifer* as a model species. Previous work has shown that nutrition can alter selenium toxicity in this species (Conley et al. 2011). Many groups have reared *N. triangulifer* on natural periphyton (Jackson and Funk 2019, Orr and Buchwalter 2020, Orr et al. 2021), whereas others have used laboratory-grown diatoms (Weaver et al. 2014, Soucek and Dickinson 2015, Raby et al. 2018). A more heterogeneous, natural mixture of periphyton may provide better nutrition but is impossible to standardize. On the other hand, cultured diatoms may not provide optimal nutrition but may better reflect ecological realities in some settings. Because we posit that high TDS impose energetic costs to

developing larvae, we recommend that researchers using *N. triangulifer* report the mass of suitably sized controls when possible so that results can be interpreted in the context of nutritional status.

Further, this species is relatively small compared with other species of mayflies and aquatic insects (Jackson and Funk 2019), which affects their use in toxicity tests. Morphologically, these animals undergo major changes from hatchlings with no gills and few ionocytes to the penultimate larval stage with 14 large gills and thousands of ionocytes concentrated on the medial part of the gills (Fig. 3.2A–C). Thus, it appears that the number of cells is commensurate with size of the larvae. Notably, most stages of this species' larval life cycle can only be worked with experimentally using a microscope. For these reasons, we often use mature larvae close to the end of their larval cycle for ease of use and to maximize biomass, which is important in radiotracer studies that use a minimal amount of radioactivity for safety concerns (Scheibener et al. 2017, Orr and Buchwalter 2020), among other experiments. It is clear from our results that mature larvae are slightly more tolerant of major ions and, thus, experimental larval stage should be carefully considered in future studies. However, it appears that the egg stage of *N. triangulifer* is relatively intolerant to salinity stress (D. Funk, Stroud Water Research Center, Avondale, Pennsylvania, 2021, personal communication). We speculate that early larval life stages in aquatic organisms may be more sensitive because of greater surface area-to-volume ratios, faster turnover rate of essential ions, and underdeveloped antioxidant and immune systems.

In this study, we tested for sensitivity differences between larval life stages of *N. triangulifer* in a laboratory setting, but we acknowledge that acute toxicity tests, conducted in the absence of other biotic and abiotic stressors, are not a reasonable proxy for insect sensitivity in



nature (Kefford et al. 2004, Hassell et al. 2006, Vellemu et al. 2017). However, lab-based approaches allow us to perform controlled experiments to isolate the effects of different major ions and broaden our understanding of how they affect survivorship and other physiological endpoints. We found apparent trends of slightly increased major ion sensitivity in younger larvae. We also found weak differences in Na, SO<sub>4</sub>, or Ca uptake rates between 2 larval stages of *N. triangulifer*. Additional in-situ stressors (e.g., temperature, weathering patterns, predation, food limitation) may exacerbate major ion toxicity. In the future, experimental studies will work in conjunction with field studies to understand the individual effects of ions on aquatic insects and the combined effects of salinization with interacting environmental stressors. Moving forward, it is clear that larval stage should be an important consideration when planning experiments studying the physiological effects and toxicity of salinity with *N. triangulifer*.

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Author contributions: SEO oversaw and helped conduct the research, analyzed the data, and wrote the manuscript. JKC, IGW, RWG, and GEO helped conduct the research. DBB conceived the work and provided editorial assistance.

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**Figure 3.1** Larval stages of *Neocloeon triangulifer*. One-d-old hatchling (A), ~20-d-old larvae (B), and ~25-d-old larvae with dark wing pads (white arrow) (C).

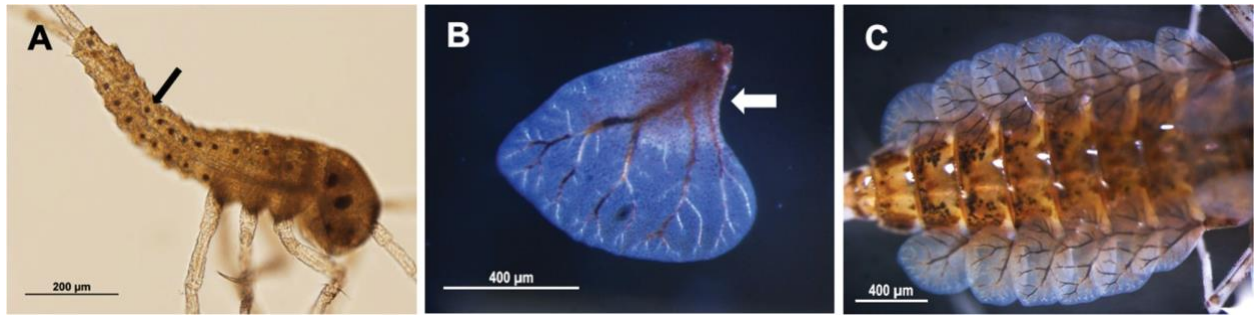
**Table 3.1** Water chemistries (mg/L) for all experimental solutions. All waters had several ions verified by inductively coupled plasma mass spectrometry.

Measured concentrations are listed in parentheses next to nominal concentrations. AWS = artificial soft water, VSW = very soft water.

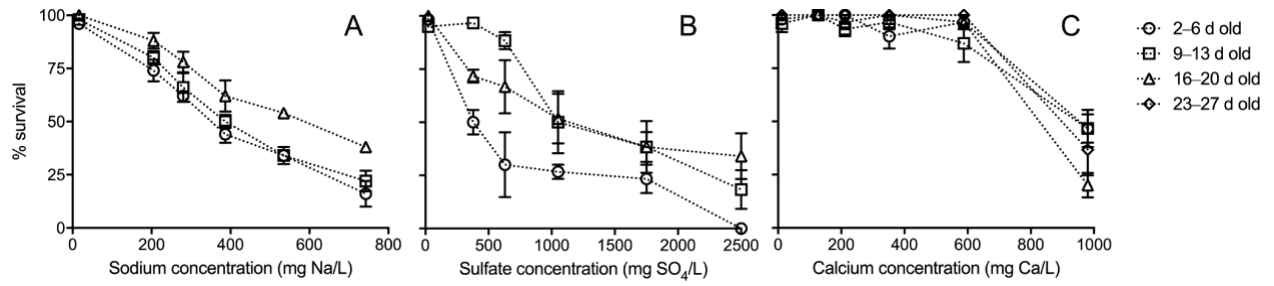
Solution	Na		Ca		Mg		K		S		SO <sub>4</sub>	Cl	CO <sub>3</sub>	pH
	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured				
Control (ASW)	15.3	15.5	12.7	11.6	3.4	3.3	1.4	1.9	7.78	7.9	23.3	14.1	42.6	7.0
Control (VSW)	15.3	15.3	4.1	4.68	0.9	0.9	1.4	–	1.8	1.9	5.4	4.6	12.3	7.8
127 Ca	15.3	15.3	127	124	3.4	3.3	1.4	–	7.78	7.9	23.3	201	42.6	7.3
212 Ca	15.3	14.7	212	203	3.4	3.8	1.4	–	7.78	7.1	23.3	335	42.6	7.3
352 Ca	15.3	15.1	352	330	3.4	3.3	1.4	–	7.78	7.5	23.3	559	42.6	7.1
588 Ca	15.3	15.5	588	546	3.4	3.6	1.4	–	7.78	7.8	23.3	933	42.6	7.0
980 Ca	15.3	15.5	980	915	3.4	3.8	1.4	–	7.78	7.9	23.3	1555	42.6	6.9
205 Na	205	228	12.7	11.56	3.4	–	1.4	–	7.78	7.9	23.3	334	42.6	7.7
280 Na	280	298	12.7	12.26	3.4	–	1.4	–	7.78	8.7	23.3	452	42.6	7.8
387 Na	387	414	12.7	11.69	3.4	–	1.4	–	7.78	8.2	23.3	615	42.6	7.6
535 Na	535	559	12.7	11.83	3.4	–	1.4	–	7.78	8.7	23.3	844	42.6	7.8
743 Na	743	915	12.7	11.99	3.4	–	1.4	–	7.78	8.6	23.3	1165	42.6	7.9
378 SO <sub>4</sub>	15.3	16.6	53.2	52.6	63.9	60.9	1.4	–	126	128	378	4.6	12.3	7.5
630 SO <sub>4</sub>	15.3	16.8	88.7	85.1	107	103.0	1.4	–	210	212	630	4.6	12.3	7.4
1050 SO <sub>4</sub>	15.3	17.0	148	143.6	178	178.1	1.4	–	351	365	1050	4.6	12.3	7.4
1750 SO <sub>4</sub>	15.3	17.4	246	232.5	296	291.4	1.4	–	585	601	1750	4.6	12.3	7.3
2500 SO <sub>4</sub>	15.3	17.1	351	327.0	423	414.5	1.4	–	836	862	2500	4.6	12.3	7.3

**Table 3.2** General linear model analysis of the effects of ion type, larvae age, and ion concentration on survivorship of *N. triangulifer* larvae.

Analysis of variance	SS	df	MS	<i>F</i>	<i>p</i>
Regression	47.27	5	9.454	111.4	<0.0001
Ion	7.67	2	3.833	45.18	<0.0001
Age	0.87	2	0.437	5.15	0.0065
Concentration	34.08	1	34.08	401.6	<0.0001
Residual	19.60	231	0.084	–	–
Total	66.87	236	–	–	–

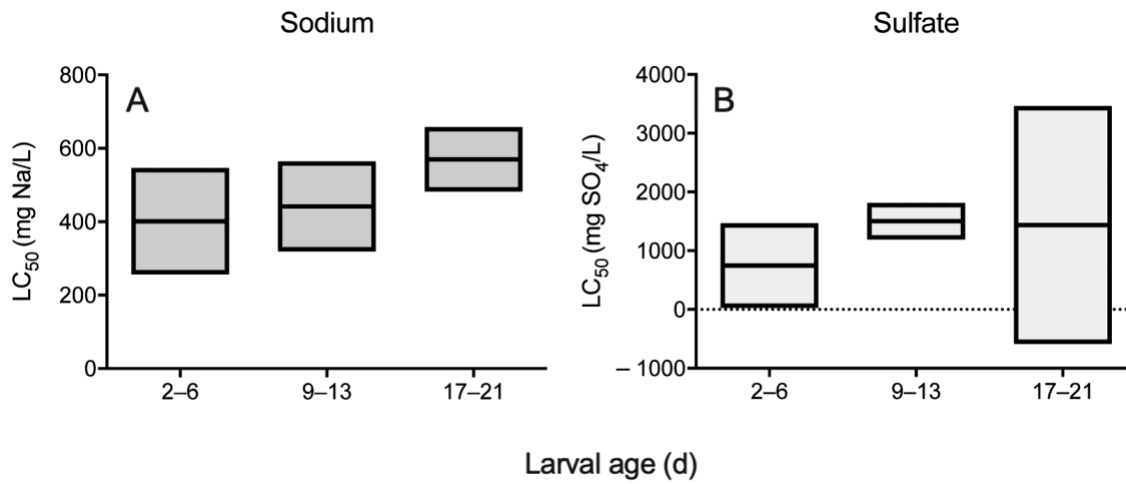


**Figure 3.2** Ionocyte staining of *Neocloeon triangulifer* larvae. One-d-old larva without gills with several large ionocytes along the thorax (black arrow) and abdomen (A), a single gill plucked off a mature (~25 d old) larva, revealing hundreds of ionocytes concentrated at the medial part of the gill (white arrow) (B), and the abdomen of a mature larvae with 14 large gills (C).

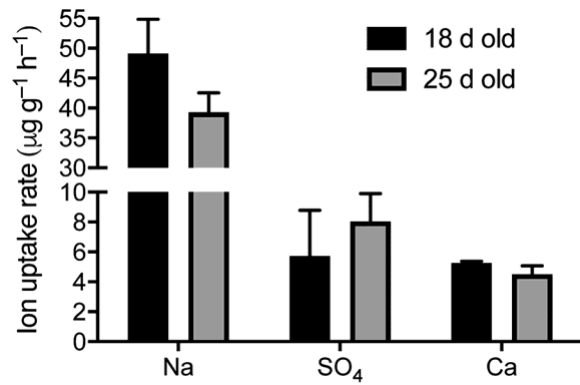


**Figure 3.3** % survival of different ages of *Neocloeon triangulifer* larvae after 96 h of exposure to sodium (NaCl)

(A), sulfate (a mixture of CaSO<sub>4</sub> and MgSO<sub>4</sub>) (B), or calcium (CaCl<sub>2</sub>) (C). Error bars represent SE of the mean.



**Figure 3.4** Calculated median lethal concentrations (LC<sub>50</sub> values) for 3 different ages of *Neocloeon triangulifer* larvae exposed to sodium in NaCl exposures (A) or sulfate in Ca/MgSO<sub>4</sub> exposures (B). Error bars represent 95% confidence limits.



**Figure 3.5** Ion uptake rates in artificial soft water (ASW) of *Neocloeon triangulifer* larvae at 18 d and 25 d old ( $n = 6$ ;  $p = 0.17$ ,  $p = 0.53$ , and  $p = 0.22$  for Na, SO<sub>4</sub>, and Ca, respectively). Error bars represent the SE of the mean. Note the break and change in scale of the y-axis.



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## CHAPTER FOUR

### **PHYSIOLOGICAL PLASTICITY AND ACCLIMATORY RESPONSES TO SALINITY STRESS ARE ION-SPECIFIC IN THE MAYFLY, *NEOCLOEON TRIANGULIFER***

Sarah E. Orr<sup>1</sup>, Tatiane Terumi Negrão Watanabe<sup>2</sup>, and David B. Buchwalter<sup>1</sup>

<sup>1</sup>Department of Biological Sciences, North Carolina State University; <sup>2</sup>Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607

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## Abstract

Freshwater salinization is a rapidly emerging ecological issue and is correlated with significant declines in aquatic biodiversity. It remains unclear how changing salinity regimes affect the physiology of sensitive aquatic insects. We used the parthenogenetic mayfly, *Neocloeon triangulifer*, to ask how ionic exposure history alters physiological processes and responses to subsequent major ion exposures. Using radiotracers ( $^{22}\text{Na}$ ,  $^{35}\text{SO}_4$ , and  $^{45}\text{Ca}$ ), we observed that mayflies chronically reared in elevated sodium or sulfate ( $157 \text{ mg L}^{-1} \text{ Na}$  or  $667 \text{ mg L}^{-1} \text{ SO}_4$ ) had 2-fold ( $p < 0.0001$ ) and 8-fold ( $p < 0.0001$ ) lower ion uptake rates than mayflies reared in dilute control water ( $16 \text{ mg L}^{-1} \text{ Na}$  and  $23 \text{ mg L}^{-1} \text{ SO}_4$ ) and subsequently transferred to elevated salinities, respectively. These acclimatory ion transport changes provided protection in 96-hour toxicity bioassays for sodium, but not sulfate. Interestingly, calcium uptake was uniformly much lower and minimally influenced by exposure history, but was poorly tolerated in the toxicity bioassays. With qRT-PCR, we observed that the expression of many ion transporter genes in mayflies was influenced by elevated salinity in an ion-specific manner (upregulation in response to sulfate, downregulation in response to calcium). Elevated sodium exposure had minimal influence on the same genes. Finally, we provide novel light microscopic evidence of histomorphological changes within the epithelium of the Malpighian tubules (insect primary excretory system) that undergoes cellular degeneration and necrosis secondary to calcium toxicity. We conclude that physiological plasticity to salinity stress is ion-specific and provide evidence for ion-specific toxicity mechanisms in *N. triangulifer*.

## Introduction

Many freshwater ecosystems are becoming increasingly salty throughout the world (Canedo-Arguelles et al., 2016; Kaushal et al., 2018, 2005). Various human activities such as road deicing, resource extraction, and irrigation of arid landscapes are some of the activities that contribute to elevated major ion concentrations in freshwaters (Entrekin et al., 2011; Pond et al., 2008a). Changes in precipitation patterns, drought, and seawater intrusion are additional climate driven changes to freshwater salinity regimes (Barlow and Reichard, 2010; Kinzelbach et al., 2003; Mosley, 2017). Because different environmental scenarios can lead to the elevation of specific major ions (e.g., NaCl in road-deicing, SO<sub>4</sub> and Ca in mountain-top coal mining), it is critical to understand how various ion matrices lead to different toxic effects in aquatic organisms (Griffith, 2017a; Kunz et al., 2013).

Ecologists report that sensitive aquatic organisms such as mayflies, are disproportionately affected by freshwater salinization (Beermann et al., 2018; Kefford, 2018; Pond et al., 2008a). Mayflies and other aquatic insects play an essential role in freshwater ecosystem function and serve as biological indicators (Hawkins, 2006; Kenney et al., 2009). Scientists have only recently begun to study the physiological mechanisms behind salinity stress in aquatic insects to better understand this ecological dilemma. Previous work demonstrates that ion influx rates increase with increasing concentration (Scheibener et al., 2017) and temperature (Orr and Buchwalter, 2020) in aquatic insects. However, our limited evidence suggests that these increased flux rates do not result in systemic dysregulation of the elements at the whole body level (Buchwalter et al., 2019; Scheibener et al., 2017) or alter hemolymph osmolality (Kefford, 2018; Verberk et al., 2020). Thus, our limited evidence to date points towards mayflies as strict osmoregulators and we hypothesize, at least in the case of sulfate toxicity, that bioenergetic cost of this regulation (the



ATP expense of excessive ion turnover) may explain delayed development, decreases in population fitness observed in the lab (Buchwalter et al., 2019; Hassell et al., 2006; Johnson et al., 2015; Soucek and Dickinson, 2015; Verberk et al., 2020), and ultimately the loss of species that have been observed in the field (Cormier et al., 2013b, 2013a; Pond et al., 2008b).

