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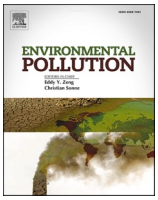

Salinity-induced ionoregulatory changes in the gill proteome of the mayfly, *Neocloeon triangulifer*☆

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ABSTRACT

Ecologists have observed declines in the biodiversity of sensitive freshwater organisms in response to increasing concentrations of major ions (salinization). Yet, how changing salinities physiologically challenge aquatic organisms, such as mayflies, remains remarkably understudied. Moreover, it is not well understood the degree to which species respond and acclimate to salinity changes. Our lab is developing the Baetid mayfly, *N. triangulifer*, as a model organism for physiological research. We have previously described acclimatory changes in both ion flux rates and altered mRNA transcript levels in response to chronic exposures to elevated major ion concentrations at the whole animal level. In the present study, we use shotgun proteomics to identify the specific proteins associated with apical ion transport and how their abundance changes in response to chronic salinity exposures in gills. Gills were isolated from the penultimate nymphal stage of *N. triangulifer* reared under control culture conditions, elevated NaCl (157 mg L^{-1} Na), elevated $CaCl_2$ (121 mg L^{-1} Ca), elevated Ca/$MgSO_4$ (735 mg L^{-1} SO_4). These conditions mirrored those from previously published physiological work. We also acutely exposed nymphs to dilute (50% dilution of culture water with deionized water) to explore proteomic changes in the gills in response to dilute conditions. We report 710 unique peptide sequences among treatment groups, including important apical ion transporters such as Ca-ATPase, Na/K ATPase, and V-ATPase. Treatment with elevated NaCl and Ca/$MgSO_4$ appeared to cause more significant differential protein expression (452 and 345, respectively) compared to $CaCl_2$ and dilute groups (134 and 17, respectively). Finally, we demonstrated the breadth of physiological functions in gills by exploring non-transport related pathways found in our dataset, including ATP synthesis, calcium signaling, and oxidative stress response. We discuss our results in the context of freshwater salinization and the challenges of working with non-model species without fully sequenced and annotated genomes.

1. Introduction

Freshwater salinization (Kaushal et al., 2005, 2018) 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) are a few of the common human activities that increase major ion concentrations in nearby freshwaters. However, our physiological understanding of how changing salinities impact aquatic organisms, such as mayflies, is not well understood.

Freshwater communities typically include species with hypothesized marine origins (e.g., fish, mollusks, and crustaceans), but are usually dominated by insects, who are secondarily aquatic and evolved from terrestrial ancestors multiple times independently (Griffith, 2017; Kristensen, 1981; Misof et al., 2014). In general, insects thrive under relatively dilute conditions, and remarkably few species have evolved to tolerate brackish, marine, or hypersaline conditions (Albers and Bradley, 2011; Maddrell, 1998). Consequentially, freshwater insects have diverse strategies of osmoregulation in dilute environments in order to mitigate the ionic gradients that drive ion loss and water influx (Bradley

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et al., 2009). In general, they actively transport ions with structures on apical surfaces (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 osmolalities below that of insect hemolymph has been reported (Dowse et al., 2017), suggesting a challenge for maintaining ion and water homeostasis when the ionic gradient favors the loss of ions rather than uptake and retention. Several hypotheses have been proposed to explain toxicity at osmolalities below that of insect hemolymph, including energetics of ion uptake, loss of pH regulation, and localized ion poisoning (Kefford, 2019). Osmoregulatory physiology has been well-studied in mosquitoes (Bradley, 1987; 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.

Until recently, little was known about how aquatic insects acclimate to changing salinity regimes. In a companion study to the work presented here (Orr et al., 2021), we showed that *N. triangulifer* responded to chronic exposures to elevated NaCl and Ca/$MgSO_4$ by significantly decreasing their uptake rates of Na, and SO_4, respectively. Prior exposure to elevated $CaCl_2$ resulted in only modest decreases of already relatively low rates of Ca uptake; relatively low rates of apical Ca uptake appear common in aquatic insects (Orr and Buchwalter, 2020; Orr et al., 2022; Poteat and Buchwalter, 2014; Poteat et al., 2012). We speculated that physiological changes in the gill, might be responsible for these observed flux rate differences because the gill contains an abundance of ion-transporting cells (i.e., chloride cells or ionocytes) (Komnick, 1977; Wichard and Komnick, 1974) in addition to its role in oxygen uptake (Craig, 1990). From a morphological perspective, there is evidence for plasticity in the expression of chloride cells in response to changing salinity (Kumar et al., 2020; Wichard et al., 1973), including a reduction in gill chloride cell numbers under ion rich conditions and a proliferation of gill chloride cells when nymphs are subjected to extremely dilute conditions in *N. triangulifer* (Cochran and Buchwalter, 2022) (but also see Berrill et al., 1991 and Nowghani et al., 2017 where such changes were not observed).

