

# It's all about the fluxes: Temperature influences ion transport and toxicity in aquatic insects

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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, *Ischnychia sayi* and *Maccaffertium* sp., suggesting species-specific differences in the thermal sensitivity of ion transport. Finally, we demonstrated that the toxicity of  $\text{SO}_4^{2-}$  was influenced by temperature profoundly in a 96-h bioassay. Under the saltiest conditions (1500 mg L<sup>-1</sup>  $\text{SO}_4^{2-}$ ), 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.

## 1. 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 mountaintop 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

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(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.

## 2. Methods

### 2.1. 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 14 h:10 h 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 January and February 2019. The mayflies, *Isonychia sayi* and *Maccaffertium* sp., and the caddisfly, *Hydropsyche sparna*, are common facultative taxa in North Carolina. Larvae 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 h before experiments began. Voucher specimens were preserved in 70 % ethanol and identified using morphological features with a dichotomous key (Morse et al., 2017).

### 2.2. Radioactivity measurement

$^{35}\text{S}$ ,  $^{22}\text{Na}$ , and  $^{45}\text{Ca}$  isotopes were obtained as Na<sub>2</sub> $^{35}\text{SO}_4$ ,  $^{22}\text{NaCl}$ , and  $^{45}\text{CaCl}_2$ , 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 for 48–72 hours. Digestates were then 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.

### 2.3. Assessing temperature effects on ion uptake rates

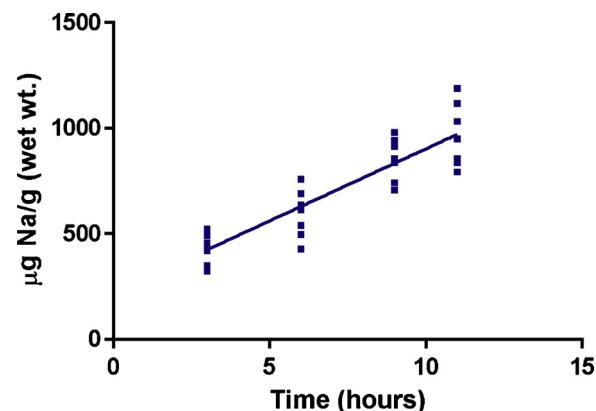
Flux experiments were performed in 100 mL high-density polyethylene (HDPE) beakers with 20 mL of treatment solution. All beakers had an air line with gentle aeration, a Teflon square substrate, and a ParaFilm™ cover to avoid evaporative loss. Temperatures (15, 20, or 25 °C) were controlled by incubators and monitored with a HOBO™ 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 uni-directional linear uptake rates, which were taken as the slopes of radioactivity acquisition vs time plots (Fig. 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.

### 2.4. 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 h before each experiment. They were individually placed in test chambers (1.28 ± 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 s for flush, hold, and measure phases) at the appropriate temperature (15, 20, or 25 °C) subtracting blank chambers as background.

### 2.5. Life history outcomes across salinities

To assess the effect of temperature on sulfate toxicity, we performed an acute 96-h bioassay with 10 day old *N. triangulifer* larvae in 6-well plates. Before each experiment, waters were measured for conductivity



**Fig. 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 °C. Linear regression analysis is based on the mean values for each time point. These particular data are presented in Fig. 2B and indicated by an arrow.

( $\mu\text{S cm}^{-1}$ ), 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 with deionized water, then filled with 8 mL of 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 L<sup>-1</sup>): 23, 360, 515, 735, 1050, and 1500. Larvae were acclimated to various temperatures (15, 20, or 25 °C) 24 h prior to the beginning of the experiment. Temperatures were controlled by incubators with 14 h:10 h 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 s /well) and larvae were visually assessed using a Leica DFC480 microscope. Mortalities were recorded and removed daily with a glass pipette. After 48 h, 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 h, 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 23 × 6.4 × 0.16 cm acrylic plates from SWRC. Three treatment waters (ASW, 665 mg L<sup>-1</sup>  $\text{SO}_4$ , and 1300 mg L<sup>-1</sup>  $\text{SO}_4$ ) 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 subimagoes were collected daily in a mesh lid and development time was recorded.

## 2.6. Data analysis

GraphPad Prism (v6, GraphPad Software, La Jolla, CA, USA) was used for all data analysis. Errors bars represent mean ± 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. All data was analyzed for normality.

