IONOREGULATION IN *DAPHNIA MAGNA*; MECHANISMS OF MAJOR ION TOXICITY IN ADULTS AND PHYSIOLOGY OF IONOREGULATION IN JUVENILES

IONOREGULATION IN *DAPHNIA MAGNA*; MECHANISMS OF MAJOR ION TOXICITY IN ADULTS AND PHYSIOLOGY OF IONOREGULATION IN JUVENILES

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ABSTRACT

Elevations in major ions (sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations paired with chloride (Cl^-), sulphate (SO_4^{2-}) and (bi)carbonate (HCO_3^-/CO_3^{2-}) anions) in freshwater environments through anthropogenic activities cause physiological disturbances in freshwater animals. Because these animals are adapted for active ion uptake to combat passive ion loss to the external environment, increases in ambient major ion concentrations can alter ionoregulation. There has been a concerted research effort into toxicity of major ions and the development of predictive models, which require extensive physiological data, to support the development of comprehensive regulations surrounding pollution by major ions. The branchiopod crustacean, *Daphnia magna* has been the focus of many of these toxicological studies. The physiological effects of elevated ambient major ion concentrations in adults were investigated. Transepithelial potential (TEP) and hemolymph ion concentrations were altered in animals exposed to elevated ion concentrations approaching previously described LC50 values. These changes in TEP and hemolymph ion concentrations are indicative of physiological disturbance and may be indicators of toxicity. Diffusional gradients and active ion pumps were found to contribute to TEP in *D. magna*, unlike in freshwater fish. Notable differences between adult and juvenile *D. magna* in sensitivity to ionic composition of the water and ionoregulation have been described. These differences suggest that both juveniles and adults should be considered in studies focused on monitoring major ion pollution. Ion transport through the nuchal organ in embryo and neonate *D. magna* was directly measured. Influx of Na^+ and efflux of NH_4^+, H^+ and Cl^- was observed. K^+ flux is dependent on developmental stage. The results from the evaluation of the physiological effects of increased ambient major ions in adults and mechanisms of ion transport in juveniles will aid in establishing environmental regulations for major ions in aquatic ecosystems.

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$[ion]_i$	Ion activity in the extracellular fluid of organism mM
$[ion]_o$	Ion activity in external water in mM
$[ion]_{sample}$	Ion concentration of the hemolymph sample
°C	Degrees Celsius
ΔV	Change in voltage
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
Ba	Barium
C	Concentration
Ca	Calcium
Cl	Chloride
CN	Cyanide
DHTW	Dechlorinated Hamilton tap water
D_I	Diffusion coefficient
DIDS	4,4,' diisothiocyano-2,2'-stilbenedisulfonic acid
DL	Dissolution limit
DMSO	Dimethylsulfoxide
DNP	2,4, -dinitrophenol
DPC	Diphenylamine-2-carboxylic acid
EPRI	Electric Power Research Institute
F	Faraday constant (9.649 x 10^4 Columb per mole)

GHK Equation	Goldman-Hodgkin Katz Equation
h	Hour
HCO_3/CO_3	(Bi)carbonate
IAA	Iodoacetic acid
J_I	Net flux of ion in pmol cm^{-2} s^{-1}
K	Potassium
L	Litre
LC50	Median lethal concentration
Mg	Magnesium
mg l^{-1}	Milligram per litre
MIT	Multi-ion toxicity model
mmol/L	Millimole per litre
mV	Millivolts
N	Sample size
Na	Sodium
pH	Negative log of the hydrogen ion concentration
p_{ion}	Permeability of subscripted ion in meters per second
R	Universal gas constant which is 8.314 joules/mole/ ºK
r^2	Coefficient of determination
S	Slope
SEM	Standard error of the mean
SIET	Scanning ion-selective electrode technique
SO_4	Sulphate

T	Absolute temperature in ºK (temperature in ºC+273)
TEP	Transepithelial potential in volts
µm	micrometer

THESIS ORGANIZATION AND FORMAT

This thesis is organized in a sandwich format and consists of 5 chapters. Chapter 1 is a general introduction outlining the background information and the objectives of the research. Chapters 2 and 3 are manuscripts that have been prepared for submission to peer-reviewed journals and Chapter 4 has been published in a peer-reviewed journal. The final chapter is a general discussion that summarizes the main findings in the context of the current body of literature and proposes future directions for this research.