Aquatic insect studies are critical in the face of freshwater salinization, as mayflies are unusually sensitive to salinity. Ionocytes found on the gill and body surface of mayfly nymphs express two primary energy-consuming proteins: Na/K ATPase and V-Type ATPase (Nowghani et al., 2017, 2019), and their activities can be measured using ouabain and bafilomycin enzymatic assays (Jonusaite et al., 2011). However, most ionoregulatory studies have been performed with aquatic Dipteran taxa that lack gills, and other mayfly-specific transporters have yet to be identified. Previous work in mosquitoes has explored ionoregulation in various tissues including anal papillae (Donini and O'Donnell, 2005; Patrick et al., 2006), Malpighian tubules (Bradley et al., 1982; Dow et al., 2021; Jonusaite et al., 2017), gastric caecum (D'Silva and O'Donnell, 2017), and midgut (Boudko et al., 2001; Jagadeshwaran et al., 2010; Onken et al., 2008). Some scientists have even developed custom antibodies to localize specific transporters including V-Type ATPase, Na/K ATPase, and carbonic anhydrase using immunohistochemistry in mosquito larvae (Patrick et al., 2006; Smith et al., 2008; Zhuang et al., 1999). Pullikuth and colleagues also identified a sodium/proton exchanger and potassium/proton exchanger in mosquito larvae's anal papillae and midgut (Pullikuth et al., 2003, 2006). The exact transporters involved in the regulation of other essential major ions (e.g., magnesium, bicarbonate, calcium, and sulfate) in aquatic insects remain unknown (Griffith, 2017; Silver and Donini, 2021). From an evolutionary perspective, mosquitoes are distantly related to other aquatic insects as they independently evolved to life in freshwater, and unlike mayflies, are not particularly salt sensitive. Thus, the relevance of mosquitoes for ecological inference is highly questionable, and there is clearly a deficit of molecular understanding of ionoregulation in sensitive aquatic insects, like mayflies.

The goals of the present study were threefold. First, we asked which specific ion-transport proteins are expressed in gill tissue of *N. triangulifer* nymphs. Further, we examined how exposure history to elevated major ions (NaCl, $CaCl_2$, or Ca/$MgSO_4$) or lack thereof (dilute treatment) affected the expression of these gill proteins. Finally, we investigated other proteins and pathways expressed on the gill that were not directly related to transport or ionoregulation. Together, our findings provide new insights into the physiology of the mayfly gill as freshwater becomes more salinized worldwide.

2. Materials and methods

2.1. Mayfly culture

The baetid mayfly, *Neocloeon triangulifer*, was originally found in White Clay Creek (240 μS cm^{-1}) 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 nymph food. *N. triangulifer* hatchings (<1-d old) were seeded into glass Pyrex containers with approximately 4 L of constantly aerated 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 rearing and control water conditions and as the base of all amended waters.

2.2. Scanning electron microscopy

Mature *N. triangulifer* nymphs (~21-d old) were preserved in 2% glutaraldehyde and stored at 4 °C.After the initial fixation, insects werewashed in buffer solution (0.1 M Sodium cacodylate buffer) 3 × 10-m. Then, samples were post fixed in 2% Osmium tetroxide for 2-h followed by 3 × 10 buffer washes. A dehydration series of Ethanol: 70%, 95%, 2 × 100% (10-m each) and two changes of hexamethyldisilazane 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. Only control nymphs were analyzed with SEM because of cost limitations.