Extremely elevated salinities can be detrimental (Griffith, 2017a; Johnson et al., 2015), but are sensitive aquatic organisms able to acclimate to changing salinity regimes? This question has been assessed in some organisms such as daphnids (Chen and Stillman, 2012; Coldsnow et al., 2017) and a species of mayfly (Dowse et al., 2017). However, mechanisms of acclimation (e.g., flux rates of specific major ions) remain unknown in aquatic insects. We hypothesize that mayflies can acclimate by making subtle physiological changes and better survive in a stressfully salty environment over time.

Here, we capitalize on recent advances in the establishment of the baetid mayfly, *Neocloeon triangulifer* as a laboratory model (Chou et al., 2018, 2017; Funk et al., 2006; Kim et al., 2017). We ask how major ion exposure history affects subsequent physiological responses to different ionic conditions. To specifically ask if and how *N. triangulifer* can acclimate to different salinity regimes, we used radiotracers to assess how exposure history affects ion transport rates for sodium, sulfate, and calcium. We also developed a suite of RT-qPCR probes for several genes related to ion transport to assess how exposure history affects gene expression at the mRNA level. Additionally, we ask if and how these physiological changes associated with exposure history are manifested at the whole organism level by comparing the survival of larvae with different exposure histories to acute salinity challenge. Finally, we performed routine histological evaluation on mayfly larvae to assess tissue morphological changes in the important ion regulatory organ, the Malpighian tubules.

## Methods

The overarching strategy for these studies was to compare how *N. triangulifer* larvae reared under different ionic conditions respond to changing salinity regimes (See Supplementary Fig. 1). Artificial soft water (ASW) (Table 1) serves as our routine culture media, and was the control treatment for these experiments, in addition to being the base water to which all ions of interest were added. Pre-exposure concentrations (Supplementary Table 2) were intended to be challenging, but not acutely lethal to increase potential acclimatory effects. The elevated sulfate concentrations consisted of a blend of Ca- and Mg-SO<sub>4</sub> and were chosen based on previously published full life cycle studies for this ion (Buchwalter et al., 2019). The elevated sodium pre-exposure concentrations were chosen based on previously published studies of NaCl toxicity in this species (Jackson and Funk, 2019; Soucek and Dickinson, 2015). Finally, calcium pre-exposure concentrations were raised in similar magnitudes, due to lack of previous toxicity data.

### *Mayfly Husbandry*

The parthenogenetic baetid mayfly, *Neocloeon triangulifer*, was originally collected from White Clay Creek in Pennsylvania (WCC-2 clone) and gifted to us by the Stroud Water Research Center (SWRC; Avondale, PA) (Sweeney and Vannote, 1984). *N. triangulifer* larvae were reared in laboratory conditions in 4-quart glass Pyrex dishes at room temperature (21-23°C) and a 14:10 h light:dark photoperiod. Collaborators at the SWRC also gifted us natural periphyton cultured on plastic plates as nutrition for the mayfly larvae.

### *Ion Flux Experiments*

To assess the plasticity of ion transport, mayfly hatchlings were seeded into either control (ASW) or one of the three elevated salinity treatments (157 mg L<sup>-1</sup> Na, 735 mg L<sup>-1</sup> SO<sub>4</sub>, or 122 mg L<sup>-1</sup> Ca) within 24 hours of hatching. After rearing for approximately 21 days, mature larvae were placed in experimental waters to perform the transplant ion flux measurements (Fig. 4.1).

Radioactive experimental waters were made in ASW with <sup>45</sup>CaCl<sub>2</sub> or dual-labeled with <sup>22</sup>NaCl and Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> (PerkinElmer, Billerica, MA, USA) with exposure activities ranging from 156 to 260 Bq mL<sup>-1</sup>. Exposure waters were measured with the Beckman LS6500 Multipurpose Scintillation Counter (Beckman Coulter, Brea, CA) directly before the experiments. Experiments were performed in 100 mL high-density polyethylene beakers with 15 mL of exposure water, gentle aeration, and sealed with a ParaFilm™ covering. All experiments had 8 replicates (*n*=8) for each of the 3 time points used to calculate mass-specific, unidirectional uptake rates. Details of this calculation method can be found in previous publications (Orr and Buchwalter, 2020) and Supplementary Figure 2.

After each timepoint, mayflies were removed from the radioactive exposure waters, rinsed in two consecutive baths of ASW to remove any loosely adsorbed ions from the exoskeleton and weighed. In the <sup>45</sup>Ca experiments, mayflies were additionally rinsed with 0.05 M EDTA and 0.1 M l-ascorbic acid sodium salt due to the adsorptive nature of Ca on insect exoskeletons (Poteat and Buchwalter, 2014a). After rinsing, mayflies were blotted dry, weighed, and digested in 500 μL of Soluene 350 (PerkinElmer) in a 20 mL glass vial for 48 hours. Digestates were neutralized with 500 μL of glacial acetic acid and 12 mL of scintillation cocktail (Perkin Elmer Ultima Gold uLLT) was added to measure radioactivity by liquid scintillation counting. We applied appropriate

corrections for spill-over and quench, and only measurements with lumex values <5% and error rates <10% were used in analyses.

### *Gene Expression*

We examined the expression of eight unique genes in *N. triangulifer* that we hypothesized may be affected by salinity (e.g., ion transporters). The gene names and primer information are listed in Supplementary Table 2. Primers lacking accession numbers have been verified through NCBI BLAST to ensure high similarity (> 90%) between other more established species (e.g., *Aedes aegypti*, *Aedes gambiae*, and/or *Drosophila melanogaster*). *N. triangulifer* hatchlings were seeded into either control (ASW) or one of three elevated salinity (735 mg L<sup>-1</sup> SO<sub>4</sub>, 157 mg L<sup>-1</sup> Na, or 121 mg L<sup>-1</sup> Ca) waters within 24 hours of hatching. After rearing for approximately 21 days, mature larvae were placed in experimental waters to perform a full transplant assessment (i.e., all four combinations of exposures for each major ion of interest) for an acute 8-hour exposure (Supplementary Fig. 1). To assess the relative gene expression of important transporters, we randomly sampled mayfly larvae at the end of each exposure and flash froze 2 composited larvae/replicate in liquid nitrogen ( $n=5$ ). Samples were stored in a -80°C freezer until RNA extraction was performed using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions and quantified using a NanoDrop<sup>TM</sup> 1000 (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized using the MultiScribe<sup>TM</sup> MuLV reverse transcriptase and random primers (Applied Biosystems, Carlsbad, CA) in 20 µL reaction tubes using a Bio-Rad iCycler (Bio-Rad, Hercules, CA).

Quantitative real-time PCR (qRT-PCR) was performed using diluted cDNA, diluted primers, and SYBR Green Master Mix (Bio-Rad, Hercules, CA) on a QuantStudio<sup>TM</sup> 3 machine

(Thermo Fisher Scientific, Waltham, MA) in 10  $\mu\text{L}$  reactions with technical triplicates. Custom primers were designed through Integrated DNA Technologies (IDT, Coralville, Iowa) and are listed in Supplementary Table 2. Standard PCR parameters were used and followed by a melt curve to ensure high quality of all samples.

### *96 Hour Toxicity Bioassay*

To assess survival across treatment groups with differential exposure histories, we seeded *N. triangulifer* hatchlings into either control (ASW) or elevated salinity (205  $\text{mg L}^{-1}$  Na, 360  $\text{mg L}^{-1}$   $\text{SO}_4$ , or 480  $\text{mg L}^{-1}$  Ca) waters within 24 hours of hatching. After approximately 10-12 days of rearing, middle-aged larvae were seeded into 6-well plates across a series of concentrations for each ion of interest: 15.3 (ASW), 205, 280, 387, 535, or 743  $\text{mg L}^{-1}$  Na, or 23.3 (ASW), 360, 515, 735, 1050, 1500  $\text{mg L}^{-1}$   $\text{SO}_4$ , or 12.7 (ASW), 480, 686, 980, 1400, 2000  $\text{mg L}^{-1}$  Ca. All waters were sampled and verified by North Carolina State University's Environmental and Agriculture Testing Services Lab (ICP-EATS) and concentrations were within 10% of nominal values, except 743  $\text{mg L}^{-1}$  Na, which had a 21% error.

Each replicate well contained 10 larvae and each treatment was represented by 3 replicates ( $n=3$ ) or 6 ( $n=6$ , for control groups only). Sterile 6-well plates were filled with 8 mL (approximately 75% full) of each water and aerated fully. Wells were given 2 drops ( $\sim 200 \mu\text{L}$ ) of food slurry (0.1 g periphyton per mL of corresponding water) to prevent alterations of salinity concentrations within the wells. All plates were aerated (60 seconds/well) and inspected daily with a Leica MZ 16F stereoscope and any mortalities were recorded and removed. At 48 hours, a 50% water change was performed to remove excess debris/feces and replace it with new test solution. Additional food was added from corresponding food slurries as needed. Previous 96-hour

bioassays in our lab have indicated that these methods prevent substantial changes to water conductivities and pH (Orr and Buchwalter, 2020).

### *Microscopic Pathology*

Mature *N. triangulifer* larvae were treated with various salinity treatments for 96 hours: control (ASW,  $n=5$ ), sodium ( $674 \text{ mg L}^{-1} \text{ Na as NaCl}$ ;  $n=7$ ), sulfate ( $735 \text{ mg L}^{-1} \text{ SO}_4 \text{ as CaSO}_4$  and  $\text{MgSO}_4$ ;  $n=5$ ), or calcium ( $980 \text{ mg L}^{-1} \text{ Ca as CaCl}_2$ ;  $n=7$ ). After the exposure, mayflies were immediately rinsed, fixed in 10% neutral buffered formalin for 24h, transferred to ethanol 70%, embedded in paraffin in sagittal orientation, sectioned into 4-5  $\mu\text{m}$ -thick, and stained with hematoxylin and eosin (HE) at the histology laboratory at the North Carolina State University Histopathology core facility. Multiple serial sagittal HE sections of mature *N. triangulifer* larvae from each group were blindly examined by one American College of Veterinary Pathologists (ACVP) board-certified anatomic pathologist to determine and characterize any histological alterations including inflammatory and/or degenerative processes on targeted organs, and lesion severity with light microscopy.

### *Data Analysis*

Flux rates were determined by linear regression analysis using GraphPad Prism (v6, GraphPad Software, La Jolla, CA, USA). Uptake rates were calculated based on the slopes of linear regression analysis of each time course based on wet weights. Comparisons of flux rates among groups were performed via one-way ANOVA with Tukey's multiple comparisons test for all experiments. All data were also analyzed for normality.

For gene expression experiments, the delta-delta CT method (Pfaffl, 2001) was used to calculate relative expression and normalized to the housekeeping gene,  $\beta$ -actin. Data were

normalized again to the control samples and relative fold change was analyzed with a one-way ANOVA and Tukey's multiple comparisons. Finally, the toxicity bioassays were also analyzed using a one-way ANOVA with Tukey's multiple comparisons and LC50 values were calculated using probit analysis. For all plots, error bars represent mean  $\pm$  SEM and a p-value of 0.05 was chosen *a priori*.

## Results

### *Ion Flux Experiments*

Flux rates in control reared larvae were concentration dependent for sodium and sulfate, but not calcium (Fig. 4.1). For example, control mayflies (23.3 mg L<sup>-1</sup> SO<sub>4</sub>) had a basal sulfate uptake rate of  $2.4 \pm 0.1 \mu\text{g SO}_4 \text{ g}^{-1} \text{ hr}^{-1}$ , but increased sulfate uptake to  $272.1 \pm 8.1 \mu\text{g SO}_4 \text{ g}^{-1} \text{ hr}^{-1}$  when placed in elevated sulfate (735 mg L<sup>-1</sup> SO<sub>4</sub>) ( $p < 0.0001$ ) (Fig. 4.1A). Similarly, control mayflies (16 mg L<sup>-1</sup> Na) had a sodium uptake rate of  $57.4 \pm 1.0 \mu\text{g Na g}^{-1} \text{ hr}^{-1}$ , but increased sodium uptake to  $144.9 \pm 7.9 \mu\text{g Na g}^{-1} \text{ hr}^{-1}$  in elevated sodium (157 mg L<sup>-1</sup> Na) ( $p < 0.0001$ ) (Fig. 4.1B). Interestingly, calcium uptake rates changed minimally between treatment waters. Control mayflies (12.7 mg L<sup>-1</sup> Ca) had mean calcium uptake rates of  $5.934 \pm 2.8 \mu\text{g Ca g}^{-1} \text{ hr}^{-1}$  in control water and  $8.1 \pm 1.7 \mu\text{g Ca g}^{-1} \text{ hr}^{-1}$  in elevated calcium (121 mg L<sup>-1</sup> Ca) waters ( $p = 0.21$ ) (Fig. 4.1C).

Previous exposure history generally decreased ion uptake rates within each exposure water (control or elevated concentration). Control mayflies exposed to elevated sulfate (735 mg L<sup>-1</sup> SO<sub>4</sub>) waters had a sulfate uptake rate of  $272.1 \pm 8.1 \mu\text{g SO}_4 \text{ g}^{-1} \text{ hr}^{-1}$ , but mayflies that were reared in that elevated sulfate concentration had 8-fold slower sulfate uptake rate at  $30.1 \pm 36.8 \mu\text{g SO}_4 \text{ g}^{-1} \text{ hr}^{-1}$  ( $p < 0.0001$ ). Similarly, pre-exposed mayflies reduced their sulfate uptake rate by 1.7-fold from  $2.4 \pm 0.1 \mu\text{g SO}_4 \text{ g}^{-1} \text{ hr}^{-1}$  to  $1.3 \pm 1.3 \mu\text{g SO}_4 \text{ g}^{-1} \text{ hr}^{-1}$ , but this change was not statistically significant ( $p = 0.54$ ) (Fig. 4.1A).

We observed similar patterns in larvae reared under elevated sodium conditions (157 mg L<sup>-1</sup> Na). Mayflies that were naïve to this salinity challenge had a sodium uptake rate of  $144.9 \pm 7.9 \mu\text{g Na g}^{-1} \text{ hr}^{-1}$ , but pre-exposed mayflies reduced their sodium uptake rate 2-fold to  $73.0 \pm 7.0 \mu\text{g Na g}^{-1} \text{ hr}^{-1}$  ( $p < 0.0001$ ). Pre-exposed mayflies also reduced their sodium uptake rate 1.5-fold in control waters to  $39.7 \pm 5.5 \mu\text{g Na g}^{-1} \text{ hr}^{-1}$  compared to control counterparts with a sodium uptake



rate of  $57.4 \pm 1.0 \mu\text{g Na g}^{-1} \text{hr}^{-1}$  ( $p < 0.01$ ) (Fig. 4.1B). Mayflies presented with an elevated calcium challenge had little differences in calcium uptake rate between rearing histories ( $p = 0.10$ ). However, pre-exposed mayflies were able to reduce their calcium uptake rate 2.7-fold in control water to  $2.2 \pm 0.3 \mu\text{g Ca g}^{-1} \text{hr}^{-1}$  compared to  $5.9 \pm 2.8 \mu\text{g Ca g}^{-1} \text{hr}^{-1}$  of naïve mayflies ( $p < 0.0001$ ) (Fig. 4.1C).

### *Gene Expression*

We assessed changes in relative mRNA expression of 6 genes of interest across all four treatment combinations for each major ion of interest (Fig. 4.2). Control-reared mayflies that were placed into elevated sulfate ( $735 \text{ mg L}^{-1}$ ) water had no statistically significant changes in expression in any of the selected genes relative to controls. However, mayflies that had been reared in elevated sulfate and were subsequently exposed to control water for 8 hours, experienced a 1.9-fold upregulation in the  $\text{Ca}^{2+}$  ATPase gene ( $p < 0.05$ ). Mayflies that had been reared in and exposed to elevated sulfate had the largest response with significant upregulation in  $\text{SO}_4^-$  Transporter (9.8-fold,  $p < 0.05$ ), Na-independent  $\text{SO}_4^-$  Transporter (10.7-fold,  $p < 0.001$ ),  $\text{NaHCO}_3$  Cotransporter (6.1-fold,  $p < 0.01$ ), and  $\text{Ca}^{2+}$  ATPase (3.9-fold,  $p < 0.01$ ) (Fig. 4.2A).

Sodium challenge had seemingly little effect on mRNA transcript levels; only one gene in a single treatment group was significantly changed. Mayflies that had elevated sodium ( $157 \text{ mg L}^{-1} \text{ Na}$ ) rearing histories and were subsequently exposed to control water significantly reduced the expression of  $\text{NaHCO}_3$  Cotransporter to 0.4-fold ( $p < 0.05$ ) (Fig. 4.2B).