CHAPTER 1	**GENERAL INTRODUCTION**
CHAPTER 2	**ALTERATIONS IN HEMOLYMPH ION CONCENTRATIONS AND PH IN ADULT *DAPHNIA MAGNA* IN RESPONSE TO ELEVATIONS IN MAJOR ION CONCENTRATIONS IN FRESHWATER** Authors: Carolyn Morris, Michael Sakarya, Odelia Koh and Michael J. O'Donnell Contributions: CM performed all experiments, analyzed all data, and drafted the chapter under the supervision of MJO. MS and OK were supervised by CM and MJO and contributed some of the experimental data. Status: to be submitted to a peer-reviewed journal Journal: *Environmental Toxicology and Chemistry*

CHAPTER 3 **TRANSEPITHELIAL PONENTIAL (TEP) RESPONSES TO INCREASED AMBIENT CONCENTRATIONS OF MAJOR IONS IN ADULT *DAPHNIA MAGNA***

Authors: Carolyn Morris and Michael O'Donnell

Status: to be submitted to industry partner (EPRI) prior to being submitted to a peer-reviewed journal

CHAPTER 4 **MULTIPLE FUNCTIONS OF ION TRANSPORT BY THE NUCHAL ORGAN IN EMBRYOS AND NEONATES OF THE FRESHWATER BRANCHIOPOD CRUSTACEAN, *DAPHNIA MAGNA***

Authors: Carolyn Morris and Michael O'Donnell

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Contributions: CM and MJO performed all experiments, analyzed all data, and drafted the chapter.

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CHAPTER 5 **GENERAL DISCUSSION**

Chapter 1

GENERAL INTRODUCTION

Ionoregulation by Freshwater Animals

Animals living in a hypo-osmotic medium face osmotic influx of water and diffusive loss of salts to the external environment (Hazon et al., 2012; Aladin and Potts, 1995; Larsen et al., 2014). To combat this overhydration and passive ion loss while minimizing the expenditure of energy, hyper-regulators employ active ion uptake, have lower permeability of the body wall to ions and water, decreased oral or anal drinking, and increased rate of urine production (Potts and Parry 1964; Rudy 1967; Fox 1952; Robertson 1960; Lockwood 1976, 1977; Mantel and Farmer 1983).

Salinization of Freshwater Ecosystems

Salinization of freshwater environments is of growing concern (Cañedo-Argüelles et al., 2013; Findlay and Kelly, 2011; Goodfellow et al., 2000; Herbert et al., 2015). The global contamination by major ions (sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations paired with chloride (Cl^-), sulphate (SO_4^{2-}) and (bi)carbonate (HCO_3^-/CO_3^{2-}) anions) of freshwater poses a threat to function, structure and biodiversity of aquatic ecosystems (Abbaspour et al., 2012; Balushkina et al., 2009; Carrasco and Perissinotto, 2012; Mirabdullayev et al., 2004; Velasco et al., 2006; Cañedo-Argüelles et al., 2013; Findlay and Kelly, 2011; Goodfellow et al., 2000; Herbert et al., 2015). Although major ions are critical for physiological functions, alterations of ion concentrations within hypo-osmotic habitats cause osmoregulatory

stress and disturbance to hydromineral homeostasis in freshwater animals. Rock weathering and saline groundwater contribute to salts that are naturally found in freshwater (Schuler et al., 2019). However, over the last two decades, focus has been placed on anthropogenic activities that introduce increased concentrations of major ions in freshwater systems. These activities include irrigation runoff from agriculture (Smedema & Shiati, 2002), saline oil-field discharges (Boelter, Lamming, Farag & Bergman, 1992), road de-icing salt application (Findlay and Kelly, 2011), mountain-top coal mining (Pond et al., 2008), and fracking fluid spills (Blewett et al., 2017). A varying level of risk is introduced by each of these activities, as they deposit different ion species, concentrations and combinations (Mount et al., 2016). For example, the dominant ions associated with road de-icing are Na^+, Cl^-, Ca^{2+}, Mg^{2+} (Forman and Alexander, 1998; Kaushal et al., 2005; Kelting et al., 2012) whereas, the dominant ions associated with mountaintop coal mining are Ca^{2+}, Mg^{2+}, HCO_3^- and SO_4^{2-} (Griffith et al., 2012).