2.3. Experimental exposure to elevated concentrations of different salts or dilute media

To characterize the specific ion transporters present on the mayfly gill and to assess the plasticity of their expression, nymphs 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 laboratory (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^{-1} Na), elevated $CaCl_2$ (121 mg L^{-1} Ca), and elevated Ca/$MgSO_4$ (735 mg L^{-1} SO_4) (Table 1). Animals were exposed chronically to all treatments for the majority of their nymph life cycle (~21-25-d) in 3 4-L glass pans per treatment with constant aeration. Additionally, we included an acute, 48-h dilute treatment of mature nymphs, consisting of a 50% dilution of control ASW with deionized water. All exposures took place on the benchtop under standard laboratory conditions of 14:10 light:dark photoperiod and 21–23 °C. 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 nymphs were removed and randomly divided into groups of approximately 20. Each nymph 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. Approximately 280 gills were dissected from each group of 20 nymphs to obtain sufficient protein

Table 1
Water chemistry for all experimental exposure waters. Cations (Na, Ca, Mg, K) and anions (SO_4, Cl, CO_3) are all listed in mg L^{-1}. 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 ion concentrations are listed in parentheses beside nominal ion concentrations.

	Na	Ca	Mg	K	S	SO_4	Cl	CO_3	pH	Cond. (μS/cm)
Control (ASW)	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	130.7
Dilute (50% ASW)	7.65	6.35	1.7	0.7	3.89	11.7	7.05	21.3	7.99	68.3
Elevated $CaCl_2$	15.3	121 (113)	3.4	1.4	7.78	23.3	97	42.6	8.07	529
Elevated NaCl	157 (152)	12.7	3.4	1.4	7.78	23.3	255	42.6	7.67	777
Elevated Ca/$MgSO_4$	15.3	193.7	79.1	1.4	245 (256)	735	14.1	42.6	8.06	995

yields for each sample to undergo proteomic analysis at the NCSU Molecular Education, Technology and Research Innovation Center (METRIC). Treatments had varying numbers of grouped samples based on survival (n = 8–14).

2.4. Shotgun proteomics

Gills were thawed on ice and combined to achieve 4 total replicates per treatment group with a 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™ bicinchoninic acid (BCA) Kit (ThermoFisher). Samples were evaporated to dryness in a vacuum concentrator and stored in −20 °C freezer. On day of digestion assay, 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). Then, each sample was rinsed 3 times with 2 M urea, 10 mM $CaCl_2$ in 100 mM pH 8 tris buffer, then 3 times with 100 mM pH 7.5 tris buffer for a total of 6 rinses before digestion. Next, 100 μL Trypsin (4 μg) and 100 μL chymotrypsin (2.5 μg) were added to each sample. This combination enhanced membrane protein coverage (Fischer and Poetsch, 2006). After samples were rinsed with quench buffer, they were dried and stored at −20 °C.

To perform proteomics analysis, samples were reconstituted in 200 μL mobile phase A and injected 2 μL for nano liquid chromatography with tandem mass spectrometry (LC-MS/MS). Elution of peptides from the trap and analytical columns was performed using a gradient of mobile phase A and mobile phase B. 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 μm, 75 μm × 20 mm and the analytical column was EASY-Spray™, 2 μm, 75 μm × 250 mm.

2.5. Data analysis

Raw nanoLC-MS/MS data were processed with Proteome Discoverer 2.4.305 software ((PD), Thermo Scientific, Bremen, DE) using a label-free quantification workflow to evaluate relative differences in protein abundances between treated and control sample groups. Data were processed twice to obtain protein identifications from a collection of 3 different protein sequence databases, because *N. triangulifer* does not currently have a *de novo* reference genome. The first processing iteration employed a home-built, custom protein database derived from a 6-open-reading-frame (ORF) translation of a partial *N. triangulifer* genome (45,035 sequences), which was built using a previous transcriptomics dataset (Chou et al., 2020). The second processing iteration employed two databases obtained from UniProt: the entire *Metazoa* un-reviewed database from TrEMBL (taxon 33,208 including sub-taxonomies, 22, 923, 323 sequences) and the entire reviewed Swiss-Prot database (all taxa, 477,917 sequences), to maximize our ability to annotate the data properly. Duplicate sequences were removed automatically by Proteome Discoverer. A custom, home-built contaminants database was used to identify reagent enzyme peptides and human keratins.