Finally, we examined gene expression changes in mayflies that had experienced calcium challenge. Control-reared mayflies that were exposed to elevated calcium ( $121 \text{ mg L}^{-1} \text{ Ca}$ ) had a significant 4.3-fold upregulation in the Na-independent  $\text{SO}_4^-$  Transporter gene ( $p < 0.001$ ).

Similarly, pre-exposed mayflies that were exposed to control water also had a 2.7-fold upregulation in this gene ( $p < 0.05$ ). Most interestingly, all treatment groups with any amount of elevated calcium exposure had a significant downregulation in  $\text{SO}_4$  Transporter ( $p < 0.001$ ), Na/K ATPase ( $p < 0.001$ ), V-Type ATPase ( $p < 0.01$ ), and Carbonic Anhydrase ( $p < 0.01$ ) (Fig. 4.2C).

### *Toxicity Bioassays*

To evaluate whether any of the physiological or molecular acclimatory responses described above could influence survival we performed 96-hour toxicity bioassays for each major ion of interest (sulfate, sodium, and calcium) (Fig. 4.3). Mayfly larvae that had been acclimated to elevated sulfate, performed worse than their control counterparts (Fig. 4.3A). Control mayflies had no significant changes in survival at any concentration of sulfate, however, pre-exposed mayflies experienced mortalities at the three highest concentrations tested (735, 1050, and 1500  $\text{mg L}^{-1}$   $\text{SO}_4$ ). The most challenging concentration, 1500  $\text{mg L}^{-1}$ , only had a 33% survival rate in acclimated mayfly group, compared to the near perfect survival of control mayflies in the same treatment group ( $p < 0.01$ ). Because of these results, we calculated an acute LC50 of 1939  $\text{mg L}^{-1}$   $\text{SO}_4$  for only the pre-treated group.

In contrast, larvae with previous exposure history to elevated Na unaffected by even extremely high Na exposures (up to 1500  $\text{mg L}^{-1}$ ). Control larvae experienced significant mortalities in the three highest concentrations of sodium: 73% survival in 387  $\text{mg L}^{-1}$  Na ( $p < 0.05$ ), 63% survival in 535  $\text{mg L}^{-1}$  Na ( $p < 0.001$ ), and 67% survival in 743  $\text{mg L}^{-1}$  Na ( $p < 0.001$ ) (Fig. 4.3B). Control-reared mayflies had an acute LC50 of 1169  $\text{mg L}^{-1}$  Na.

Finally, pre-exposure to elevated calcium resulted in poor performance at all concentrations tested excluding the control (15  $\text{mg L}^{-1}$ ) (Fig. 4.3C). The control reared mayflies

performed better than the Ca-pre-exposed mayflies, at the two mid-range concentrations (480 and 696 mg L<sup>-1</sup> Ca). Larvae with a history of previous exposure only had a 17% and 27% survival at these two concentrations, respectively. For 980 and 1400 mg L<sup>-1</sup> Ca treatment groups, both acclimated and control larvae survived poorly and in the highest concentrations (2000 mg L<sup>-1</sup> Ca), not a single larva from either group survived. An acute LC50 value for the control-reared group was calculated to be 901 mg L<sup>-1</sup> Ca.

### *Microscopic Pathology*

The histological results were similar in the different groups: control, sodium, and sulfate treated groups. Microscopic lesions were not detected in any of the assessed tissues including the cephalic, or organs within the thoracic and the abdominal cavities. A comparison of the control and treated mayfly larvae did not provide any evidence of toxic lesions by light microscopy. Interestingly, within all samples from the calcium treated group, the epithelial cells of the Malpighian tubules had multifocally acute cellular degeneration and/or necrosis (Fig. 4.4). Acute cellular degeneration (hydropic degeneration) was characterized by the evidence of cellular swelling, rounded edges, and feathery to vacuolated cytoplasm. Necrotic cells showed fragmented cytoplasm with associated fade basophilic nuclei (karyolysis) or nuclear fragmentation (karyorrhexis).

## Discussion

To our knowledge, the question of how ionic exposure history affects physiological processes and/or toxicological outcomes has been unexplored in aquatic insects. This question is important because these organisms are used routinely to evaluate ecological conditions in nature (Cormier et al., 2013a; Hawkins, 2006; Hawkins et al., 2010; Johnson et al., 1993) where they are typically exposed over generations. In contrast, toxicity evaluations are almost always performed with naïve animals, and data for relevant insect surrogate species remain relatively scarce (Buchwalter et al., 2019; Hassell et al., 2006; Johnson et al., 2015; Scheibener et al., 2017, 2016; Soucek and Dickinson, 2015; Soucek et al., 2018). Here, we take a first step in assessing how exposure history influences physiological and organismal performance in a relevant insect model, *N. triangulifer* (Buchwalter et al., 2019; Chou et al., 2020, 2018, 2017; Funk et al., 2006; Kim et al., 2017; Orr and Buchwalter, 2020; Soucek et al., 2018; Soucek and Dickinson, 2015; Sweeney and Vannote, 1984) in the context of three different major ions: sodium, sulfate, and calcium. For the first time, we demonstrate that exposure history to salinity can significantly influence ion transport rates in a mayfly.

### *Ion Flux Experiments*

Our data show that mayfly larvae that had been reared in an elevated major ion concentration generally decreased their uptake rates compared to naïvely exposed counterparts. The marked reduction we observed in both sulfate and sodium transport rates seems to be an appropriate acclimatory response. We previously observed that exposure to elevated sulfate delayed development (Buchwalter et al., 2019), and we speculated that the energetic cost of maintaining hemolymph homeostasis was responsible for developmental delays and toxicity.

Other researchers have also observed similar developmental delays (Johnson et al., 2015; Soucek and Dickinson, 2015). Thus, a reduction in flux rates would serve to minimize the energy costs of maintenance. Interestingly, calcium uptake rates are remarkably slow in this species (Orr and Buchwalter, 2020) as they are for other aquatic insects (Poteat et al., 2012; Poteat and Buchwalter, 2014), relative to other ions. Even when concentrations are raised considerably, *N. triangulifer* maintains very slow uptake of calcium. Under control exposure conditions, the elevated calcium reared larvae clearly down-regulated uptake, but a high degree of interindividual variability under that higher concentrations precluded the finding of statistical significance in this comparison. Nevertheless, the apparent reduction of calcium influx rates also appears to be a response to calcium challenge.

While literature is relatively sparse for salinity acclimation ion flux studies, our findings are consistent with other aquatic taxa. One study found that freshwater mussels (*Toxolasma texasensis*) acclimated to 10% seawater significantly reduced renal sulfate reabsorption compared to controls that conserved sulfate (Dietz et al., 2000). Similarly, other researchers found that calcium uptake was reduced significantly in rainbow trout that had been acclimated to waters amended by  $\text{Ca}(\text{NO}_3)_2$  for 50 days both *in vivo* and *in vitro* (Perry and Wood, 1985). Shorter acclimation periods (3 d) to elevated calcium in tilapia larvae also reduced calcium uptake rates compared to larvae reared in low calcium (Hwang et al., 1996; Lin et al., 2016). Although our results are in support of these studies, it is important to note that calcium may play different roles in freshwater vertebrates that rely heavily on the ion for maintaining healthy bony skeletons.

## *Gene Expression*

At the gene expression level, we observed some stark differences in between pre-exposed and naïve larvae. For example, we observed that the pre-exposed group left in elevated sulfate dramatically upregulated the mRNA expression of two sulfate transporting genes, likely in tissues associated with efflux, such as the Malpighian tubules. We also observed unexpected crosstalk with other ion transporters, NaHCO<sub>3</sub> cotransporter and Ca ATPase. In contrast, larvae reared under elevated sodium appeared to have a stimulation of V-Type ATPase expression but a high degree of variability in those data impedes us from making definitive conclusions. Finally, calcium pre-exposure generally decreased expression of several genes. This could be due to an attempt to reduce uptake, or alternatively, could result from a forfeiture of gene regulation because of the severe toxicity of calcium observed in our toxicity bioassays. We were somewhat handicapped in this line of inquiry by the very small size of *N. triangulifer* larvae, and our lack of understanding of the tissue specific distributions of these genes of interest. Thus, this “whole organism” level of expression represents an exploratory effort that we can hopefully refine in the future.

Few studies have analyzed gene expression changes of mayflies in response to salinity stress. Our lab has previously demonstrated a slight decrease in the mRNA expression of a sulfate transporter and a Na-independent sulfate transporter in *N. triangulifer* in response to 24-hours of sulfate stress (Buchwalter et al., 2019). However, the majority of literature on aquatic insect salinity-induced gene expression focused on mosquitoes due to their importance as a human disease vector. Several studies report changes in *A. aegypti* mRNA expression in response to salinity stress including genes such as P-type Na/K ATPase and V-type H-ATPase (Patrick et al., 2006), sodium-hydrogen antiporter 3 (Durant and Donini, 2019), aquaporins (Akhter et al., 2017; Misyura et al., 2020), and snakeskin and mesh homeostasis-related genes (Jonusaite et al., 2016).

In addition, RNA-Seq experiments in *Anopheles coluzzii* and *Anopheles merus* larvae have indicated significant changes in the mRNA expression of AgJNKa (MAPK gene) and AgAE2 (ion transport candidate gene) in response to seawater exposure (Uyhelji et al., 2016). Studies for osmoregulatory gene expression in response to elevated salinity have been conducted in other aquatic taxa such as Palaemonid prawns (Rahi et al., 2020), Hawaiian Anchialine shrimp (Havird et al., 2014), Chinese mitten crab (Yang et al., 2019), alewife fish (Velotta et al., 2017), among others (Havird et al., 2013; Rahi et al., 2018). Unfortunately, most of the aforementioned studies were only performed using seawater and thus, ion-specific conclusions cannot be drawn.

### *Toxicity Bioassays*

To characterize the survival and acute toxicity of major ions, we performed toxicity bioassays with a range of relevant concentrations chosen based on both prior work and environmental values. Mayflies that were reared in moderately elevated salinities performed better in sodium challenge, but worse in sulfate challenge. This surprising finding may reveal why aquatic insects are disappearing in nature, especially in sites that have particularly high levels of sulfate, such as West Virginia streams (Cormier et al., 2013a; Pond et al., 2008a). Due to the extremely slow uptake of calcium, we chose a higher concentration for the acclimation period before the calcium bioassays. However, the calcium exposures were significantly more harmful than the other salt exposures and the larvae appeared visibly less healthy than the larvae acclimated to sodium or sulfate. Notably, the “acclimatory” conditions were moderately elevated salinities and it is possible that chronic exposure to lower concentrations may be more beneficial. We acknowledge that our choice of higher calcium concentrations may require future investigations to determine where thresholds occur.

We find our toxicity data to be relatively consistent with other labs. In the present study, acute LC50 values of control-reared mayflies were calculated for sodium (1169 mg L<sup>-1</sup> Na) and calcium (901 mg L<sup>-1</sup> Ca). For sulfate, however, only pre-exposed animals were used to calculate the LC50 value (1939 mg L<sup>-1</sup> SO<sub>4</sub>), which may be more reflective of natural exposures, but less comparable to other naïve laboratory exposures. Similarly, other groups have calculated LC50s for Cl (1062 mg L<sup>-1</sup>), SO<sub>4</sub> (1227 mg L<sup>-1</sup>) (Soucek and Dickinson, 2015), and NaCl (2755 mg L<sup>-1</sup>) (equivalent to 1084 mg L<sup>-1</sup> Na) (Jackson and Funk, 2019). Toxicity data such as these are essential for establishing ion-specific environmental regulations and protecting freshwater ecosystems.

### *Microscopic Pathology*

There has been limited work to examine tissue morphological changes caused by increased salinity in aquatic insects and scarce publications regarding the normal anatomy and histology of mayfly larvae. We hypothesized that increases in salinity would alter the tissue morphology of important osmoregulatory organs and the primary excretory system (e.g., gills and/or Malpighian tubules). Insects rely on Malpighian tubules for osmoregulatory homeostasis and urine formation, which may be impacted by elevated major ion exposures (Larsen et al., 2014). Through a blind approach, we observed that only calcium-treated mayflies had significant cellular degeneration and necrosis of the Malpighian tubules. We recognize that our calcium concentration was relatively high compared to most natural waters (Cormier et al., 2013a), but our microscopic pathology concentration choices were chosen for mechanistic exploratory purposes. Due to the particularly low calcium uptake rates, we were surprised by the severity of histopathological effects detected. These observations led us to consider alternate mechanisms of toxicity for calcium, specifically. Salinity-induced histological changes have been demonstrated in other



species of aquatic organisms including yellow perch (Nero et al., 2006), snow trout (Shahriari Moghadam et al., 2018), and sea bass (Hamedi et al., 2016), but none of these studies specifically examined the effects of calcium. Furthermore, histopathological studies have been performed in mayflies (Liarte et al., 2014), but did not directly correlate with salinity-induced changes as seen herein.

### *Ion-Specific Mechanisms of Toxicity*

Our study specifically examined three major ions: sodium, sulfate, and calcium, but ions cannot be studied in complete isolation. Rather, we used NaCl, CaSO<sub>4</sub>/MgSO<sub>4</sub>, or CaCl<sub>2</sub> to manipulate our water chemistries experimentally. Our sulfate exposures were balanced with two cations, calcium and magnesium, to minimize cation influence. Although our waters elevated with sodium and calcium had the same anion, chloride, our acute toxicity values were dramatically different. In fact, our sodium elevated waters with chloride concentrations ranging from 255 to 1165 mg L<sup>-1</sup>, were significantly less toxic than our calcium elevated waters with 97 to 525 mg L<sup>-1</sup> Cl (Table 1). These results may suggest that the cations of interest, rather than chloride, influenced our observed results in mRNA expression, ion flux rates, Malpighian tubules hydropic degeneration and necrosis, and acute toxicity.

There are many hypotheses regarding major ion toxicity in sensitive aquatic insects (Kefford, 2018), but data produced from our lab has largely supported the energy depletion theory (Buchwalter et al., 2019). The cost of active ion transport across epithelia makes up a large portion of an aquatic insect's energy budget, but is exacerbated by salinity stress (see Verberk et al., 2020). However, the uniformly low uptake of calcium and extreme toxicity provokes us to reconsider the mechanism of toxicity for this ion. Like most major ions, calcium is essential to numerous

biological functions, and low concentrations are often reported to be especially dangerous for aquatic life (Dam et al., 2010). One group found that calcium stimulates sodium uptake in *D. magna* (Glover and Wood, 2005). Could the presence of calcium increase the uptake and consequential toxicity of other ions present (e.g., Cl)? Although there is little evidence for dysregulation in mayflies (Dowse et al., 2017), other groups suggest that hemolymph acidosis may be the root of salt stress in mosquito larvae (Durant and Donini, 2019). Recently, Wood and colleagues found that changes in trans-epithelial potential across the gills correlate with major ion toxicity in several species of fishes (Po and Wood, 2021; Wood et al., 2020). These authors speculate that the mechanism of toxicity may be related to metabolic acidosis. We suggest the mechanisms of toxicity for salinity may differ between ions and taxa.