Salinity fluctuations can be detrimental to the function and biodiversity of aquatic ecosystems (Abbaspour et al., 2012; Balushkina et al., 2009; Carrasco and Perissinotto, 2012; Mirabdullayev et al., 2004; Velasco et al., 2006), but the extent of the impact is not completely understood. There has been a concerted research effort into the toxicological effects of elevations of major ions and their interactions (Cormier and Suter, 2013; Cormier et al., 2013; Erickson et al., 2017; Mount et al., 2016; Pond et al., 2008). Toxicity studies have shown that both acute and chronic exposure to single salts or binary salt mixtures have detrimental effects on many freshwater organisms (Erickson et al., 2017; Mount et al., 2016; Mount et al., 2019). Ionic species and concentrations of ions as well as the ratio of different ions in mixtures in the water have all been described as contributing to major ion toxicity (Erickson et al., 2017; Mount et al., 1997; Mount et al., 2016; Mount et al., 2019). The contribution of cation concentration versus

anion concentration to major ion toxicity has been tested and recently, it has been suggested that the cation plays the largest role in toxicity (Erickson et al., 2017; Mount et al., 2016). Binary salt combinations can produce additive or mitigative effects. For example, K^+ is generally toxic but a stimultaneous increase in Na^+ concentration mitigates K^+ toxicity (Mount et al., 2016). The background water composition varies demographically and can influence the effects of increased major ions from anthropogenic activites (Mount et al., 2016).

While some jurisdictions commonly regulate Cl^- concentration, pollution by major ions (species, concentrations and ion combinations) is not widely regulated (USEPA 1998, USEPA 2016). Although conductivity, total dissolved solids, and total salinity have been suggested as regulatory endpoints, no agreement has been made (USEPA 1998, USEPA 2016). The current regulations are inadequate because they are not ion-specific, vary greatly between regions and lack legal consequences (Schuler et al., 2019). The ability to predict toxicity and other physiological disturbances caused by major ion pollution is lacking as there are gaps in knowledge of the associated physiological mechanisms. Exploring these mechanisms may aid in the creation of effective environmental regulations. The Electric Power Research Institute (EPRI) has developed a predictive toxicity model, the Multi-ion Toxicity (MIT) model, that aims to model the differential toxicity of major ions (EPRI, 2018). Electrical gradients are differentially disturbed by major ions across biological membranes, at the site of ion transport (*e.g.* the gills of freshwater animals), thus disrupting ionoregulation and leading to toxicity and even death. As such, this sophisticated and selective approach has the potential to monitor major ions in aquatic environments. Hemolymph ion regulation is influenced by ion transporting pumps and leak channels which affect transepithelial potential (TEP). The model therefore requires knowledge of hemolymph ion concentrations and is based on the notion that a change in

TEP is predictive of mortality. TEP can be predicted by the Goldman-Hodgkin Katz (GHK) Equation.

Equation:

$$TEP = \frac{RT}{F}\ln\left(\frac{p_k[K^+]_o + p_{Na}[Na^+]_o + p_{Cl}[Cl^-]_i}{p_k[K^+]_i + p_{Na}[Na^+]_i + p_{Cl}[Cl^-]_o}\right)$$

where:

R: universal gas constant, which is 8.314 joules/mole/ºK

T: absolute temperature, in ºK (*i.e.* Temperature in ºC + 273)

F: Faraday constant, which is 9.649×10^4 Coulombs per mol

TEP: transepithelial potential, in volts (equivalent to joules per Coulomb)

p_{ion}: permeability for subscripted ion, in meters per second

$[ion]_o$: ion activity in the external water in mM

$[ion]_i$: ion activity in the extracellular fluid of the organism (blood plasma or hemolymph) in mmol per L

This equation was originally developed to predict cellular membrane potential (Goldman, 1943; Hodgkin and Katz, 1949) and takes into account both monovalent and divalent ions. EPRI has modified the GHK equation to predict TEP across the biological membranes as a proxy of toxicity. The MIT model has been built on electrochemical theory and toxicity data but has had no experimental or physiological validation to date.

The effect of salinization of freshwater systems on freshwater crustaceans

Increased salinity in freshwater systems has complex and synergistic effects on freshwater animals. Species richness and diversity decreases in increased salinity (Hart et al., 1991) and several studies measuring endpoints of survival, growth, hemolymph/blood ion concentration and metabolic rate have reported a decrease in individual performance of some freshwater crustacean and fish (Hart et al., 1991; Velasco et al., 2019). When faced with increased salinity, freshwater fish are remarkably better equipped to employ coping mechanisms, including avoidance or tolerance, compared to freshwater crustaceans (Hart et al., 1991). However, crustaceans have been found to initiate molecular changes as well as to alter their behavior, morphology and physiology.