The mass spectrometry data analysis program, SEQUEST HT search node of PD, used the following parameters for protein identification: maximum of 3 missed cleavage sites; minimum peptide length of 6 amino acids; 5 ppm precursor mass tolerance; 0.02 Da fragment mass tolerance; maximum of 4 dynamic oxidations of methionine; dynamic protein terminus modifications of acetyl addition, methionine loss, or both; and static carbamidomethylation of cysteine. The original mayfly gill protein samples were digested with a cocktail of trypsin and chymotrypsin, so a custom cleavage reagent was made that combined trypsin and chymotrypsin cleavage sites at phenylalanine, lysine, leucine, arginine, tryptophan, and tyrosine at the C-terminus with proline as an inhibitor. Only *y* and *b* ions were included for spectrum matching. In both iterations of processing, peptides were validated by the Percolator node with a *q*-value set to 0.05 and a strict false discovery rate (FDR) set to 0.01. The strict parsimony rule was applied for protein grouping.

The Feature Mapper and Precursor Ions Quantifier nodes in the PD consensus workflow were set up purposefully to reduce or eliminate adjustments to raw data before identifying and quantifying features. Retention time alignment was set to "false" in the Feature Mapper node. Fields for retention time tolerance and mass tolerance were set to 0 for the automatic determination of these values. Normalization mode and scaling mode fields in the Precursor Ions Quantifier node were both set to "none." Precursor abundances were based on intensity with protein abundances calculated using the summed abundances of precursors. Analysis of variance (ANOVA) was used to calculate *p*-values for quantification ratios.

The master annotated peptide file was sorted by keyword to pare down the relevant ion transporting proteins. Peptides were grouped and explored manually based on previous findings and generated hypotheses. Further, we explored phylogenetic relationships based on reference species with the highest confidence match based on the lowest q-values and posterior error probability. We created a Venn diagram of differentially expressed peptides using the free Bioinformatics and Evolutionary Genomics software from the website: https://bioinformatics.psb.ugent.be/webtools/Venn/.

3. Results

We assessed the microstructure of the gills and abdomen by imaging them with scanning electron microscopy and discovered two distinct forms of ionoctyes: bulbiform and coniform. Interestingly, only coniform ioncytes were observed on the gills, while both forms were found on the abdomen (Fig. 1).

We identified 710 unique peptide sequences with high confidence among all gill samples (Supplementary Table 1). Significant differences in protein expression was observed among treatment groups. Compared to controls, dilute, $CaCl_2$, Ca/$MgSO_4$, and NaCl-treated mayflies had 17, 134, 452, and 345 differentially expressed peptides, respectively. The overlap of expression among treatment groups is represented by a Venn diagram (Fig. 2). The abundance ratios (relative to controls) and associated p-values for the salinity exposures are in Supplementary Tables 2–5.

A primary goal of the study was to identify transport proteins expressed on the gill of *N. triangulifer* nymphs. We identified several

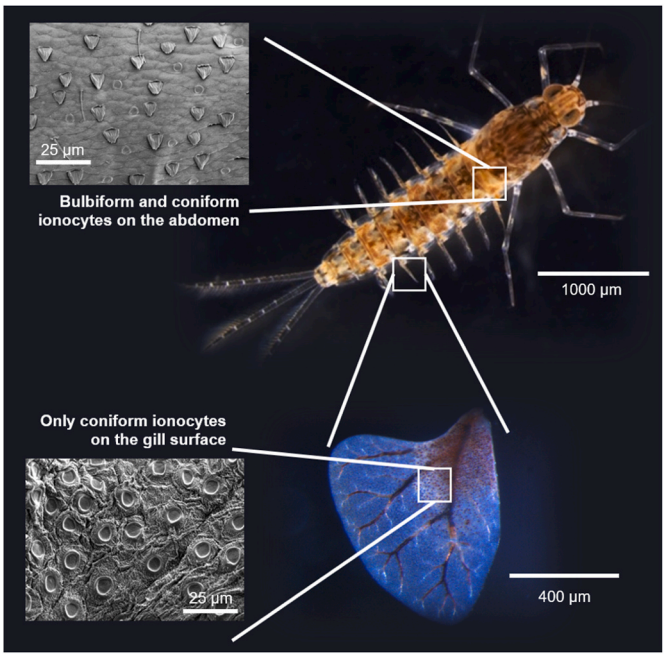

Fig. 1. Scanning electron microscopy of the ionocytes on the gill and abdomen of a mature *N. triangulifer* larvae.