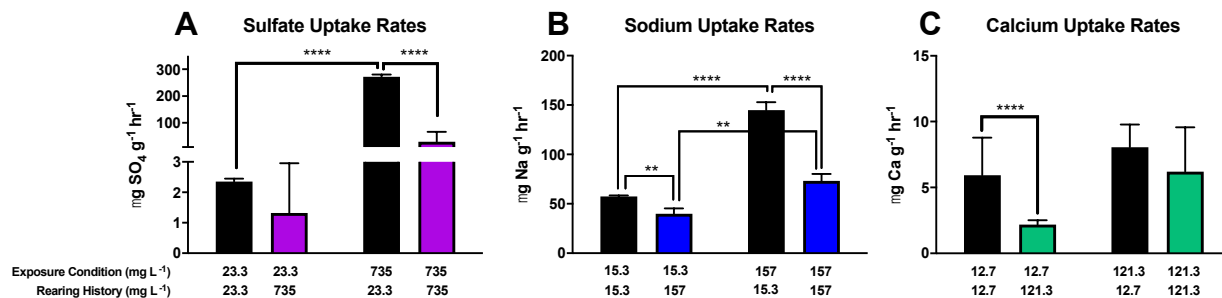
### *Conclusions*

Historically, ecological assessments are often performed with conductivity as the surrogate descriptor of the salinity. However, it is now known that the elevations of major ions differ between environmental scenarios (Cormier et al., 2013c) and that these ions affect organisms differently (Clements and Kotalik, 2016; Griffith, 2017b; Kunz et al., 2013). Indeed, in the present study we show significant evidence for ion-specific effects. In summary, our data suggests that the history of previous exposure to elevated ions in mayflies reduces ion uptake rates. Further, we found ion-specific changes in gene expression, tissue morphology, and survival of more challenging concentrations of major ions. Our results support the idea of acclimation to elevated sodium, but not to sulfate or calcium in *N. triangulifer* larvae. We believe that elevated sulfate imposes steep energetic consequences on mayflies, while calcium may have a completely different mechanism

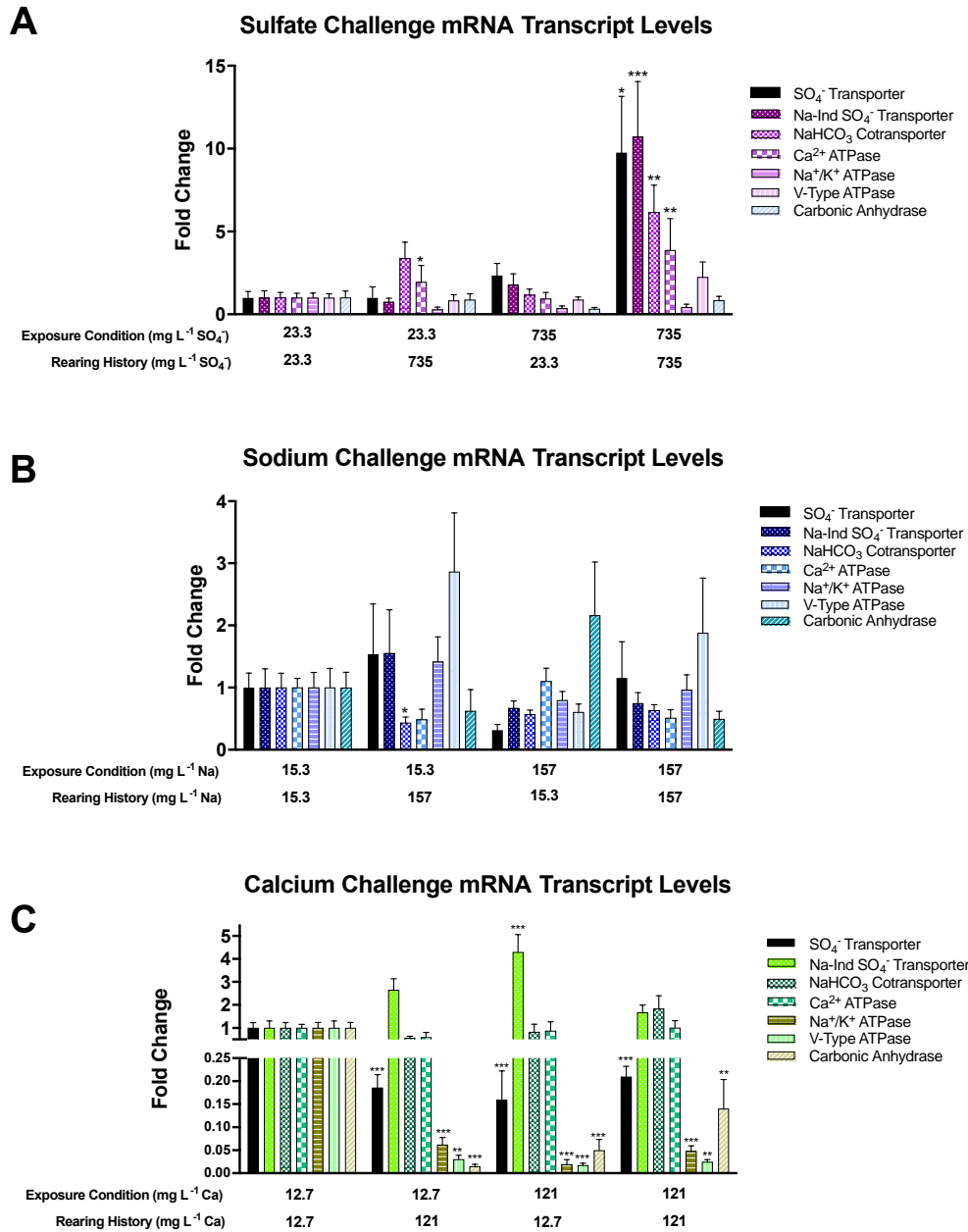
of toxicity. Understanding the osmoregulatory physiology of important aquatic insects, such as mayflies, is an essential step to improve regulatory efforts and protect our freshwater ecosystems.

**Table 4.1** Water chemistry for all experimental exposure waters. Cations (Na, Ca, Mg, K) and anions (SO<sub>4</sub>, Cl, CO<sub>3</sub>) are all listed in mg L<sup>-1</sup>. All waters were sampled, filtered, and verified by North Carolina State University's Environmental and Agriculture Testing Services Lab (ICP-EATS) and concentrations were within 10% of nominal values except for 743 Na, which had a 21% error. Measured values are listed in parentheses beside nominal values.

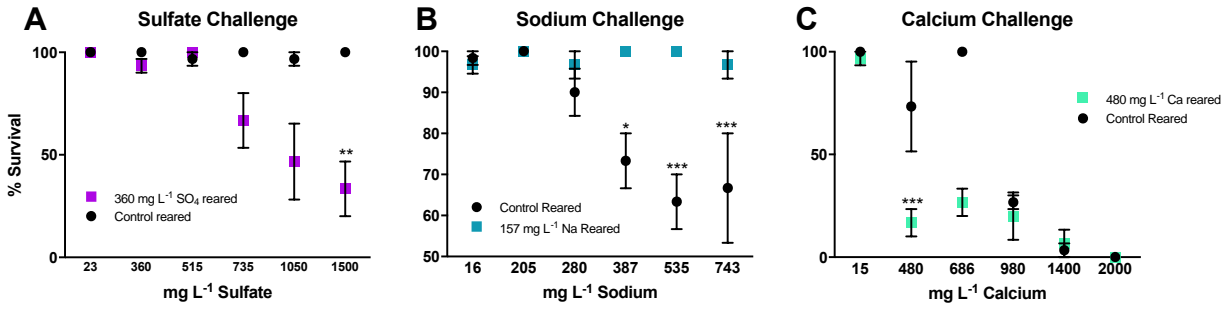
	Na	Ca	Mg	K	S	SO <sub>4</sub>	Cl	CO <sub>3</sub>
<b>Control (ASW)</b>	15.3 (16.5)	12.7 (11.6)	3.4	1.4	7.78 (7.9)	23.3	14.1	42.6
<b>121 Ca</b>	15.3	121 (113)	3.4	1.4	7.78	23.3	97	42.6
<b>480 Ca</b>	15.3	480 (471)	3.4	1.4	7.78	23.3	158	42.6
<b>686 Ca</b>	15.3	686 (646)	3.4	1.4	7.78	23.3	208	42.6
<b>980 Ca</b>	15.3	980 (933)	3.4	1.4	7.78	23.3	280	42.6
<b>1400 Ca</b>	15.3	1400 (1338)	3.4	1.4	7.78	23.3	380	42.6
<b>2000 Ca</b>	15.3	2000 (1896)	3.4	1.4	7.78	23.3	525	42.6
<b>157 Na</b>	157 (152)	12.7	3.4	1.4	7.78	23.3	255	42.6
<b>205 Na</b>	205 (228)	12.7	3.4	1.4	7.78	23.3	334	42.6
<b>280 Na</b>	280 (298)	12.7	3.4	1.4	7.78	23.3	452	42.6
<b>387 Na</b>	387 (414)	12.7	3.4	1.4	7.78	23.3	615	42.6
<b>535 Na</b>	535 (559)	12.7	3.4	1.4	7.78	23.3	844	42.6
<b>743 Na</b>	743 (915)	12.7	3.4	1.4	7.78	23.3	1165	42.6
<b>360 SO<sub>4</sub></b>	15.3	101	40.4	1.4	120 (129)	360	14.1	42.6
<b>515 SO<sub>4</sub></b>	15.3	139.2	56.4	1.4	172 (173)	515	14.1	42.6
<b>735 SO<sub>4</sub></b>	15.3	193.7	79.1	1.4	245 (256)	735	14.1	42.6
<b>1050 SO<sub>4</sub></b>	15.3	271.8	111.5	1.4	351 (383)	1050	14.1	42.6
<b>1500 SO<sub>4</sub></b>	15.3	383	157.9	1.4	501 (532)	1500	14.1	42.6



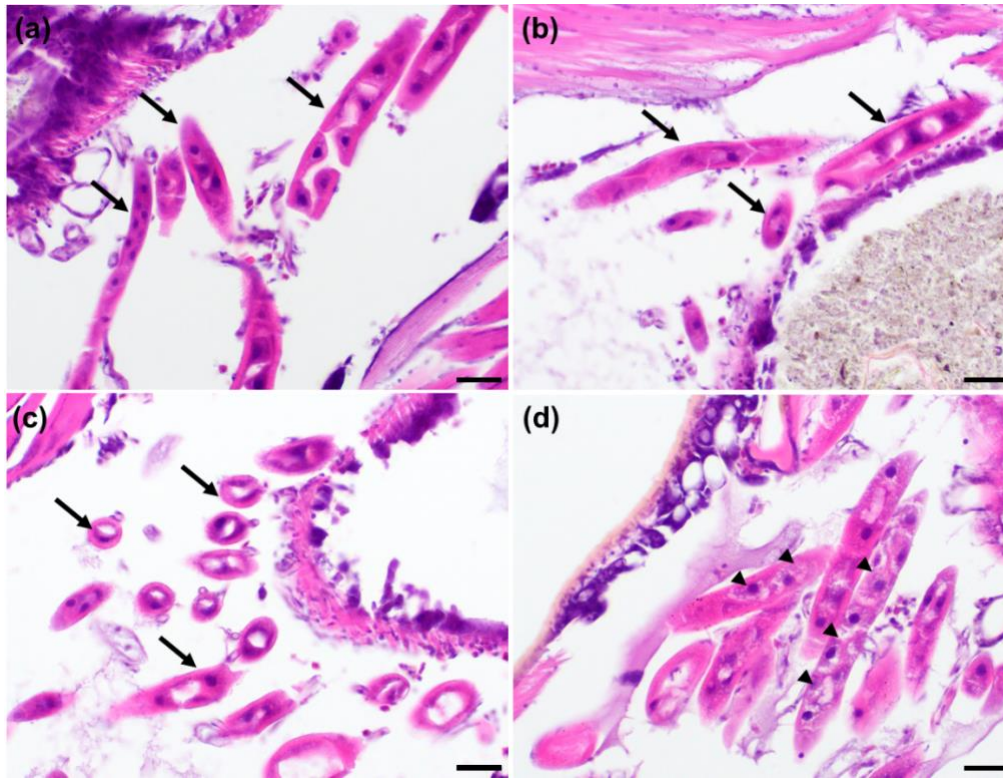
**Figure 4.1** Ion uptake rates in *N. triangulifer* for sulfate (A), sodium (B), and calcium (C). The y-axis indicates both rearing history and exposure condition for each major ion of interest. Each column represents a time-course study demonstrated in Supplementary Figure 2. Errors bars represent standard error from the mean ( $n=8$ ). A one-way ANOVA with Tukey's multiple comparisons was performed and asterisks indicate statistically significant differences between groups (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , \*\*\* indicates  $p < 0.001$ , and \*\*\*\* indicates  $p < 0.0001$ ).



**Figure 4.2** mRNA transcript levels in *N. triangulifer* exposed to sulfate (A), sodium (B), or calcium (C). Errors bars represent standard error from the mean ( $n=5$ ). A one-way ANOVA with Tukey's multiple comparisons was performed and asterisks indicate statistically significant differences from the control group (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , and \*\*\* indicates  $p < 0.001$ ).



**Figure 4.3** Survival of *N. triangulifer* in 96-hour toxicity bioassays to examine the effects of previous exposure to sulfate (A), sodium (B), or calcium (C). Errors bars represent standard error from the mean ( $n=3$ ). A one-way ANOVA with Tukey's multiple comparisons was performed and asterisks indicate statistically significant differences of % survival between the control reared or pre-exposed mayfly larvae (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , and \*\*\* indicates  $p < 0.001$ ).



**Figure 4.4** Sagittal sections of *N. triangulifer* larvae. Hematoxylin and eosin (HE), bar = 20  $\mu$ m. The cellular epithelium of the Malpighian tubules from the control (a), sodium-treated (b), and sulfate-treated (c) groups is within normal limits (arrow). The small and luminal tubules are lined by a simple cuboidal epithelial on a thin basement membrane with lack of muscular layer. The Malpighian tubules (arrowhead) in the calcium-treated group (d) are multifocally undergoing cellular degeneration and necrosis.



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**CHAPTER FIVE**

**SALINITY-INDUCED OSMOREGULATORY CHANGES IN THE GILL OF THE  
MAYFLY, *NEOCLOEON TRIANGULIFER***

Sarah E. Orr, Dereje D. Jima, and David B. Buchwalter

Department of Biological Sciences, North Carolina State University, Raleigh, NC 27607

## Abstract

Freshwater salinization has become an increasing concern due to the decline of sensitive aquatic organisms observed by ecologists. Our physiological understanding of how changing salinities impact aquatic organisms, such as mayflies, is not well understood. Our lab is developing the small, Baetid mayfly, *N. triangulifer*, as a model organism for physiological research. We have previously described ion flux rates and altered mRNA transcript levels in response to major ion concentrations at the whole animal level. However, the specific proteins associated with apical ion transport have never been described in mayflies, where ionocytes are heavily concentrated on the gills. In the present study, we chronically reared *N. triangulifer* larvae to the penultimate larval stage in either control (ASW), elevated NaCl (157 mg L<sup>-1</sup> Na), elevated CaCl<sub>2</sub> (121 mg L<sup>-1</sup> Ca), elevated Ca/MgSO<sub>4</sub> (735 mg L<sup>-1</sup> SO<sub>4</sub>), or dilute (50% ASW diluted with DI water) conditions, before dissecting gills and performing shotgun proteomics. Preliminary analysis revealed over 33,000 unique peptide sequences among samples. The majority of proteins were common among all treatment groups, but there were also significant differences in protein expression in each condition. Ongoing analysis will identify unique ion transporters involved in osmoregulation and apical transport on the gill surface. Finally, we demonstrated the breadth of physiological functions in gills by exploring non-transport related pathways found in our proteomics dataset, including ATP synthesis, angiogenesis, and TGF-beta signaling. We discuss our results in the context of freshwater salinization and propose more -omics based molecular studies in sensitive freshwater insects. To our knowledge, we are the first to use a proteomic-approach to study gill proteins in mayflies and this project has tentatively demonstrated salinity induced osmoregulatory changes on the gill of the mayfly, *N. triangulifer*.



## **Introduction**

Freshwater salinization has become an increasing concern due to the alarming decline of sensitive aquatic organisms observed by ecologists (Cormier and Suter, 2013; Kaushal et al., 2005, 2018; Pond et al., 2008). Anthropogenic activities such as road de-icing (Jackson and Funk, 2019; Kotalik et al., 2017) and resource extraction (Entrekin et al., 2011; Kunz et al., 2013; Pond et al., 2008) have increased major ion concentrations in nearby freshwaters. Our physiological understanding of how changing salinities impact aquatic organisms, such as mayflies, is not well understood.

Freshwater insects are secondarily aquatic and groups evolved from terrestrial ancestors multiple times independently (Misof et al., 2014a). Consequentially, aquatic insects have diverse strategies of osmoregulation in dilute environments in order to prevent ion loss and water influx (Bradley et al., 2009). In general, they actively transport ions with epithelial tissues (e.g., gills) and consistently excrete dilute urine in order to maintain homeostasis in freshwaters (Bradley, 1987; Jonusaite et al., 2016; Nowghani et al., 2017). However, major ion toxicity at concentrations below that of insect hemolymph has been reported (Dowse et al., 2017), suggesting a complex physiological dilemma for sensitive organisms. Osmoregulatory physiology has been well-studied in mosquitoes (Silver and Donini, 2021) due to their impact as a human disease vector, but less is known about primitive and more sensitive groups, such as mayflies.

Early work in aquatic insect osmoregulation revealed the importance of ion-transporting cells (i.e., chloride cells or ionocytes) on the surface of aquatic insects (Komnick, 1977; Wichard and Komnick, 1974). Since then, there has been conflicting evidence on how salinity or other environmental factors affect the density and size of these ionocytes in aquatic organisms; some groups found no changes (Berrill et al., 1991; Nowghani et al., 2017), while others reported

significant differences (Kumar et al., 2020; Wichard et al., 1973). Ionocytes can be found on different structures including the abdomen, legs, and most importantly, gills (Griffith, 2017; Shaw and Stobbart, 1963; Wigglesworth, 1933). Mayfly gills are flat, movable epithelia bilayers with branching trachea that allow functional oxygen uptake and ion transport (Craig, 1990).

Our lab is helping to develop the small, Baetid mayfly, *N. triangulifer*, into a model organism for ecotoxicological research. Increases in major ion concentrations have induced physiological changes in *N. triangulifer*, including elevated ion flux rates and altered mRNA transcript levels (Buchwalter et al., 2018; Chou et al., 2018; Orr and Buchwalter, 2020; Scheibener et al., 2017). We have also found ion-specific effects on mRNA transcript levels of important ion transporters, albeit only at the whole-organism level due to small body size (Orr et al., 2021). One group has been able to generate tissue-specific mRNA expression data for the mayfly, *Cloeon dipterum* (Kamsoi et al., 2021). However, mRNA levels are not always directly correlated to protein expression (Maier et al., 2009), which emphasizes the importance of proteomic approaches. From this perspective, the exact ion transporters expressed and actively functioning on the gills of *N. triangulifer* and other mayflies are unknown.