## 3. Results

### 3.1. Sulfate, sodium, and calcium uptake rates in *N. triangulifer* and field-collected aquatic insects across three temperatures (15, 20, 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  $\mu\text{g g}^{-1} \text{h}^{-1}$  sodium uptake rate observed at 15 °C (Fig. 2A). This non-linear rate of increase was also observed for sulfate. At 15 °C, sulfate uptake rate was 2.5  $\mu\text{g g}^{-1} \text{h}^{-1}$ , but increased 23 % at 20 °C and 60 % at 25 °C (Fig. 2B). Interestingly, calcium uptake rates changed very little (< 5 %) across temperatures (Fig. 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  $\mu\text{g g}^{-1} \text{h}^{-1}$  at 15 °C (Fig. 2D). Sodium uptake rates increased 32 % and 37 % at 20 °C and 25 °C, respectively, relative to 13.3  $\mu\text{g g}^{-1} \text{h}^{-1}$  at 15 °C (p < 0.05) (Fig. 2E). *I. sayi* calcium uptake rates were 0.3  $\mu\text{g g}^{-1} \text{h}^{-1}$  at 15 °C, but increased 99 % at 20 °C and 88 % at 25 °C (p < 0.05) (Fig. 2F). We observed similar patterns in *Maccaffertium* sp.; sulfate uptake rates increased 81 % at 25 °C relative to 7.8  $\mu\text{g g}^{-1} \text{h}^{-1}$  at 15 °C (Fig. 2G), while sodium uptake rates increased 63 % at 25 °C relative to 9.8  $\mu\text{g g}^{-1} \text{h}^{-1}$  at 15 °C (Fig. 2H). However, calcium uptake rates at 15 °C were 0.4  $\mu\text{g g}^{-1} \text{h}^{-1}$  and increased 75 % at 25 °C (p < 0.01) (Fig. 2I). Sulfate uptake rate in *Hydropsyche sparna* was 2.9  $\mu\text{g g}^{-1} \text{h}^{-1}$  at 15 °C and increased 2.4-fold at 25 °C (p < 0.01) (Fig. 2J). Similarly, sodium uptake rates increased 4.3-fold at 25 °C relative to the 1.0  $\mu\text{g g}^{-1} \text{h}^{-1}$  observed at 15 °C (p < 0.0001) (Fig. 2K). Calcium uptake rates also increased in *H. sparna* at 25 °C, albeit not significantly (Fig. 2L).

### 3.2. 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  $\mu\text{g O}_2/\text{g/h}$  and increased 27 % to 686  $\mu\text{g O}_2/\text{g/h}$  at 20 °C and 2.2-fold to 1188  $\mu\text{g O}_2/\text{g/h}$  at 25 °C (p < 0.0001) (Fig. 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 (Fig. 3B and 3C), suggesting that for a given ionic concentration, thermally driven changes in transport rates scale with metabolic rates in this species.