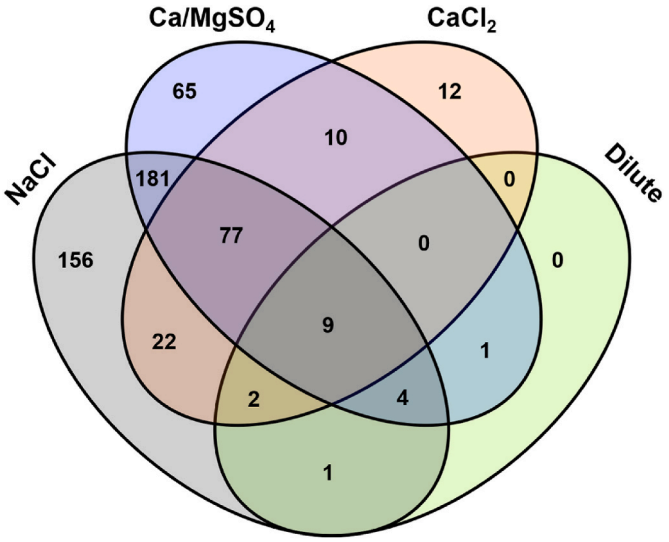

Fig. 2. Venn diagram analysis of peptides found in the gills of *N. triangulifer* after chronic exposure to different water ionic challenges.

subunits of three important ion transporters: Calcium-Transporting ATPase, Na/K ATPase, and V-Type ATPase. Several different subunits were identified for V-Type ATPase including A, B and H, which are located in the V_1 domain responsible for ATP hydrolysis (Pamarthy et al., 2018). We also found other relevant proteins of interest involved in stress signaling (heat shock proteins (HSP) and mitogen-activated protein kinase (MAPK)), energetics (ATP Synthase, Glycogen Synthase), oxidative stress (Catalase, Superoxide Dismutase) and calcium-binding messaging (Calmodulin). An ordinary one-way ANOVA with Dunnett's multiple comparisons test revealed significant differences among treatment groups for many of these proteins (Fig. 3).

Na/K ATPase was significantly downregulated in response to $CaCl_2$ ($p = 0.015$), NaCl ($p = 0.001$) and $Ca/MgSO_4$ ($p = 0.014$), but not to dilute treatment ($p = 0.99$). We also found that Ca-ATPase was significantly downregulated by NaCl treatment ($p = 0.017$), but not by any

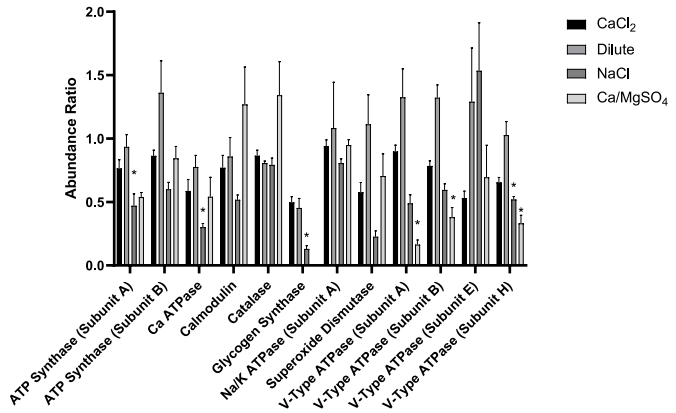

Fig. 3. Abundance ratios of protein expression for proteins of interest among treatment groups compared to controls ($n = 4$).

other treatment. We observed a significant downregulation of V-Type ATPase in mayflies exposed to elevated NaCl ($p = 0.01$) or $Ca/MgSO_4$ ($p < 0.0001$). Heatmaps represent our correlation analysis to show patterns in the dataset (Fig. 4), which are annotated for proteins of the most interest in the present study (Calcium-transporting ATPase, Sodium/Potassium ATPase, V-type ATPase).