The goals of the present study were threefold. First, we asked which ion-transport proteins are expressed on gill tissue of *N. triangulifer* larvae. Further, we examined how elevations of different major ions (NaCl, CaCl<sub>2</sub>, or Ca/MgSO<sub>4</sub>) or lack thereof (dilute treatment) affected the expression of gill proteins. Finally, we investigated other proteins and pathways expressed on the gill that were not directly related to transport or osmoregulation. This project closely aligns with previous physiological observations using the same water chemistries (Orr et al., 2021). To our knowledge, we are the first to use a proteomic-approach to study gill proteins in any species of

mayfly and this project is a step in better understanding osmoregulation of sensitive aquatic insects in a changing world.

## Methods

### *Mayfly Culture*

The baetid mayfly, *Neocloeon triangulifer*, was originally found in White Clay Creek in Pennsylvania by the Stroud Water Research Center (SWRC; Avondale, PA) (Sweeney and Vannote, 1984). Collaborators at SWRC have shared both this WCC-2 clone and naturally grown periphyton from stream water on plastic plates as mayfly larvae food. *N. triangulifer* hatchings (<1-d old) were seeded into glass Pyrex containers with approximately 4 L of water in a 14:10 h light:dark photoperiod at room temperature (21-23°C). Artificial soft water (ASW) (Table 1) was used as our control water conditions and the base of all amended waters.

### *Scanning Electron Microscopy*

Older *N. triangulifer* larvae (~21-d old) were preserved in 2% glutaraldehyde and stored at 4°C. Following the initial fixation, insects were then washed in buffer solution (0.1 M Sodium cacodylate buffer) 3X10-m. Then, samples were post fixed in 2% Osmium tetroxide for 2-h followed by 3X10 buffer washes. A dehydration series of Ethanol: 70%, 95%, 2 X 100% (10-m each) and two changes of HMDS for 15-m each. Finally, samples were air dried overnight before mounting and sputter coating for imaging on the Hitachi S3200N Scanning Electron Microscope at NCSU's Analytics Instrumentation Facility.

### *Larval Exposures for Proteomics*

To characterize the specific ion transporters present on the mayfly gill and to assess the plasticity of their expression, larvae were reared chronically under different elevated ionic conditions previously used for physiology experiments (Orr et al., 2021). The specific

concentrations of exposure waters were chosen based on previous toxicity results in our lab (Buchwalter et al., 2018) and others (Soucek and Dickinson, 2015; Soucek et al., 2018). The exposure waters consisted of: control (ASW), elevated NaCl (157 mg L<sup>-1</sup> Na), elevated CaCl<sub>2</sub> (121 mg L<sup>-1</sup> Ca), and elevated Ca/MgSO<sub>4</sub> (735 mg L<sup>-1</sup> SO<sub>4</sub>) (Table 5.1). Animals were exposed chronically to all treatments for the majority of their larval life cycle (~21-25-d). Additionally, we included an acute, 48-hour dilute treatment of mature larvae which consisted of a 50% dilution of control ASW with deionized water. All waters were sampled, filtered, and measured by North Carolina State University's Environmental and Agriculture Testing Services Lab (ICP-EATS) and concentrations were within 10% of nominal values. Additionally, the pH of each water was measured with a benchtop Orion Star pH meter (Thermo Scientific). After rearing, mayfly larvae were removed and had all 14 gills removed with forceps under the microscope. Gills were immediately placed into 0.2 mL PCR tubes with 50 mM ammonium hydrogen carbonate (ABC) with 1% sodium deoxycholate (SDC) and stored at -20°C. A total of approximately 20 larvae (280 gills) were dissected to obtain sufficient protein yields for each sample to undergo proteomic analysis at the NCSU Molecular Education, Technology and Research Innovation Center (METRIC).

### *Shotgun Proteomics*

Gills were thawed on ice and combined to achieve 4 total replicates per treatment group with similar total protein yield (100 µg, *n* =4). Samples were homogenized with Promega Protease Inhibitor Cocktail, a ceramic bead, and Geno/Grinder twice and then total protein was quantified with a Pierce™ BCA Kit (ThermoFisher). Samples were evaporated to dryness in a speedvac and stored in -20°C freezer. Next, dry samples were reconstituted and treated with dithiothreitol (to

reduce disulfide bonds), 8 M Urea 0.1 M Tris (to denature), and iodoacetamide (to alkylate cysteine residues). A series of centrifuging and rinsing was performed before adding 100  $\mu$ L Trypsin (4  $\mu$ g) and 100  $\mu$ L chymotrypsin (2.5  $\mu$ g) to each sample. This combination was used to enhance membrane protein coverage (Fischer and Poetsch, 2006). After samples were rinsed with quench buffer, they were dried and stored in  $-20^{\circ}\text{C}$ .

To perform proteomics analysis, samples were reconstituted in 200  $\mu$ L mobile phase A and injected 2  $\mu$ L for nanoLC-MS/MS. Mobile Phase A consisted of: 98% water, 2% acetonitrile, and 0.1% formic acid and mobile phase B consisted of: 80% acetonitrile, 20% water, and 0.1% formic acid. The trap column used was Acclaim™ PepMap™ 100 C18, 3  $\mu$ m, 75  $\mu$ m x 20 mm and the analytical column was EASY-Spray™, 2  $\mu$ m, 75  $\mu$ m x 250 mm. Raw data was processed with Proteome Discoverer 2.4.0.305 (ThermoFisher).

### *Data Analysis*

Annotating and analyzing the proteomic data set in a non-model organism has resulted in many challenges. The main obstacle was the lack of a reference genome to use for *N. triangulifer*. We have been working closely with bioinformatician, Dr. Dereje Jima, to annotate the proteomics data produced by METRIC. With Dr. Jima's help, we annotated over 33,000 peptides using R, which included a large amount of redundancy among proteins and species. This approach did successfully identify hundreds of relevant ion transporters that had expression levels influenced by the various salinity treatments. However, this analysis produced contradiction among protein fold changes (i.e., a protein identified from one species was upregulated in a particular treatment group, but the same protein was downregulated for a different species). This led us to attempt transforming previous *N. triangulifer* transcriptomics data (Chou et al., 2020) into a reference

proteome. Unfortunately, this approach left us with surprisingly low hits and identified almost no expected ion transporters.

Currently, we are employing METRIC to run additional analysis with our data using Proteome Discoverer software. While we are currently waiting on results, we feel optimistic that our efforts will result in usable proteomics data that will reveal gill-specific ion transporters for our developing model organism, *N. triangulifer*.

## Results and Expected Outcomes

Freshwater salinization poses an imminent threat to sensitive aquatic insects, such as mayflies (Canedo-Arguelles et al., 2018; Kaushal et al., 2018; Pond et al., 2008). Because of their importance to freshwater ecosystems, it's imperative that we better understand their physiology so that mitigation efforts can be made. Previous work in our lab revealed ion-specific mechanisms of toxicity in the mayfly, *N. triangulifer*, including salinity-induced changes in whole-body level mRNA expression of ion transporters and ion flux rates (Orr et al., 2021). *N. triangulifer* appears to be physiologically plastic and can somewhat acclimate to both dilute and rich conditions. Here, we used a proteomic-approach to investigate gill biology of *N. triangulifer* larvae exposed to different salinities with concentrations that mirrored our previous physiological study.

### *Morphologically-distinct ionocytes exist on the abdomen and gills*

SEM revealed an abundance of ionocytes on both the gill and abdomen of *N. triangulifer* larvae. Interestingly, the abdomen had two distinct types of ionocytes: coniform and bulbiform, while the gills only had coniform cells. We were unable to quantify these results confidently due to low sample size.

It is clear that freshwater habitats present a unique challenge to aquatic organisms that must osmoregulate in concentrations hypotonic to their own hemolymph. One way aquatic insects have adapted to dilute freshwater conditions is by expressing numerous ionocytes on gill and abdomen surfaces to increase the number ionic transporters (Komnick, 1977). We were surprised to find morphologically-distinct ionocytes on different tissues of *N. triangulifer* larvae through SEM (Figure 5.1). It remains unclear if these distinct ionocytes house different transport proteins or have different physiological responsibilities.



## Proteomics

We originally identified 33,573 unique peptide sequences among all samples, which made up 15,815 unique proteins. This preliminary analysis showed significantly different protein expression among treatment groups. Compared to controls, dilute, CaCl<sub>2</sub>, Ca/MgSO<sub>4</sub>, and NaCl-treated mayflies had 9,690, 10,562, 8,477, and 9,065 differentially expressed proteins, respectively (data not shown). These preliminary results suggest that many ion transporters exist on the gill tissue (Table 5.2). These include proteins we expected to find, such as Ca-transporting ATPase, sodium/proton antiporter, sulfate transporter, and V-type proton ATPase. However, other unexpected ion transporters appeared in our preliminary search, including magnesium transporter and potassium channel (Table 5.2). Although our analysis is not complete, we will discuss preliminary proteomics results here.

Osmoregulation makes up a significant portion of an aquatic insect's energy budget, which can be exacerbated in environments with salinity stress (Verberk et al., 2020). This is made clear in the present study by the numerous peptides from ATP-consuming proteins found (e.g., 52 peptides of V-ATPase). Vacuolar H<sup>+</sup>-ATPases (V-ATPases) are found ubiquitously in eukaryotic organisms and functions as a proton-pump to aid in physiological function of cells (Finbow and Harrison, 1997). The role of V-ATPases has been studied in many insects and been linked to ion and water transport in the midgut of *M. sexta* (Weihrauch et al., 2001) and malpighian tubules of several insects (Maddrell and O'Donnell, 1992).

Our preliminary results showed differential V-ATPase peptide expression in mayflies from all four treatment groups (dilute, NaCl, Ca/MgSO<sub>4</sub>, and CaCl<sub>2</sub>) compared to control mayflies. Nowghani et al. used the mayfly, *Hexagenia rigida*, to examine the role of V-ATPase in ion uptake in gills. They found that the majority of V-ATPase activity occurred in the Malpighian tubules

followed by the hindgut and tracheal gills (Nowghani et al., 2017). However, upon further experimentation they found no differences in Na uptake across different gills or different regions of the same gill. In a later study, the same group found that neither Na/K ATPase or V-ATPase activity changed in the gill of *H. rigida* after exposure to salt-contaminated (NaCl) water using protocols with ouabain or bafilomycin (Nowghani et al., 2019). However, the medial regions of each gill had decreased sodium flux when exposed to salt-contaminated water compared to freshwater (Nowghani et al., 2019).

Mosquito V-ATPase function has been well characterized in midgut and malpighian tubules (Gill et al., 1998), but these animals are air-breathers with anal papillae and are dissimilar from other water-breathing aquatic insects (Misof et al., 2014b). Many studies, including large -omics works, have been published on osmoregulation in mosquitoes (Bradley, 1987; Durant et al., 2021; Uyhelji et al., 2016), but this information not be applicable to many other sensitive aquatic taxa because of the numerous evolutionary and physiological differences.

Aquaporins are highly conserved integral membrane proteins that play a role in water movement between cells, which suggests a potentially major role in osmoregulation. Our preliminary analysis revealed 17 unique aquaporin peptide sequences among samples; the majority of which were not expressed differentially with altered major ion concentrations. Interestingly, there were no differences in aquaporin expression in *Aedes aegypti* larvae reared in either freshwater or brackish water (Misyura et al., 2020). Similarly, we have found no changes in mRNA expression of an aquaporin gene in *N. triangulifer* larvae treated with high concentrations of NaCl (1850 mg/L NaCl) (data not shown). Taken together, aquaporins are clearly present on the gills of *N. triangulifer*, but may not play a critical role in osmoregulation in stressful major ion concentrations.

Likewise, septate junctions provide cellular structure and barriers to solute diffusion in invertebrate intercellular spaces. Early work showed evidence for paracellular fluid flow in the Malpighian tubules of the mayfly, *Ecdyonurus dispar* (Nicholls, 1983) and ionocytes of the mayfly, *Coloburiscoides sp.* (Filshie and Campbell, 1984). Not surprisingly, our preliminary work here identified 2 claudin proteins and 3 tight junction/occluden peptides on the gills of *N. triangulifer*. Only some of these peptides were found to be expressed significantly different than controls among treatment groups, but results were inconsistent. Another group found an important role for septate junctions in the mayfly, *Hexagenia rigida* (Nowghani et al., 2017), and the mosquito, *A. aegypti* (Jonusaite et al., 2016). Importantly, previous work in *N. triangulifer* demonstrated that even at stressful salinities, larvae were able to maintain ionic hemolymph concentrations despite increased uptake rates (Buchwalter et al., 2018), unlike the observations in *H. rigida* (Nowghani et al., 2017). Species-specific osmoregulatory mechanisms or interpretation of alternative experimental techniques could explain these differences. We concur that paracellular transport pathways are important to consider when studying salinity stress in aquatic organisms, but suggest that the numerous ion transporters found on the gills may have a larger role in osmoregulation.

Fibroblast growth factor (FGF) appears to play a crucial role in directing the growth and branching of the trachea in the gill of the mayfly, *Cloeon dipterum* (Ruiz-Sobrino et al., 2020). Our preliminary analysis revealed 10 unique peptides of FGF or FGF-receptors in the gills of *N. triangulifer*. Further, Ruiz-Sobrino et al. identified the FGF ligand, *branchless (bnl)*, as an important regulator of gill-branching morphogenesis, but this protein was not identified in our preliminary analysis. The branching patterns of the gill trachea have been shown to be plastic to salinity exposure in early work (Wichard et al., 1973) and recent findings from our lab (data not

shown). Specifically, dilute exposures appear to elicit more extensive tracheal branching, which may indicate an increase hemolymph supply to the gill in extremely dilute conditions. Alternatively, saltier exposures reduce the amount of tracheal branching, likely to limit the ionic uptake in gill tissue. Not surprisingly, we also found evidence of highly conserved angiogenesis-related genes in our proteomic analysis including vascular endothelial growth factor (VEGF, 5 peptides), platelet-derived growth factor (PDGF, 2 peptides), and endothelial receptor tyrosine kinase (TIE-2, 2 peptides) (Risau, 1997).

Because of the high energy demand of osmoregulation, ATP synthesis is a crucial process in gill tissues active in apical transport. Thus, it is not surprising that 282 ATP synthase peptides were found in our preliminary analysis and a large portion of these peptides were affected by altered major ion concentrations. Previously, transcriptomics has revealed the induction of ATP synthase transcripts in *N. triangulifer* larvae in response to thermal stress in (Chou et al., 2020), but no other studies on ATP synthesis in response to salinity stress in aquatic insects exist.

Here, we have aimed to identify proteins expressed on the gill tissue of our developing model organism, *N. triangulifer*, to better understand the osmoregulatory biology of mayflies. Our study is unique given the difficulty of tissue-specific experiments in small animals and the lack of -omics work in sensitive aquatic insects. Although analysis is ongoing, it is clear that different major ion compositions elicited significantly differential protein expression. This finding helps explain our previous physiological observations of salinity stress using the same water chemistries as the present study (Orr et al., 2021). Further, we hope to soon finalize and highlight major ion transporting proteins found on the gill that will support a more in-depth future research on specific ion transporters. This proteomic approach is a step to better understanding mayfly osmoregulatory physiology in an increasingly salty world.

### *Author Statement*

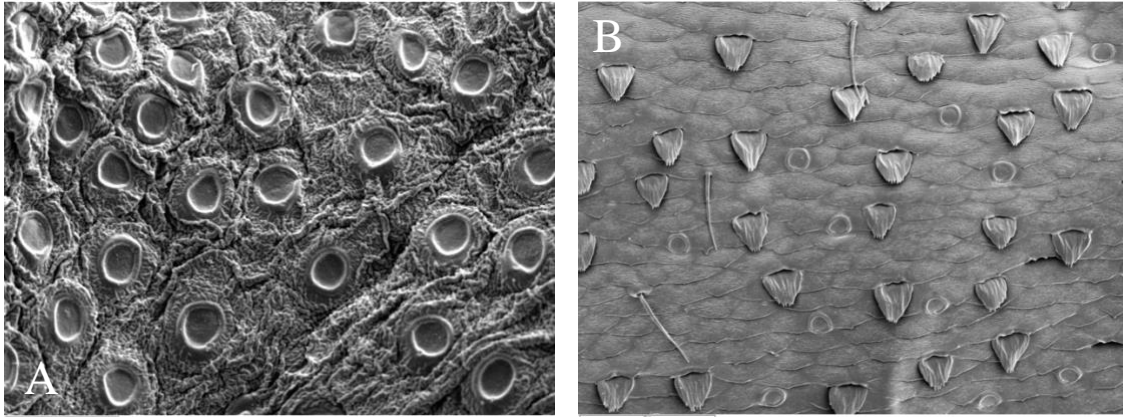
SEO and DBB conceived the concepts of the work and conducted the research. SEO wrote the manuscript. DDJ analyzed and annotated the proteomics data.

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**Table 5.1** Water chemistry for all experimental exposure waters. Cations (Na, Ca, Mg, K) and anions (SO<sub>4</sub>, Cl, CO<sub>3</sub>) are all listed in mg L<sup>-1</sup>. Waters were sampled, filtered, and verified by North Carolina State University’s Environmental and Agriculture Testing Services Lab (ICP-EATS) and concentrations were within 10% of nominal values. Measured values are listed in parentheses beside nominal values.