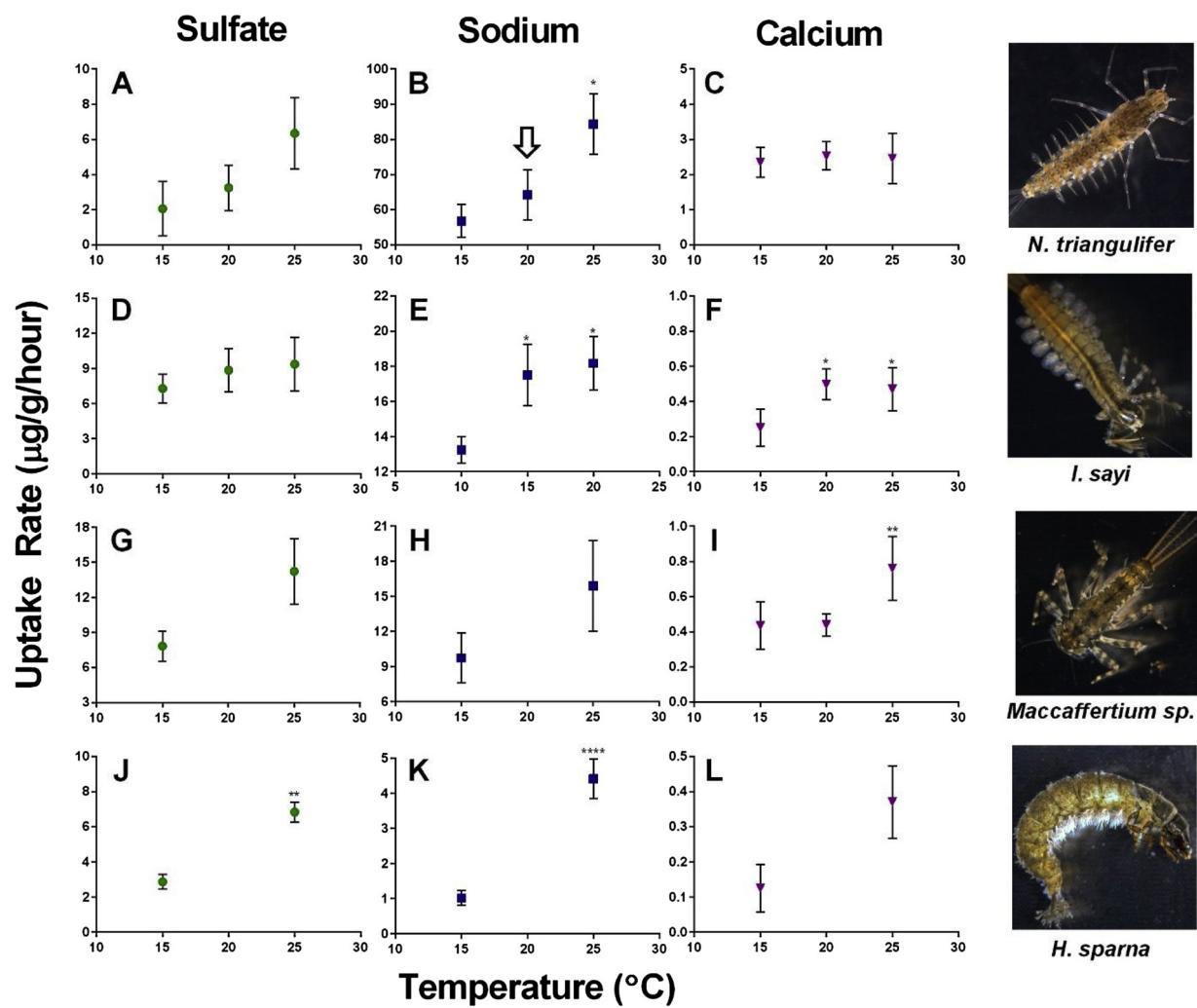
### 3.3. Temperature and salinity affect toxicity and development time

Temperature strongly influenced sulfate toxicity in a 96-h acute bioassay; 98 ± 2 % of *N. triangulifer* larvae survived in water with 1050 mg L<sup>-1</sup> sulfate at 20 °C, but in the same waters, only 84 ± 5.1 % survived at 15 °C and 70 ± 5.5 % in 25 °C (Fig. 4A). At the highest salinity, 1300 mg L<sup>-1</sup> sulfate, 94 ± 4 % of mayflies survived at 20 °C, 78 ± 6.6 % survived at 15 °C, and 44 ± 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 mg L<sup>-1</sup> sulfate caused a 4.5 % increase in development time compared to controls (ASW) (p < 0.0001) and highly-elevated sulfate waters (1300 mg L<sup>-1</sup>) caused a 27 % increase in development time (p < 0.0001). However, the magnitude of these delays was consistent across all three temperatures (Fig. 4B).

## 4. 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 h for  $\text{Ca}^{+2}$  and 24 h 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



**Fig. 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). The arrow in plot B refers to the data shown in Fig. 1.

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 1–2 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 *H. sparna* 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–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 Mota reported that freshwater-adapted eels, *Anguilla anguilla*, had a higher osmotic to diffusional