We found no significant differences in protein expression of calmodulin, catalase, or superoxide dismutase. However, we did see treatment-specific changes in two energetically relevant proteins. Elevated NaCl exposure caused a significant downregulation in ATP Synthase ($p = 0.018$) and glycogen synthase ($p = 0.001$). We searched for but did not find other proteins of interest in the gill tissue, including fibroblast growth factor, claudins, aquaporins, sulfate transporters, and chloride channels.

4. Discussion

Freshwater salinization is causing local extinctions of sensitive aquatic insects, such as mayflies (Canedo-Arguelles et al., 2018; Pond et al., 2008). However, we still understand very little about the mechanisms that determine the differential sensitivity of species to different major ion matrices. We have previously shown that 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). *N. triangulifer* appears to be physiologically plastic and can somewhat acclimate to both dilute and rich-ion conditions, as shown by altered sodium uptake rates (Cochran and Buchwalter, 2022; Orr et al., 2022). 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. Here, we used a proteomic approach to investigate the gill biology of *N. triangulifer* nymphs exposed to different salinities with concentrations that mirrored our previous physiological study.

4.1. Morphologically-distinct ionocytes exist on the abdomen and gills

Freshwater habitats present a unique challenge to aquatic organisms that must ionoregulate at water concentrations of ions that are much lower than their own hemolymph. One way aquatic insects have adapted to dilute freshwater conditions is by expressing numerous ionocytes on gill and abdomen surfaces to actively recruit ions from the surrounding dilute medium (Komnick, 1977). Ionocytes can be found on different

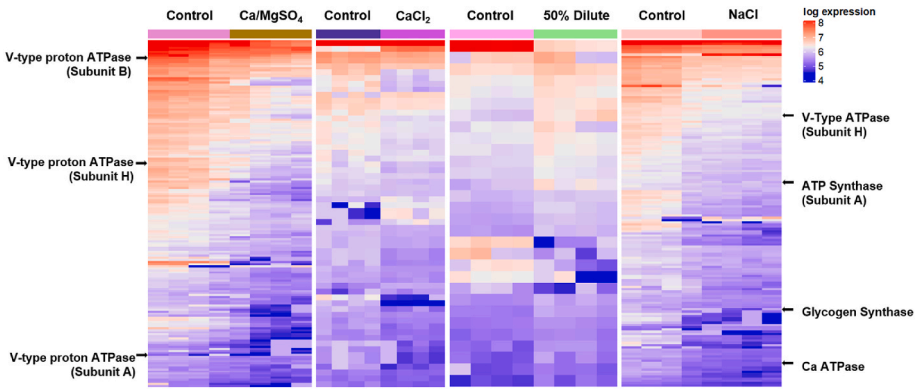

Fig. 4. Dendrogram analysis of protein expression patterns among treatment groups, where red and blue indicate high and low relative expression, respectively. Relevant proteins of interest are noted in the Ca/$MgSO_4$ and NaCl groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

structures including the abdomen, legs, and most importantly, gills (Griffith, 2017; Shaw and Stobbart, 1963; Wigglesworth, 1933). Mayfly gills are typically flat, often movable epithelia bilayers with branching trachea that allow functional oxygen uptake and ion transport. Scanning electron microscopy revealed an abundance of ionocytes on both the gill and abdomen of *N. triangulifer* nymphs (Fig. 1). Interestingly, the abdomen has two distinct types of ionocytes: coniform and bulbiform, while the gills only have coniform cells (Fig. 1). It remains unclear if these distinct ionocytes house different transport proteins or have different physiological functions.

We were somewhat surprised to identify no sulfate transporters on the gill tissue of *N. triangulifer* in any treatment group, because sulfate uptake rates are generally commensurate with external concentrations in this and other aquatic insect species (Scheibener et al., 2017). Sulfate transporters are relatively understudied, even in mammalian systems. Some groups propose that sulfate transporters function in ionoregulatory tissues to primarily support the reabsorption of other essential major ions (Markovich and Aronson, 2007). Previous mRNA expression results in *N. triangulifer* at the whole-body level also revealed an increase in sulfate transporters in response to elevated sulfate conditions (Orr et al., 2021). This finding led us to propose that the sulfate transporters were involved in the efflux of excess major ions in excretory tissues, such as the Malpighian tubules. Taken together, we hypothesize that sulfate uptake may be more prominent in bulbiform ionocytes (found only on the abdomen of this species) or through dietary means.