	<b>Na</b>	<b>Ca</b>	<b>Mg</b>	<b>K</b>	<b>S</b>	<b>SO<sub>4</sub></b>	<b>Cl</b>	<b>CO<sub>3</sub></b>	<b>pH</b>
<b>Control (ASW)</b>	15.3 (16.5)	12.7 (11.6)	3.4	1.4 (1.92)	7.78 (7.9)	23.3	14.1	42.6	8.14
<b>Dilute (50% ASW)</b>	7.65	6.35	1.7	0.7	3.89	11.65	7.05	21.3	7.99
<b>Elevated CaCl<sub>2</sub></b>	15.3	121 (113)	3.4	1.4	7.78	23.3	97	42.6	8.07
<b>Elevated NaCl</b>	157 (152)	12.7	3.4	1.4	7.78	23.3	255	42.6	7.67
<b>Elevated Ca/MgSO<sub>4</sub></b>	15.3	193.7	79.1	1.4	245 (256)	735	14.1	42.6	8.06



**Figure 5.1** Scanning electron microscopy of the ionocytes on the gill (A) and abdomen (B) of a mature *N. triangulifer* larvae.

**Table 5.2** Preliminary summary of main ion transport proteins found in gills of *N. triangulifer* larvae.

ABC transporter	Phosphate import ATP-binding protein
Acid-sensing ion channel	Potassium channel
Amiloride-sensitive sodium channel	Potassium proton antiporter
Anion exchange protein	Potassium uptake protein
ATP-binding cassette	Potassium-transporting ATPase
Calcium-activated chloride channel regulator	Sodium channel protein
Calcium-transporting ATPase	Sodium transport ATPase
Calcium-uniporter protein	Sodium/calcium exchanger
Cation channel sperm-associated protein	Sodium/glucose cotransporter
Cation-transporting P-type ATPase	Sodium/potassium ATPase
Cation/calcium exchanger	Sodium/potassium/calcium exchanger
Cation/H(+) antiporter	Sodium/proton antiporter
Cationic amino acid transporter	Sodium/sulfate cotransporter
Chloride channel	Sulfate transporter
Magnesium transporter	V-type proton ATPase



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## **CHAPTER SIX**

### **CAN PHYSIOLOGICAL TRAITS OF AQUATIC INSECTS PROVIDE CLUES ABOUT THEIR SENSITIVITIES TO CHANGING SALINITY REGIMES?**

S. E. Orr, J. K. Cochran, D. A. Dickey, and D. B. Buchwalter

North Carolina State University

## Abstract

Freshwater salinity regimes, which vary naturally, are further changing in response to anthropogenic activities and sensitive aquatic taxa are posed with physiological challenges of dealing with increasing or decreasing major ion concentrations. Some generalist species occur over a wide range of salinities, while other specialist species appear to have more narrow ranges of salinity tolerance. It is not clear what factors determine these salinity niches and how organisms may differentially respond to salinity changes in nature. We hypothesized that physiological traits, such as ion uptake rates, may explain salinity tolerances of different species of aquatic insects. Specifically, we examined the ability of different species to increase ion uptake rates under dilute conditions and decrease uptake rates under rich conditions. We used radiotracers ( $^{22}\text{Na}$ ,  $^{35}\text{SO}_4$ ,  $^{45}\text{Ca}$ , and  $^3\text{H}_2\text{O}$ ) to compare ion and water uptake rates in mayflies (*Neocloeon triangulifer*, *Isonychia* sp., *Maccaffertium modestum*, *Drunella coloradensis*, *Callibaetis floridanus*), caddisflies (*Hydropsyche betteni*), and mosquitoes (*Aedes albopictus*). We also assessed cuticular permeability by measuring the loss of previously acquired  $^{22}\text{Na}$  after 9 h of deionized water challenge in these same taxa. There are clear physiological differences in transport dynamics (e.g., 19-fold difference in Ca uptake rate across 5 taxa in control water) and permeability among species (e.g., *N. triangulifer* lost 29% of its  $^{22}\text{Na}$  label after 9 h DI water challenge while *H. betteni* lost only 11%). Further, water influx happened rapidly, but our experiments revealed an interesting concept of an “exchangeable pool of body water” that varied among species (68% in *A. albopictus* and 44% in *N. triangulifer*). We also found that water flux remained unchanged by salinity challenge. This work highlights the unique osmoregulation strategies evolved by different aquatic insect taxa and how these physiological differences contribute to major ion toxicity in a changing world.

## **Introduction**

The fascinating and convoluted evolution of insects has resulted in the most specious, diverse group of animals on Earth (Bradley et al., 2009). Aquatic insects are particularly interesting because of their multiple invasions of freshwater over millions of years (Misof et al., 2014). Aquatic insects have evolved various strategies for gas exchange in aquatic environments and osmoregulation in hypotonic freshwaters (Resh et al., 2008; Silver and Donini, 2021). The extreme diversity among freshwater insects results in a wide range of ecological niches. Some tolerant groups, such as mosquitoes, can thrive in environments with high concentrations of pollutants and a wide range of temperatures, oxygen levels, and salinity concentrations (Bradley, 1987). Other specialist taxa, like mayflies, are known to be particularly sensitive to pollutants (Brinkman and Johnston, 2008; Kim et al., 2012; Xie and Buchwalter, 2011) and abiotic factors (Jackson and Funk, 2019; Kefford, 2018; Orr et al., 2021). Understanding this range in sensitivities is particularly relevant in the Anthropocene because organisms are exposed to toxicants and dramatic changes in abiotic factors, like temperature and salinity.

Freshwater ionic composition varies spatially with geology, and further fluctuates with rainfall, evaporation and hyporheic influences (Canedo-Arguelles et al., 2016; Iglesias, 2020). However, recently, anthropogenic freshwater salinization has emerged as an ecological dilemma worldwide (Canedo-Arguelles et al., 2016; Kaushal et al., 2018). Anthropogenic activities such as fracking (Entrekin et al., 2011), mountain top coal mining (Pond et al., 2008), and road de-icing (Corsi et al., 2010) can significantly impact the concentrations of major ions in nearby freshwaters. Further, changes in precipitation, acid deposition, and weathering are decreasing the major ion content of freshwater systems elsewhere. (Driscoll et al., 2001; Jeffries et al., 2003; Johnson et al., 1969; Likens and Buso, 2012). In general, salinity is a key factor in determining where species can



thrive (Cormier & Suter, 2013) and scientists have observed decreases in biodiversity correlated with freshwater salinization and sensitive aquatic insects, such as mayflies, appear to be disproportionately affected (Cormier et al., 2013a; Pond et al., 2008). Different groups have proposed physiological explanations for salt sensitivity including pH imbalance, ionic poisoning, energetic costs, or a combination of these (Kefford, 2018; Verberk et al., 2020). Previous work in our lab has strongly supported the energetic cost hypothesis (Buchwalter et al., 2018; Orr et al., 2021), but the mechanistic explanations for why some species thrive and others disappear remains elusive.

Aquatic insect taxa have various strategies for regulating salt and water balance in dilute freshwater environments. For example, mosquitoes have anal papillae specialized for ion uptake (Durant et al., 2021), while mayflies have gills that vary in size and shape to obtain oxygen and major ions (Sartori and Brittain, 2015). Further, some species of caddisflies express ionoregulatory epithelial tissues on their abdomens (Wichard and Komnick, 1973). Mitochondrial-rich ionocytes expressed on osmoregulatory tissues can vary in number and size depending on species and environment (Komnick, 1977; Wichard et al., 1973). However, it is unclear to what degree different morphological and physiological characteristics play a role in salinity tolerance among taxa.

Freshwater organisms are typically found in environments in which they are hypertonic to the external medium and preventing excessive water uptake is an osmoregulatory priority. A previous study used tritium to show that bigger, air-breathing aquatic insects had slower water accumulation than smaller, dissolved oxygen breathers (Buchwalter et al., 2002). These findings suggest that smaller, gill-breathing aquatic taxa may be more permeable to their environment and susceptible to osmoregulatory stress. Similarly, we hypothesized that measuring water

accumulation would provide important information on the osmoregulatory mechanisms and tolerance to salinity changes among multiple aquatic taxa.

Here, we aimed to characterize the osmoregulatory physiology of several distinct species of aquatic insects in a common garden experiment. We used radiotracer experiments to estimate ion uptake rates ( $^{22}\text{Na}$ ,  $^{35}\text{SO}_4$ ,  $^{45}\text{Ca}$ ) across dilute and salty test solutions. Next, we measured water uptake with tritiated water ( $^3\text{H}_2\text{O}$ ) and discuss the relatively novel idea of species-specific exchangeable pools of body water. We also examined differences in Na loss in deionized water via radiotracer efflux experiments. Finally, we discuss our findings in the context of salinity niches in a changing world and the critical need for updated major ion water quality criteria.

## Methods

### *Animal collection and rearing*

Parthenogenetic *N. triangulifer* mayflies were reared in the laboratory and originally obtained from White Clay Creek in Pennsylvania (Clone WCC-2) by collaborators at Stroud Water Research Center (SWRC; Avondale, Pennsylvania) (Sweeney and Vannote, 1984). *N. triangulifer* eggs were incubated at room temperature (21-23°C) until hatched and were then seeded into glass pyrex dishes with control water [artificial soft water (ASW); Table 6.1]. ASW was made with recipes from the United States Environmental Protection Agency (D. Mount, EPA, Duluth, Minnesota, 2017, personal communication) using a base of pure distilled water (18.0 megohm) and laboratory-grade salts (Thermo Fisher Scientific, Waltham, Massachusetts). Larvae were reared on the benchtop at room temperature with gentle aeration, parafilm cover, and a 14:10-h light:dark photoperiod. We fed *N. triangulifer* larvae with natural periphyton ad libitum on acrylic plates (6.5 x 23 x 0.15 cm) from SWRC. Mature larvae (~25 d rearing) were then removed from rearing dishes and used in experiments.

Asian tiger mosquitoes, *A. albopictus*, were originally collected from Augusta, GA and have been kept as a laboratory colony at NC State University in the Reiskind lab. Mosquito larvae were hatched into rearing trays with 1 L of ASW and 1g ground Wardley Pond Pellets fish food. Larvae were kept at 27.5°C, 50% relative humidity, and 14:10 light:dark photoperiod. On day 3, 1<sup>st</sup> instars were separated into new rearing trays, with each tray receiving 100 larvae, 1 L ASW, and 1g food. After 6 days, 4<sup>th</sup> instars were removed for experiments right before pupation.

*D. coloradensis* mayflies were collected from Logan, Utah in a mountain stream in Temple Fork (41.827931°, -111.578736°) by removing larvae from rock bottoms with forceps. Live larvae

were packaged with ice packs and shipped overnight to NC State University for experimental use. Larvae were acclimated in ASW and experiments were performed in our walk-in cooler at 15°C.

*C. floridanus* mayfly larvae were collected from a pond at Worton, Maryland (39.333942°, -76.150896°), which historically has large fluctuations in salinity because of seawater intrusion. The pond had a conductivity of 7500  $\mu\text{S}/\text{cm}$ , caused by marine influx. Larvae were reared at SWRC in natural stream water and the adults were mated manually. Gravid adult females were transferred to NC State University where we obtained fresh hatchlings and reared in ASW at room temperature. *C. floridanus* larvae were treated identically to *N. triangulifer* larvae as described above. However, *C. floridanus* develop rapidly compared to *N. triangulifer* (~10 days at room temperature) and are sexually dimorphic. Male and female larvae were randomly mixed for all experiments.

*Isonychia sp.* and *M. modestum* were collected from the Eno River in Durham, North Carolina (36.081°, -79.140°) in multiple collecting trips during fall or winter. A D-frame kick net was used to collect mayflies in riffle areas of the river and animals were transferred back to NC State University in an aerated cooler. Because these both *Isonychia sp.* and *M. modestum* were collected in cold seasons, animals were acclimated and experiments were performed in our walk-in cooler at an appropriate temperature (5-15°C). *H. betteni* caddisflies were collected from a riffle flowing out of Yates Mill Pond in Raleigh, North Carolina (35.7180517°, -78.6861007°) by removing larvae from rock bottoms with forceps. Animals were transferred back to NC State University in a mason jar with mesh substrate and acclimated in ASW at room temperature. *Glaenocorisa sp.* water boatman were collected with a D-frame kick net by skimming the surface of lentic water on the edge of Yates Mill Pond (35.7205498°, -78.6891648°). Animals were transferred back to NC State University and acclimated to ASW at room temperature. All field

collected animals were starved and allowed to acclimate in aerated ASW in laboratory conditions for 24-48 hours before experiments began.

To characterize evolutionary relationships among taxa, we used NCBI taxonomy identifiers and PhyloT (v2) to create a dendrogram. We also live imaged all organisms studied on a MZ 16F stereoscope (Leica, Wetzlar, Germany) to illustrate morphological differences among taxa.

### *Ion flux experiments*

To characterize osmoregulatory physiology among species and water chemistries, we chose to use 5 waters for each major ion of interest (Na, Ca, and SO<sub>4</sub>; Table 6.1). Uptake rates for each ion were measured in control (ASW), 2 dilute waters, and 2 salty waters. The control and dilute waters (6.25% control and 25% control) were the same among all experiments. For each ion, we amended the salty waters with NaCl, CaCl<sub>2</sub>, and Ca/MgSO<sub>4</sub> for Na, Ca, and SO<sub>4</sub> experiments, respectively. We chose concentrations of major ions based on relevant environmental major ion concentrations and previous laboratory studies (Orr et al., 2021). For each experiment, our “medium” concentrations were 387, 480, and 630 mg/L Na, Ca, and SO<sub>4</sub> and our “high” concentrations were 743, 980, and 1050 mg/L Na, Ca, and SO<sub>4</sub>, respectively (Table 6.1).

To characterize ion uptake rates across species and waters with varying salinities, we used radiotracers with exposure activities ranging from 156 to 260 Bq/mL. Experiments were appropriately replicated into multiple spatially randomized 100 mL high-density polyethylene beakers with 15-20 mL of experimental solution and a parafilm cover. Gentle aeration was provided to all species except *A. albopictus*, which thrive in lentic environments. Because <sup>22</sup>Na is a gamma-emitting isotope, we used the Wizard 3 Gamma Counter (Perkin Elmer, Billerica,

Massachusetts), which can accurately count non-homogenized samples including live animals. Thus, some Na uptake experiments followed the same individual animal over a short time course (3, 6, 9 h). For the beta-emitting  $^{35}\text{SO}_4$  and  $^{35}\text{Ca}$  experiments, we used the LS6500 multipurpose scintillation counter (Beckman Coulter, Brea, California), which requires animals to be euthanized and fully homogenized before counting. Sulfate uptake experiments used a short time course (3, 6, 9 h), but calcium uptake experiments were much longer (12, 18, 24, or 36 h) because calcium moves into the body much slower than Na or  $\text{SO}_4$ .

At each time point, animals were removed from radioactive water and prepared for analysis. We rinsed animals in two consecutive baths of clean water to remove any ions adsorbed to the exoskeletons. For Ca uptake experiments, we additionally rinsed animals in 0.05 M ethylenediaminetetraacetic acid and 0.1 M L-ascorbic acid sodium salt because Ca tends to be more adsorbent than other ions (Poteat and Buchwalter, 2014). For Na uptake experiments, animals were counted in 20 mL glass vials with clean water and then immediately put back into their experiment solutions. For Ca and  $\text{SO}_4$  uptake experiments, animals were patted dry, weighed, and digested with 500  $\mu\text{L}$  of Soluene 350 (PerkinElmer) in 20 mL glass vials for 48 h in a dark, 28°C incubator. After digestion was complete, we neutralized samples with 500  $\mu\text{L}$  of glacial acetic acid, added 12 mL of scintillation cocktail (PerkinElmer Ultima Gold uLLT), and counted each sample with the Beckman LS6500 multipurpose scintillation counter.

All measurements were corrected for chemical quench and counting efficiency, which varies among isotopes. We only included measurements with lumex (non-radioactive luminescence) values <5%. All data was normalized by the wet mass of each sample, to account for size variability among replicates. Ongoing statistical analysis will incorporate the effect of sample mass to tease out possible size/weight influence.

### *Water flux experiments*

To characterize water flux, we made tritiated water ( $^3\text{H}_2\text{O}$ ) (Perkin Elmer) in our control water (ASW) with high exposure activities (400 to 450 Bq/mL) because of low counting efficiency. Experiments were performed in clean, 100 mL high-density polyethylene beakers with 20 mL of experimental water and sealed with parafilm to prevent evaporation. Gentle aeration was provided to appropriate species only, excluding *A. albopictus* and *Glaenocorisca sp.*, which thrive in lentic environments. We hypothesized that water flux would happen rapidly and thus, a short time course of 2, 4, and 6-h was chosen to try and capture uptake. After we realized that there was an apparent “exchangeable pool” of body water, we performed a long time course experiment (2, 4, 6, 12, 24, and 36-h) with *H. betteni* to determine if the total water replace would slowly increase over time.