At the molecular level, distinct modification in protein patterns have been identified in copepods (Gonzalez and Bradley, 1994). In daphnids, an increase in heat shock proteins which protect and repair native proteins has been described (Werner and Hinton, 2000), as well as adjustments in internal levels of free amino acids that potentially lower the osmotic gradient between the individual and the external environment (Weider and Hebert., 1987). Increases in aggregation behavior in zooplankton and decreased swimming speed in daphnids are notable behavioral responses to increased salinity (Baillieut and Blust., 1999; Harder 1968). Changes in salinity can lead to modification in gill morphology, particularly in mitochondria rich dark cells found in the gills and digestive system of *Daphnia* (Kikuchi, 1983). It is probable that these dark cells play a significant role in osmoregulation (Grzesiuk and Mikulski, 2006). Respiration rates and ammonium excretion rates in *Daphnia* increase in salinities outside of their optimal range (Arner and Kiovisto, 1993). *Daphnia* exposed to oil-sand process affected water, likely with increased salinity, exhibited increased O_2 consumption and decreased activity and hemoglobin

content (likely by employing macromolecule recycling) (Lari et al., 2017). Among decapod crustaceans, marine lobsters are able to increase their mobility to avoid salinity while freshwater crayfish are not yet known to have this capability (Dufort et al., 2001). Both the rusty crayfish, *Orconectes rusticus*, and the northern clearwater crayfish, *Orconectes propinquus*, showed increased hemolymph Na^+, Cl^-, free amino acids and osmotic pressure at moderately high salinity (Bazer et al., 2016). Salinity stress is associated with variation in hemolymph parameters, including increased glucose, in crayfish, *Astacus leptodactylus* (Yavuzcan Yildiz et al., 2004).

Overall, individual performance has been found to be lower in a salinity challenge than when faced with other stressors (Velasco et al., 2019). The rate of increased salinity may be faster than animals are able to evolve or adapt (Nielsen et al., 2003), highlighting the need for further regulation of pollution by major ions (Velasco et al., 2019).

Daphnia magna as a toxicology model

Daphnia magna, a branchiopod crustacean in the order Cladocera, is a commonly studied biological model and is used for studies of: phenotypic plasticity, behavior, evolution of reproduction and ecotoxicology (Ebert D., 2005). They are often the focus of toxicology experiments of different endpoints. *Daphnia magna* are an excellent research species because they mature in just a few days, exhibit parthenogenetic reproduction and require simple laboratory care. They are planktonic filter feeders that inhabit rocky freshwater or brackish habitats, including rivers, lakes, and pools along the Atlantic coastline of north-eastern US and northern Eurasia (Ebert D., 2005). They use thoracic appendages to produce a water current for a filtering apparatus and their bodies are enclosed by a shell-like carapace (Ebert D., 2005). The

carapace is largely made of chitin, is hardened by Ca^{2+} and has a double wall, between which hemolymph flows (Ebert D., 2005; Giardini et al., 2015).

Daphnids are able to produce live young through asexual parthenogenesis in which unfertilized eggs are released into the brood chamber, develop into embryos and emerge as free swimming neonates (Mittmann et al., 2014). Female *Daphnia magna* are approximately 5-10 days old when they first hold eggs in their brood chamber and they will produce a clutch of eggs every 4-6 days (Ebert D., 2005). When environmental conditions are unfavorable (*e.g.* increased species competition, reduced food availability, decreased temperature or day length, etc.) and survival is uncertain, daphnids are able to reproduce by a pattern of sexual reproduction termed gametogenesis (Alekseev and Lampert, 2001; Ebert D., 2005). A resting egg called an ephippium is fertilized by an individual male and hatches when environmental conditions have improved (Ebert D., 2005).

Daphnids are extremely sensitive to changes in water chemistry, ionic composition and pollutants, making them an ideal organism to study major ion toxicity. Sensitivity of freshwater species to ionoregulatory toxicants depends on body size and surface area to volume ratio. As such, daphnids are extremely sensitive to acute and chronic exposures to toxicants like copper and silver (Bianchini & Wood, 2002; Grosell et al., 2002). They are more sensitive to environmental toxicants including increased ambient major salt concentrations, than freshwater fish and have been the central focus of toxicity studies and environmental threshold modeling efforts (Erickson et al., 2018; Mount et al., 1997; Mount et al., 2016; EPRI 2018., Tietge et al., 1997). The sensitivity of daphnids will be important in driving new environmental regulations for pollution by major salts so it is critically important that their physiological responses are integrated into models such as the EPRI MIT model.