4.2. Differential major ion transporter protein expression is induced by salinity

Ionoregulation 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 (Na/K ATPase, V-ATPase, and Ca-ATPase). Here we discuss the importance of these transporters and their response to salinity stress in the gill of *N. triangulifer*.

The ubiquitous Na/K ATPase transports three sodium ions out and two potassium ions in concurrently, which maintains the ionic gradient across cell membranes that in turn, powers a plethora of other cellular functions. Here, we observed a significant decrease in Na/K ATPase expression in mayfly nymphs exposed to NaCl, $CaCl_2$, and Ca/$MgSO_4$. None of these water exposures contained elevated K ions, and only NaCl treatment contained elevated Na ions. Physiological acclimation has been demonstrated previously in *N. triangulifer* through substantial changes in ion transport rates in response to previous salinity exposure. Interestingly, acclimated mayfly nymphs experienced a 49.6% decrease in sodium uptake rates compared to naïve nymphs when exposed to elevated NaCl (Orr et al., 2021). This finding is partially supported by the 19.3% decrease in Na/K ATPase gill protein levels in response to the same NaCl exposure found in the present study. Likely, the animals adjust the movement of ions to prevent Na from entering the hemolymph, relieving the Malpighian tubules and hindgut from energetically costly efforts. It is plausible that other gill sodium transport proteins went undetected in this study, more significant Na ionoregulation takes place in the Malpighian tubules, or both.

Vacuolar H^+-ATPases (V-ATPases) are found ubiquitously in eukaryotic organisms and function as a proton-pump to aid in the physiological function of cells (Finbow and Harrison, 1997). The role of V-ATPases has been studied in many insects and has 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) by pumping protons into cells. Our results showed decreases in V-ATPase peptide expression in mayflies from NaCl and Ca/$MgSO_4$ groups compared to control mayflies. Nowghani et al. (2017) 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). 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). V-ATPase has broad implications on homeostasis and cellular function, but we suggest that elevated salinity exposures caused a downregulation in V-ATPase expression to prevent excessive acidification of intracellular compartments, facilitate the export of other elevated ions (i.e., Na^+ or Ca^{2+}), or both.

Plasma membrane Ca-ATPase is an essential and ubiquitous transport protein with various isoforms found on both apical and basolateral membrane of eukaryotic cells andthat keeps intracellular Ca^{2+} concentrations low (Carafoli, 1991; Stafford et al., 2017). We were surprised to find that $CaCl_2$ and Ca/$MgSO_4$ exposures had no significant effect on Ca-ATPase protein expression in *N. triangulifer* nymphs. Interestingly, NaCl exposure caused a significant decreased in Ca-ATPase, despite containing no elevated Ca ions. NaCl exposures clearly had the largest impact on gill protein expression and we posit that elevated NaCl may have caused dysregulation and loss of ionoregulatory mechanisms at the gill surface.

4.3. Aquaporins and cellular junction proteins play a lesser role in salinity acclimation

Aquaporins are highly conserved integral membrane proteins that

play a role in water movement between cells, which suggests a potentially significant role in homeostasis. Our proteomic analysis revealed no aquaporin peptides on the gill of *N. triangulifer*. It is unlikely that aquaporins are absent from gill tissue, but perhaps our proteomic approach could not detect low concentrations or alternatively, no reference genome was similar enough to annotate the aquaporin of *N. triangulifer* accurately. Interestingly, we have found no changes in mRNA expression of an aquaporin gene in *N. triangulifer* nymphs treated with high concentrations of NaCl (1850 mg/L NaCl) (data not shown). Similarly, there were no differences in aquaporin expression in *Aedes aegypti* nymphs reared in either freshwater or brackish water (Misyura et al., 2020). Taken together, aquaporins may only be present in other osmoregulatory tissues (e.g., Malpighian tubules) or not play a critical role in homeostasis 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, *Edcyonurus dispar* (Nicholls, 1983) and ionocytes of the mayfly, *Coloburiscoides* sp. (Filshie and Campbell, 1984). The present study found no claudins or septate junctions on the gill tissue of *N. triangulifer*. 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). Notably, previous work in *N. triangulifer* demonstrated that even at stressful salinities, nymphs 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 ion transport proteins found on the gills may have a more significant role in osmoregulation.