### *Efflux experiments*

Larvae were placed in a 200 mL high-density polyethylene beaker with 25 mL radioactive standard water for 48 hours to acquire a strong  $^{22}\text{Na}$  signal (average 14 Bq per mg insect wet weight). All beakers were gently aerated (except for *A. albopictus* experiments) and sealed with ParaFilm™. At 48 hours, larvae were removed from the radioactive exposure waters by gently pipetting into a mesh strainer (collecting any residual radioactive water in a waste container) and blotting dry. The larvae were then rinsed in two consecutive water baths of the corresponding exposure water to remove loosely adsorbed ions from the exoskeleton. An individual larva was then placed in a 20 mL glass vial with 3 mL of deionized water and counted with the PerkinElmer Wallac Wizard 1480 Automatic Gamma Counter (Shelton, CT) for 3 minutes. The larva was then placed in a 100 mL high-density polyethylene beaker with 100 mL of deionized water. At 3 and 9

h, larvae were removed from the experimental cup by gently pipetting into a 20 mL glass vial with 3 mL of deionized water and counted with the PerkinElmer Wallac Wizard 1480 Automatic Gamma Counter (Shelton, CT) for 3 minutes. The experimental cups were refreshed with 100 mL of fresh deionized water while the larvae were counted to minimize re-uptake of lost ions. The same larva was followed across the full experiment.

### *Data analysis*

Size and shape vary dramatically among the species we used in this work. We posit that a robust statistical analysis is necessary to appropriately tease out mass effects and reveal true physiological differences among species. We are currently working with statistician, Dr. David A. Dickey, to analyze our data properly. We also hope to reveal relationships of ion transport rates within species (i.e., if a species transports a lot of Na, do they also transport a lot of Ca and SO<sub>4</sub>?). While analysis is ongoing, strong differences between species have emerged already and we are optimistic that this effort will be an interesting and important contribution to the field of comparative physiology.



## Results and Expected Outcomes

The emergence of freshwater salinization has led to severe decreases in aquatic biodiversity in affected areas (Cormier et al., 2013a; Kaushal et al., 2018; Pond et al., 2008). In particular, mayfly species (Ephemeroptera) appear to be disproportionately affected by elevated major ion concentrations compared to more tolerant taxa, such as mosquitoes (Cormier et al., 2013a), but the mechanistic explanations are unclear. Interestingly, aquatic insects evolved through multiple invasions of freshwater (Misof et al., 2014), which has led to a diverse set of strategies for osmoregulation among taxa. Here, we hypothesized that differences in sensitivity to salinity changes may be best explained by physiological traits, including ion uptake rates. We measured uptake rates of Na, Ca, SO<sub>4</sub>, and H<sub>2</sub>O in several different waters, ranging from dilute to salty, and Na loss in deionized water using radiotracers with several different species of aquatic insects. This common garden experiment has begun to reveal obvious species-specific differences of osmoregulatory traits that cannot be explained by size alone and provides important information about physiological limitations in an increasingly salty world. Although our statistical analysis is ongoing, we will discuss our preliminary results here.

### *Species descriptions*

This project examined physiological traits of many different aquatic insects, but included 5 distinct species of mayflies (Ephemeroptera). We also studied three “outgroups” from Diptera (*A. albopictus*), Hemiptera (*Glaenocorisa sp.*) and Trichoptera (*H. betteni*). The 2 closest related species in our project were *N. triangulifer* and *C. floridanus*, which are both members of the family Baetidae, but have drastically different sensitivities to elevated major ion concentrations based on previous *N. triangulifer* work (Orr et al., 2021) and the source water conductivity *C. floridanus*

was collected from. The dendrogram of the primary 7 taxa used in our experiments reveals important evolutionary relationships among species (Figure 6.1).

We also illustrated morphological differences by imaging live larvae (Figure 6.2). Relevant information including order, respiration strategy, and average mass is summarized in Table 6.2. Mass is an especially important description because two of the most physiologically different species (*A. albopictus* and *N. triangulifer*) were the two smallest animals on average (~3 mg wet weight).

#### *Ion uptake rates vary among salinities and species*

We performed a series of ion uptake experiments across multiple salinities and species to characterize osmoregulatory traits in a common garden experiment. For each major ion of interest (Na, SO<sub>4</sub>, and Ca), we characterized ion uptake for 5 different concentrations (Table 6.1, Table 6.3). For all major ions of interest, Na is transported at the highest rates in control and dilute waters among all species. Generally, uptake rates for Na > SO<sub>4</sub> ~ Ca for all species, which has been demonstrated in other studies as well (Orr and Buchwalter, 2020; Orr et al., 2021). However, as we increase the major ion concentrations, SO<sub>4</sub> uptake rates increase exponentially and sometimes exceed Na uptake rates in comparable waters (for *H. betteni* and *A. albopictus*) (Table 6.3). For both Na and Ca, species had ion uptake rates that were relatively similar among all water chemistries. Generally, Na and Ca uptake rates for *C. floridanus* > *N. triangulifer* > *D. coloradensis* > *Isonychia sp.* > *M. modestum* > *A. albopictus* > *H. betteni*. For SO<sub>4</sub>, uptake rates for species varied and were not considerably different. Surprisingly, *A. albopictus* had the greatest SO<sub>4</sub> uptake rate in all water chemistries compared to the other species.

### *Na loss in DI water*

To characterize the cuticular permeability and ability for animals to resist ion loss in DI water, we performed a Na efflux experiment. Species varied in their ability to retain newly acquired  $^{22}\text{Na}$  over the 9 hour experiment (Figure 6.1). Not surprisingly, these results appeared to correlate with the Na uptake rates (Table 6.3). In general,  $^{22}\text{Na}$  was lost in *C. floridanus* ( $38.5 \pm 4.1\%$ ) > *N. triangulifer* ( $29.0 \pm 2.6\%$ ) > *A. albopictus* ( $22.4 \pm 5.3\%$ ) > *D. coloradensis* ( $21.5 \pm 3.5\%$ ) > *M. modestum* ( $18.3 \pm 2.0\%$ ) > *Isonychia sp.* ( $17.5 \pm 4.3\%$ ) > *H. betteni* ( $10.5 \pm 2.7\%$ ).

### *Exchangeable pool of body water varies among species*

To assess the differences in water flux among species, we performed a time course assay with tritiated ASW with *M. modestum*, *Isonychia sp.*, *H. betteni*, *N. triangulifer*, *A. albopictus*, *C. floridanus*, and *Glaenocorisa sp.* Raw data revealed we had missed the unidirectional uptake of water at 2-h because of the rapid influx of water among all species. We performed a Michaelis-Menten nonlinear regression analysis to obtain  $V_{\max}$  values that would represent percent body water exchanged (Figure 6.3). Surprisingly, the  $V_{\max}$  values were lower than we expected and varied between 44.5 and 71.5% total body water exchanged. Air breathing species (*Glaenocorisa sp.* and *A. albopictus*) had the highest percentages of body water exchanged ( $71.5 \pm 6.5\%$  and  $68.2 \pm 5.8\%$ , respectively). The one caddisfly species tested, *H. betteni*, had  $52.0 \pm 8.7\%$  exchangeable body water. All of the mayfly species tested were quite similar in their exchangeable pools of body water. *M. modestum*, *Isonychia sp.*, *N. triangulifer*, and *C. floridanus* larvae had  $44.6 \pm 5.7$ ,  $52.8 \pm 8.6$ ,  $44.5 \pm 4.7$ , and  $48.9 \pm 6.4\%$  body water exchanged, respectively. We performed a One-Way ANOVA to verify that there were no statistical differences among data ( $p > 0.99$ ).

To verify that our time course was not too short, we performed a longer experiment that included 12, 24, and 36-h in *H. betteni* larvae. This experiment resulted in a similar Michaelis-Menten regression with a  $V_{\max}$  of  $50 \pm 1.8\%$  total body water exchanged (data not shown). We ran a Student's t-test to confirm that these experiments were not significantly different ( $p = 0.83$ ). Clearly, there is a large portion of body water that is not readily exchangeable ( $< 36$  h) with the external environment in freshwater insects.

We additionally performed water uptake experiments with *M. modestum*, *Isonychia sp.*, and *H. betteni* with different concentrations of  $\text{CaCl}_2$  (480 and 980 mg/L Ca) to assess the influence of salinity on water flux (data not shown). We calculated the percent body water replaced by tritiated water and performed a Michaelis-Menten nonlinear regression analysis. In *M. modestum*, the  $V_{\max}$  ( $\pm$  standard error) for 12, 480, and 980 mg/L Ca was  $44.6 (\pm 5.5)$ ,  $42.9 (\pm 6.2)$ , and  $63.25 (\pm 10.9)$ , respectively. For *Isonychia sp.*, the  $V_{\max}$  for 12, 480, and 980 mg/L Ca was  $52.8 (\pm 8.3)$ ,  $63.1 (\pm 11.8)$ , and  $37.1 (\pm 3.7)$ , respectively. For *H. betteni.*, the  $V_{\max}$  for 12, 480, and 980 mg/L Ca was  $52.0 (\pm 8.3)$ ,  $42.4 (\pm 6.3)$ , and  $30.5 (\pm 5.4)$ , respectively. Then, we used a two-way ANOVA to test if variation among % body water replaced could be explained by salinity, species, or an interactive effect. Although our model lacked significant main effects of salinity ( $F(2, 36) = 0.6$ ,  $p = 0.55$ ) and species ( $F(2, 36) = 1.3$ ,  $p = 0.28$ ), there was a significant interaction ( $F(4,36) = 3.1$ ,  $p = 0.03$ ). Thus, there is no overall effect of salinity or species on water influx, but there is a crossover interaction.

### *Analysis and summary*

The distribution of aquatic insects is controlled by various factors including both physiological and ecological restraints (Cormier et al., 2013b; Kefford, 2018). Although our

statistical analysis is ongoing, we believe that characterizing osmoregulatory physiology of aquatic insects will allow us to better understand species distributions and tolerances.

Recently, we demonstrated that *N. triangulifer* larvae were able to acclimate to challenging salinity concentrations in an ion-specific manner (Orr et al., 2021). This may help explain the extreme tolerance of *C. floridanus* mayflies that were collected from a pond with extreme seawater intrusion. Perhaps, this population has adapted to high concentrations of seawater over multiple generations of exposure. Our results show that *C. floridanus* larvae have extremely high Na and Ca uptake rates compared to other similar species. We suspect that these animals must have a large energy budget for ion turnover and have finely tuned excretory systems. Further, stressful concentrations of major ions may divert energetic costs away from other crucial functions, such as growth and reproduction (Verberk et al., 2020). Although acclimatory abilities are clearly important, there are limits to physiological plasticity and nonlethal endpoints should also be considered.

Our study demonstrates that aquatic insects have an exchangeable pool of body water that is replaced rapidly (< 1 hour), which is an idea that has not been addressed for decades. In 1975, a tritium pond mesocosm study was performed with carp, clams, crayfish, and other aquatic organisms (Strand et al., 1975). This group found that there was an initial rapid uptake by a “loose water fraction” and a secondary slower rate of uptake that did not reach equilibrium after 7-months (Strand et al., 1975). It has been suggested that tritium and water are not incorporated into organic molecules at equal rates because of the weight of hydrogen atoms in each. Similarly, rats administered tritium through water rapidly eliminated the dose through urine, while rats administered organically bound tritium through food incorporated tritium into organs with high metabolic activity (Rochalska and Szot, 1977). Another rat study speculated that tissue-bound

tritium is “firmly bound” and not continuously replaced from body water (Thompson and Ballou, 1954).

To our knowledge, very few studies have used tritium to study water fluxes in aquatic insects. One study showed that tritium uptake rapidly reached 100% steady state in some smaller, gill-breathing taxa (e.g., *C. riparius*, *Callibaetis* sp., and *Psectrotanypus* sp.), but was slower in larger, air-breathing taxa (e.g., *N. kirvyi*, *Berosus* sp., and *Pychoptera* sp.) (Buchwalter et al., 2002). These contrasting results motivated us to perform the longer time course experiment with *H. betteni*, which further demonstrated a tightly regulated exchangeable pool of body water around 50%. Although we can only speculate experimental differences between our study and this one, we believe that this important, basic biological understanding of body water should be explored further in the future.

In summary, our preliminary results suggest that functional traits of osmoregulation may help explain sensitivity differences to challenging salinities. Overall, this work highlights the unique osmoregulation strategies evolved by different aquatic insect taxa and how these physiological differences contribute to major ion toxicity. Importantly, linking key physiological traits to fitness outcomes may also allow scientists to predict future “winners” and “losers” in an increasingly salty world.

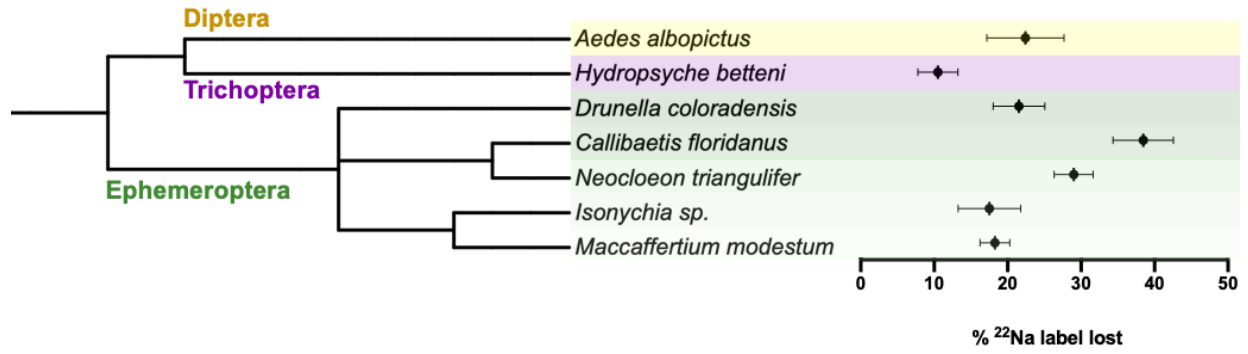
**Table 6.1** Water chemistry for all waters used in our experiments. Some ions for all waters were measured with ICP-MS and the measured concentrations are listed in parentheses next to the nominal concentration. All waters were amended from the same base water (ASW).

	Na	Ca	Mg	K	S	SO <sub>4</sub>	Cl	CO <sub>3</sub>	pH	Cond. (µS/cm)
<b>Control (ASW)</b>	15.3 (15.5)	12.7 (11.6)	3.4 (3.3)	1.4 (1.92)	7.78 (7.9)	23.3	14.1	42.6	7.37	130.7
<b>DI</b>	<0.1	<0.02	<0.1	NA	<0.01	NA	NA	NA	7.03	1.1
<b>ASW/16</b>	0.85	0.95	0.22	NA	0.63	NA	NA	NA	7.05	9.8
<b>ASW/4</b>	4.04	3.60	2.60	NA	1.14	NA	NA	NA	7.58	34.4
<b>480 Ca</b>	15.3	480 (471)	3.4 (3.3)	1.4	7.78 (7.9)	23.3	158	42.6	7.14	1394
<b>980 Ca</b>	15.3 (15.5)	980 (915)	3.4 (3.8)	1.4	7.78 (7.9)	23.3	1555	42.6	6.91	4618
<b>387 Na</b>	387 (414)	12.7 (11.69)	3.4	1.4	7.78 (8.2)	23.3	615	42.6	7.64	1204
<b>743 Na</b>	743 (915)	12.7 (11.99)	3.4	1.4	7.78 (8.6)	23.3	1165	42.6	7.89	3361
<b>630 SO<sub>4</sub></b>	15.3 (16.8)	88.7 (85.1)	107 (103.0)	1.4	210 (212)	630	4.6	12.3	7.44	562
<b>1050 SO<sub>4</sub></b>	15.3 (17.0)	148 (143.6)	178 (178.1)	1.4	351 (365)	1050	4.6	12.3	7.39	1735

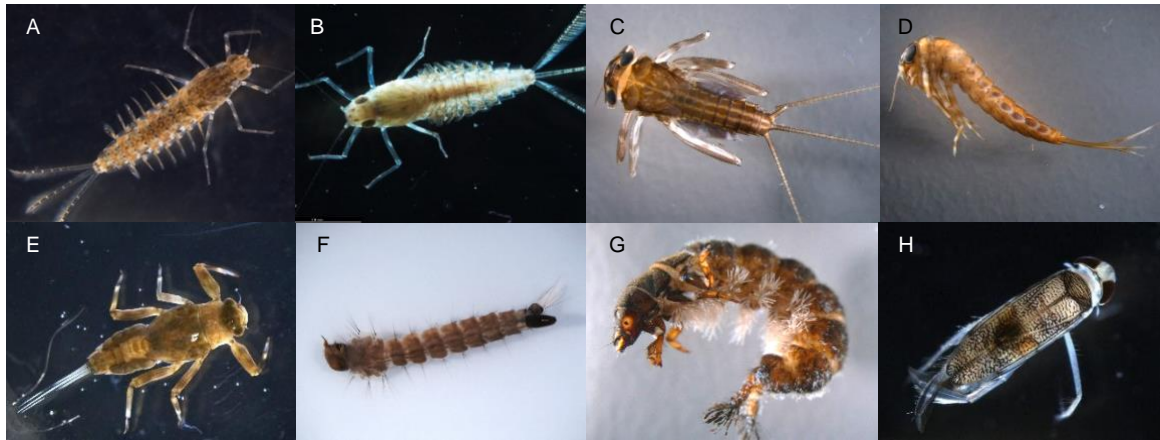
**Table 6.2** Information on all 8 species of aquatic insects used in our experiments.