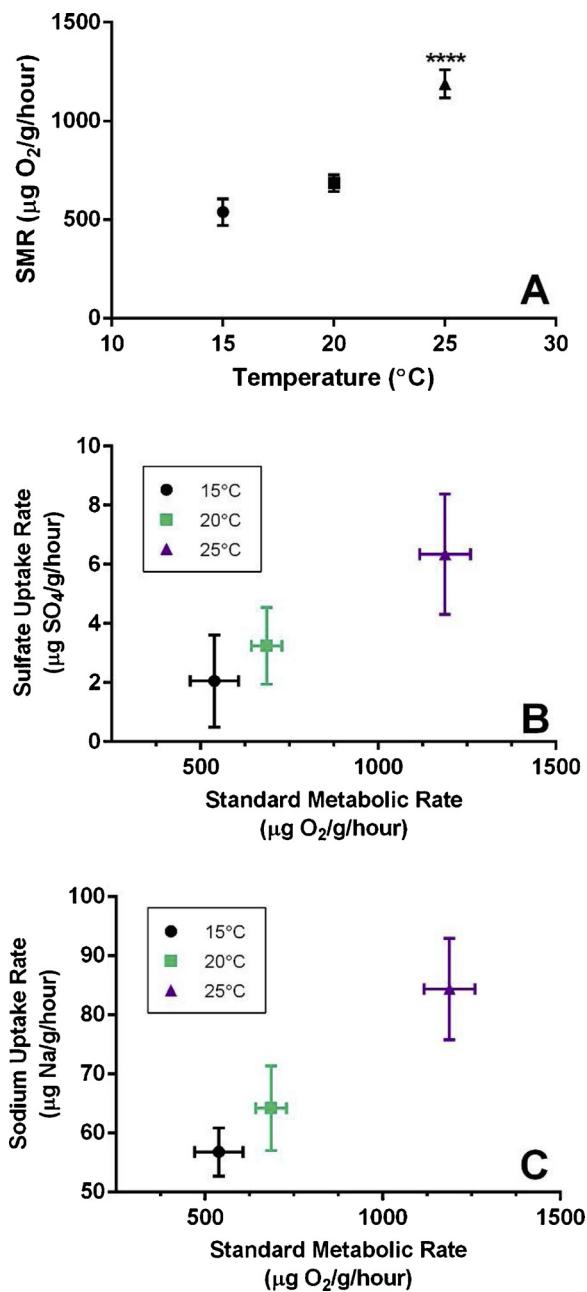


Fig. 3. (a) *N. triangulifer* standard metabolic rates (SMR) across three temperatures. Each point represents the mean of 8 individuals. (b) Sulfate uptake rate plotted against SMR ( $R^2 = 0.997$ ). (c) Sodium uptake rate plotted against SMR ( $R^2 = 0.998$ ).

permeability ratio (Mota 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.

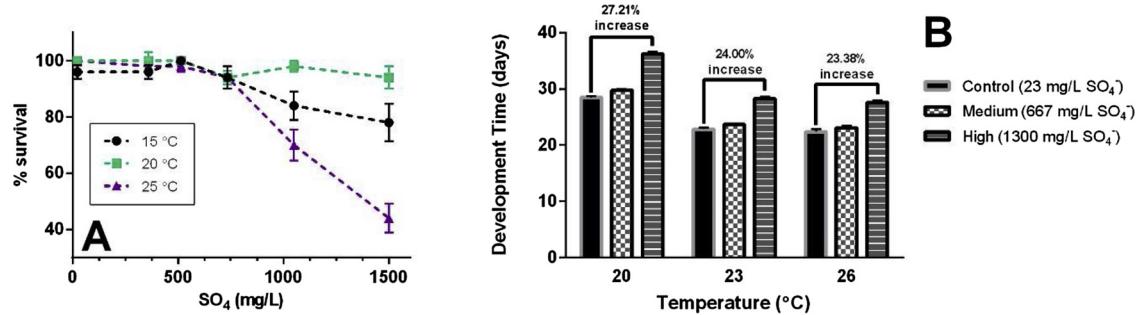
Alternatively, warmer temperature could increase ion transport rates simply by accelerating physiological processes in general. The nonlinear relationship of ion uptake across temperatures observed in *N. triangulifer* and other field-collected aquatic insects might also be

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 and Funk, 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  $^{\circ}\text{C}$  reduced survivorship to 44 % in our 96-h bioassay relative to a more ideal temperature of 20  $^{\circ}\text{C}$  where 94 % survived. However, we acknowledge that survivorship at 15  $^{\circ}\text{C}$  (78 %) was slightly reduced under salinity stress relative to 20  $^{\circ}\text{C}$ . One explanation is that 20  $^{\circ}\text{C}$  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



**Fig. 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.

should not be ignored if we want 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 or personal relationships that could have appeared to influence the work reported in this paper.

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