4.4. Non-transport related proteins also play an important role in gill biology

MAPKs are highly conserved proteins that function in osmoregulatory signal transduction in eukaryotic organisms (Kültz, 1998). Our analysis identified one MAPK peptide closely related to the mosquito, *Anopheles farauti* in only a subset of control gill samples. In a previous study, MAPK expression was altered in the gills of killifish exposed to salinity stress (Kültz and Avila, 2001). MAPK has also been characterized in *Chironomus* (Park and Choi, 2017; Wei et al., 2020) and mosquitoes (Yamamoto et al., 2013), but our study is the first to suggest an ionoregulatory role for MAPK in mayflies. Further, our dataset identified the presence of many peptides of heat shock proteins (HSPs), which function as molecular chaperones to direct the appropriate folding or proteolytic degradation of other proteins (Feder and Hofmann, 1999). HSPs have been studied as ionic stress markers in mayflies (De Jong et al., 2006) and many other aquatic species (Kultz, 1996; Yang et al., 2009). Here, we demonstrated the plasticity of HSP expression in response to salinity changes with variable up and down regulation of the different HSP peptides.

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 (Cochran and Buchwalter, 2022). Further, Ruiz-Sobrino et al. identified the FGF ligand, *branchless* (*bnl*), as an important regulator of gill-branching morphogenesis, but this protein was not found in our analysis. Perhaps, this protein is only actively expressed early in development when gill branching is rapidly developing. Specifically, dilute exposures appear to elicit more extensive tracheal branching, which may indicate an increased hemolymph supply to the gill in extremely dilute conditions. Alternatively, saltier exposures reduce the amount of tracheal branching.

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 many ATP synthase peptides were found in our analysis. Previously, transcriptomics has revealed the induction of ATP synthase transcripts in *N. triangulifer* nymphs in response to thermal stress (Chou et al., 2020), but no other studies on ATP synthesis in response to salinity stress in aquatic insects exist. In the present study, we were surprised to see a decrease in protein expression of ATP synthase in response to NaCl exposure. It is possible that the expression of other proteins (e.g., ion transporters) took priority in this overt disruption of ionoregulation and/or that energy production mechanisms broke down with the loss of appropriate proton concentration gradients across mitochondrial membranes.

4.5. Conclusions

Taken together, we have identified proteins expressed on the gill tissue of *N. triangulifer* nymphs to understand the ionoregulatory biology of mayflies better. Our study is unique given the difficulty of tissue-specific experiments in small animals and the lack of molecular work in sensitive aquatic insects. Many studies, including large transcriptomic or proteomic works, have been published on ionoregulation in mosquitoes (Bradley, 1987; Durant et al., 2021; Uyhelji et al., 2016), but this information does not apply to many other sensitive aquatic taxa because of the numerous evolutionary and physiological differences. Our analysis revealed that differention compositions elicited significantly differential protein expression; exposure to NaCl or Ca/$MgSO_4$ elicited a more significant response than the other treatments. This finding helps explain our companion study with physiological observations of salinity stress using the same water chemistries (Orr et al., 2021). These findings also support the hypothesis that toxicity caused by the energetics of ion uptake, rather than direct ion poisoning or loss of pH regulation (Kefford, 2018). Further, we highlighted major ion transporting proteins found on the gill that will support more in-depth future research on specific ion transporters.

This proteomic approach is a step to better understanding of mayfly ionoregulatory physiology and preventing the extirpation of sensitive freshwater organisms. Although mayflies clearly have a remarkable plastic response to changing environments (Orr et al., 2021), they are dramatically disappearing from streams affected by anthropogenic activities (Cormier and Suter, 2013; Pond et al., 2008). Our work bolsters the importance of studying freshwater insects at the molecular level to ameliorate ecological harm elicited by environmental change.

Author statement

SEO and DBB conceived the concepts of the work and conducted the research. SEO wrote the manuscript. DDJ and LBC analyzed and annotated the proteomics data. All authors provided editorial assistance of 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120609.

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