<b>Species</b>	<b>Order</b>	<b>Respiration Strategy</b>	<b>Wet Weight (mean <math>\pm</math> SE)</b>
<i>N. triangulifer</i>	Ephemeroptera	Gills	3.17 $\pm$ 0.48
<i>Isonychia sp.</i>	Ephemeroptera	Gills	24.14 $\pm$ 0.98
<i>M. modestum</i>	Ephemeroptera	Gills	19.21 $\pm$ 0.74
<i>D. coloradensis</i>	Ephemeroptera	Gills	28.67 $\pm$ 0.61
<i>C. floridanus</i>	Ephemeroptera	Gills	28.56 $\pm$ 0.61
<i>H. betteni</i>	Trichoptera	Gills	21.28 $\pm$ 0.7
<i>A. albopictus</i>	Diptera	Air	3.287 $\pm$ 0.07
<i>Glaenocorisa sp.</i>	Hemiptera	Air	6.75 $\pm$ 0.60





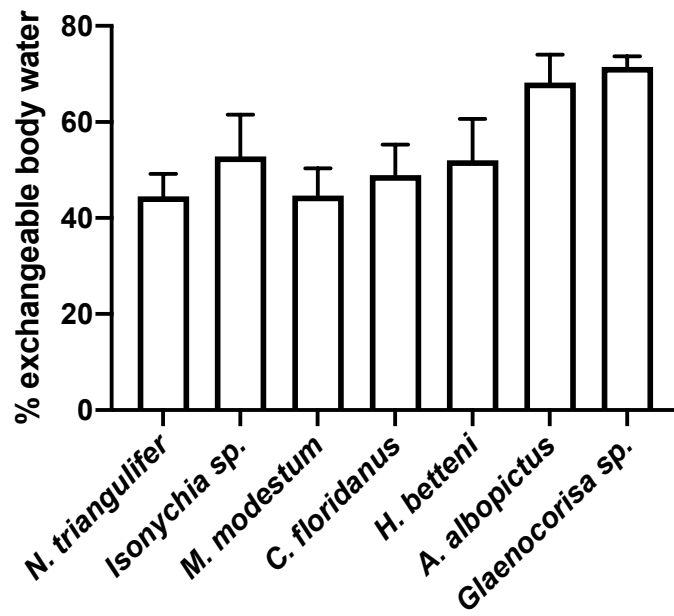
**Figure 6.1** Phylogenetic dendrogram of 7 aquatic insect species with the % <sup>22</sup>Na label lost in a 9-hour DI exposure. Dendrogram was made with PhyloT online software. Error bars represent standard error of the mean.



**Figure 6.2** Microphotographs of 8 species of aquatic insects used in our experiments: (A) *N. triangulifer*, (B) *C. floridanus*, (C) *M. modestum*, (D) *Isonychia sp.*, (E) *D. coloradensis*, (F) *A. albopictus*, (G) *H. betteni*, and (H) *Glaenocorisa sp.*

**Table 6.3** Na, Ca, and SO<sub>4</sub> uptake rates for *N. triangulifer*, *H. betteni*, *Isonychia sp.*, *M. modestum*, *A. albopictus*, *D. coloradensis*, and *C. floridanus* in 5 different waters. Average uptake rates represent the linear regression of a full time course experiment.

Species	Ion	6.25% Control 1 mg/L Na 1 mg/L Ca 1.5 mg/L SO <sub>4</sub>			25% Control 4 mg/L Na 4 mg/L Ca 5.8 mg/L SO <sub>4</sub>			Control 15 mg/L Na 15 mg/L Ca 23 mg/L SO <sub>4</sub>			Medium 387 mg/L Na 480 mg/L Ca 630 mg/L SO <sub>4</sub>			High 743 mg/L Na 980 mg/L Ca 1050 mg/L SO <sub>4</sub>		
		average (µg/g/hr)	SEM	n	average (µg/g/hr)	SEM	n	average (µg/g/hr)	SEM	n	average (µg/g/hr)	SEM	n	average (µg/g/hr)	SEM	n
<i>N. triangulifer</i>	Na	14.63	1.468	10	27.065	0.93	5	58.09	5.142	5	92.15	10.06	4	113.8	4.102	4
	Ca	0.3521	0.0868	8	1.093	0.1891	8	3.738	0.153	5	16.11	3.199	5	29.61	8.7	4
	SO <sub>4</sub>	0.3983	0.1378	6	1.434	0.4157	6	4.425	2.194	6	86.45	38.41	6	110.1	39.28	6
<i>H. betteni</i>	Na	1.67	0.2481	7	1.7837	0.484	5	7.458	1.854	5	20.41	2.541	5	35.34	7.141	5
	Ca	0.223	0.02798	7	0.9103	0.1427	6	0.889	0.2687	5	3.389	0.4119	5	6.988	1.051	5
	SO <sub>4</sub>	0.1357	0.066	8	0.4284	0.2243	8	1.778	0.5597	8	51.53	4.188	8	57.42	13.64	8
<i>Isonychia sp.</i>	Na	4.009	0.6412	8	14.94	0.807	4	16.7	2.65	5	31.98	2.012	5	49.82	5.705	5
	Ca	0.1353	0.01248	8	0.2027	0.01939	8	0.3328	0.0767	5	3.88	1.944	5	8.79	1.054	5
	SO <sub>4</sub>	0.1861	0.1579	8	0.4771	0.0751	8	1.167	0.181	8	14.52	1.357	8	30.96	3.902	8
<i>M. modestum</i>	Na	12.13	0.8955	8	14.89	0.9714	8	27.76	1.59	6	30.36	2.44	5	41.33	9.3	5
	Ca	0.2284	0.01732	8	0.526	0.05477	8	0.5318	0.0242	5	4.196	1.171	5	8.484	1.831	5
	SO <sub>4</sub>	0.09097	0.0204	8	0.1427	0.01125	8	0.8847	0.08168	8	24.13	3.335	8	43.57	5.248	8
<i>A. albopictus</i>	Na	3.315	0.6358	8	3.927	0.7434	8	19.29	3.29	12	31.98	2.012	5	49.82	5.705	5
	Ca	0.1907	0.1815	8	0.4021	0.1685	8	0.748	0.1339	7	8.145	1.12	8	14.36	1.217	8
	SO <sub>4</sub>	0.4548	0.2135	8	1.66	0.6768	7	5.408	2.538	8	151.6	42.06	8	177.2	56.39	8
<i>D. coloradensis</i>	Na	6.043	0.834	8	7.878	0.832	7	26.22	2.66	8	50.93	3.896	8	67.54	0.834	8
	Ca	0.1125	0.401	9	0.3828	0.676	8	0.8233	0.283	9	6.736	1.735	9	12.85	1.898	9
	SO <sub>4</sub>	0.1477	0.022	9	0.3884	0.0865	9	0.9926	0.1614	9	19.56	3.997	9	30.16	4.541	9
<i>C. floridanus</i>	Na	11.68	1.503	8	15.92	1.375	8	32.59	2.017	8	138.5	18.07	7	146.5	13.76	8
	Ca	2.646	0.59	8	4.871	0.97	8	6.508	0.55	8	27.96	5.96	8	48.44	9.652	8
	SO <sub>4</sub>	0.4729	0.03845	8	1.27	0.189	8	2.227	0.617	8	39.93	12.22	8	53.38	19.4	8



**Figure 6.3** Percent exchangeable pool of body water among 8 species. Error bars represent standard error of the mean.

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## CHAPTER SEVEN

### CONCLUSIONS AND FUTURE DIRECTIONS

Freshwater salinization continues to threaten the integrity of freshwater ecosystems worldwide. Sensitive aquatic insects, like mayflies, are disappearing in affected areas, but the physiological cause of their demise is not fully understood. In this work, we experimentally addressed the physiological effects of freshwater salinization in aquatic insects. These projects spanned from the molecular biology of gill tissue to life history effects of temperature and salinity stress. Our findings will be summarized here along with overarching conclusions and suggestions of future research.

#### *Summary and conclusions*

First, we explored the relationship between temperature and salinity on major ion transport and toxicity in several species of aquatic insects, detailed in chapter 2. We measured ion uptake rates in four species of aquatic insects: *N. triangulifer*, *I. sayi*, *Maccaffertium sp.*, and *H. sparna* at different temperatures. We found warmer temperatures increased the uptake rate of Na, SO<sub>4</sub>, and Ca, however, clear ion-specific differences emerged. In general, Na was transported faster than SO<sub>4</sub>, which was transported faster than Ca among all species. Increasing temperature from 15°C to 25°C significantly increased Na and SO<sub>4</sub> uptake rates by 2-fold and 4-fold in *H. sparna*. Smaller increases in uptake rates were observed for *I. sayi* and *Maccaffertium sp.*, suggesting species-specific consequences. Because ion transport is energetically expensive, we performed a 96-hour toxicity test to measure sulfate toxicity at different temperatures in *N. triangulifer*. As expected, we found that the warmest temperature (25°C) resulted in only 44% survival in 1500 mg/L SO<sub>4</sub> compared to 78% at 15°C ( $p = 0.0036$ ). We believe that the toxicity



can be explained by energy depletion from excessive ion turnover in saltier conditions. Further, we performed long-term experiments with *N. triangulifer* at various temperatures and salinities to assess the interactive chronic effects on development time. We observed a significant 27.2% increase in development time in mayflies chronically exposed to 1300 mg/L SO<sub>4</sub> compared to controls at 20°C. However, at 26°C, development time was much shorter in all salinities and only had a 23.3% increase from controls to 1300 mg/L SO<sub>4</sub>. Thus, both temperature and salinity changes should be considered for environmental protection of sensitive aquatic insects.

In chapter 3, we revealed minor, albeit significant major ion sensitivity difference among larval life stages of *N. triangulifer*. We characterized the toxicity of three different water chemistries using NaCl, CaCl<sub>2</sub>, and Ca/MgSO<sub>4</sub> among different larval life stages (2-6, 9-13, and 17-21 days) by performing traditional 96-hour toxicity tests. We found that larval sensitivity to salinity was moderately affected by age in a general linear model analysis ( $p = 0.0065$ ) among all salts. We also calculated LC<sub>50</sub> values for NaCl (401, 441, and 570 mg Na/L, respectively) and Ca/MgSO<sub>4</sub> (748, 1503, and 1439 mg SO<sub>4</sub>/L, respectively). The CaCl<sub>2</sub> toxicity tests had surprisingly high survival and thus, we were unable to calculate statistically reliable LC<sub>50</sub> values. Additionally, we used radiotracers (<sup>22</sup>Na, <sup>35</sup>SO<sub>4</sub>, or <sup>45</sup>Ca) to measure ion uptake rates of 18- and 25-day-old *N. triangulifer* larvae and found no significant differences. Importantly, our results demonstrate that younger *N. triangulifer* larvae may be more sensitive to elevated major ion concentrations than mature larvae. Because larger, late-stage larvae are often used experimentally, these findings are imperative to consider as we continue to develop *N. triangulifer* as a model organism.

Next, we asked if *N. triangulifer* mayflies were able to alter their physiology to acclimate to elevated major ion concentrations. We explored this idea in chapter 4 by rearing animals in

either control or elevated ionic conditions and performing acute experiments. First, we used radiotracers to measure ion uptake rates and found significant reductions in Na, SO<sub>4</sub>, and Ca uptake rates for animals that had been previously exposed to elevated concentrations, which was our first evidence of physiological plasticity in these animals. Then, we asked if these acclimatory changes would provide protection from major ion toxicity and performed a series of 96-hour toxicity tests. Interestingly, we found that mayflies that had been previously exposed to elevated Na performed better than controls, but the opposite pattern emerged for SO<sub>4</sub> toxicity tests. We suggest that concentration choices for the acclimation period may play a role in the threshold of beneficial pre-exposure, but also acknowledge that major ions have drastically different physiological roles. Our Ca toxicity tests revealed extreme toxicity with no significant difference in survival between pre-exposed and naïve larvae. Interestingly, we also found that Ca exposure caused cellular degeneration and necrosis in the Malpighian tubules using histomorphology techniques, while other salts did not. Further, we used qRT-PCR to examine the mRNA levels of relevant ion transporting genes and found ion-specific patterns of gene expression in response to salinity. Notably, sulfate exposure led to an increase of sulfate transporter expression in whole-body samples, suggesting a possible efflux function for these particular transporter proteins. Our results reveal the novel finding of acclimation to salinity in *N. triangulifer* and importantly, ion-specific mechanisms of toxicity.

Our acclimation results warranted a more in-depth, molecular examination of physiological changes in response to salinity. In chapter 5, we chronically exposed *N. triangulifer* larvae to a variety of salinities mirroring concentrations from the physiological work in chapter 4. We then removed gills and performed shotgun proteomics. Despite hurdles of non-model organism -omics work, we are close to identifying the exact transporters on the gill tissue

that are likely involved in apical ion transport. Our preliminary results also revealed salinity-induced changes in protein expression. This novel work is essential for developing *N. triangulifer* as a recognized model organism for sensitive aquatic insects and better understanding molecular effects of salinity challenge.

In an ongoing effort to understand the physiological traits that dictate distributions and sensitivities of aquatic insects, we have also presented important information of ion uptake rates, efflux rates, and water uptake among many species of mayflies (*C. floridanus*, *N. triangulifer*, *Isonychia sp.*, *M. modestum*, and *D. coloradensis*), a mosquito (*A. albopictus*), and a caddisfly (*H. betteni*) in chapter 6. Our preliminary results show significant differences in Na, SO<sub>4</sub>, and Ca uptake rates in different waters (ranging from very dilute to very salty) among species in a common garden experiment. We also performed experiments with tritium (<sup>3</sup>H<sub>2</sub>O) in attempt to calculate water uptake rates. With rapid water uptake (< 1 hour), we missed the unidirectional uptake in our time course, but revealed the unique idea of an exchangeable pool of body water. With longer time course experiments, we verified that tritiated water quickly reaches equilibrium in aquatic insects, but only represents 40-70% total body water. We believe this species-specific phenomena can be explained by intracellular body water that is not readily exchangeable with the external environment. We conclude that distributions and sensitivities of aquatic insects can be primarily explained by established osmoregulatory traits evolved over time.

In summary, we found that temperature profoundly influences ion uptake rates and toxicity in aquatic insects. We also found that larval life stage plays a significant role in sensitivity to salts. The implications of this work may also reach into environmental protection and regulation to help build water quality criteria for major ions in the United States. We further discovered that mayflies are physiologically plastic and can acclimate to salinity in an ion-

specific manner. Gill tissue was examined and specific apical ion transporters will soon be revealed through proteomic analysis. Finally, we characterized osmoregulatory traits among many different species of aquatic insects that may help explain sensitivity and ecological distributions. Although our work supports the idea of major ion toxicity caused by energy depletion, we were unable to directly measure this. Overall, we have revealed novel information on the physiology of aquatic insects in response to salinity stress.

### *Future directions*

This work leaves a trail of many additional questions that remain to be answered. In the future, I can image a plethora of research further examining temperature-salinity interactions. In particular, one could use gamma-emitting isotopes (e.g.,  $^{22}\text{Na}$ ) to follow a single individual's Na uptake in multiple temperatures (e.g., 15 and 25°C). This experiment would reveal a highly accurate temperature-induced change in Na uptake rate. Further, my chronic temperature-salinity experiments in chapter 2 only produced one endpoint (development time) because of poor control survival. We suggest a more robust chronic experimental setup to fully characterize the effects of temperature and salinity on survival, size, and development time.

The demonstration of acclimation to salinity in *N. triangulifer* was profound, but generates more questions on the limits of physiological plasticity. It would be interesting to perform similar acclimation experiments in different species of aquatic insects (e.g., *C. floridanus*). Further, more robust and chronic experiments could reveal adaptation to salinity over multiple generations of exposure. Because both *N. triangulifer* and *C. floridanus* have short generation times and can be reared in laboratory conditions, we suggest exposing multiple consecutive generations to a salinity challenge and measuring ion uptake rates, mRNA

expression patterns, and survival. These experiments would reveal insight into the ability for natural populations of mayflies to handle changing salinities over time.

Only two of the research chapters measured tissue-specific responses to salinity. In chapter 4, we showed histomorphological changes in the Malpighian tubules. Histology techniques are relatively inexpensive and widely accepted in biological research. Because of this, we recommend additional studies using histology to study the morphological changes in Malpighian tubules, gills, and other tissues of interest. In chapter 5, we performed shotgun proteomics on gill tissue of *N. triangulifer*, which was challenging, but not impossible. Large numbers of these small animals would be required to dissect out tissues of interest (e.g., gills, Malpighian tubules, gut, brain, etc.) and produce enough biomass for gene expression or -omics studies. However, these types of experiments could be extremely powerful and provide deeper insight into the mechanisms of aquatic insect osmoregulation.

We also posit that more rigorous physiological and molecular research needs to be done in “non-model” organisms, such as mayflies. Specifically, with the growth of -omics level studies, there are endless possibilities to better understand non-model organisms from a genetic/molecular perspective. Our proteomic approach to salinity-induced changes in the gill of *N. triangulifer* would be nicely paired with an additional transcriptomic response. Further, it would be powerful to show direct evidence of energy depletion in animals that experience major ion toxicity. We suggest that directly measuring glycogen or using Seahorse technology may allow us to demonstrate energy depletion. Taken together, our work presented here has expanded our scientific knowledge of the osmoregulatory physiology of aquatic insects, but has also generated numerous unanswered questions for future research.