Osmoregulation in Daphnia magna

Crustaceans are a diverse group found in a wide range of aquatic environments (Harris and Aladin, 1997). The success of this group lies with their ability to thrive in waters of varying ionic composition and strength. Crustaceans living in salt-water have been described as osmo-conformers, maintaining their hemolymph concentration close to that of the environmental water, while crustaceans living in estuarine or freshwater are often referenced as hyper-osmoregulators or hypo-osmoregulators (Gilles and Péqueux, 1985; Pequeux, 1995). Ionoregulatory studies have modeled ion transport in salt-transporting epithelia, such as gills in crayfish, crab and shrimp (Kirschner et al., 1973). They employ different osmoregulatory mechanisms to maintain hemolymph ion concentrations mainly through the regulation of internal NaCl concentration (Aladin and Potts, 1995; Bianchini and Wood, 2002; Bianchini and Wood, 2008; Glover et al., 2005; Stobbart et al., 1977). Crustaceans living in freshwater environments face overhydration through osmotic gain of water, as well as passive loss of osmolytes through paracellular and transcellular leakage. Therefore, they are adapted for active ion uptake to combat diffusive efflux of ions and reduction of passive ion loss by limiting the permeability properties of ion transporting epithelia and decreasing the osmotic gradient by lowering hemolymph concentration to maintain osmotic balance (Gilles and Péqueux, 1985, Bianchini and Wood, 2002; Bianchini and Wood, 2008; Glover and Wood, 2005; Ralph, 1967; Stobbart et al., 1977).

Ionoregulation is accomplished by the actions of the maxillary gland, gut and epipodites (*i.e.* gills) in adult *D. magna*. Ion-transporting epithelia within these organs have cells that are deeply folded and are endowed with abundant mitochondria, resembling cells of similar

functions in other crustaceans, insects and fish (Abel and Ellis, 1966; Copeland, 1967; Cowan, 1971; Doyle, 1960; Ernst and Ellis, 1969; Hootman and Conte, 1975). The maxillary gland, as in other freshwater branchiopods, is the excretory organ that is specialized to facilitate salt resorption from urine (Aladin and Plotnikov, 1985). Daphnids have 9 pairs of appendages, five of which are the epipodites that form an apparatus for feeding and respiration (Ebert D., 2005). Epipodites move to produce a water current for suspension feeding and have two different cell types, light and dark, that are likely of ionoregulatory function (Kikuchi, 1983). Freshwater animals generally have lower rates of salt turnover, compared to their seawater counterparts and therefore dietary salt influx is likely to be significant in most freshwater animals (Ebert D., 2005).

It has been noted that patterns and mechanisms of ionoregulation in freshwater crustaceans differ from those in freshwater fish (Hogstrand and Wood, 1998). Previous studies have found that daphnids have different ion transporters (Bianchini and Wood, 2008; Glover and Wood, 2005) and ionoregulatory mechanisms (Hogstrand and Wood, 1998) than fish. Transepithelial potential (TEP) is the voltage difference across an epithelial tissue. This potential is dependent on the permeability properties and the transport activity of the epithelial tissue layers (Potts, 1984). Diffusional and active transport of ions can influence regulation of TEP in animals (Potts, 1984). As TEP is influenced by hemolymph ion regulation through pumps and leak channels, it is likely that TEP regulation also differs between freshwater daphnids and fish (Mount et al., 1997., Tietge et al., 1997).

Ionoregulation of juvenile Daphnia magna

Different life stages of crustaceans cope with challenges imposed by variations in environmental ionic composition through structural, biochemical and physiological changes

(Bianchini and Wood, 2008). Ontogenesis of osmoregulation can occur at the embryonic or neonatal phase of development (Charmantier, Giménez, Charmantier-Daures, & Anger, 2002; Charmantier & Charmantier-Daures, 2006). Early development is highly conserved in cladoceran species and juveniles emerge following epimorphic (direct) embryonic development (Mittmann et al., 2014). Notable ionoregulatory differences between adult and neonate daphnids have been described (Giardini et al., 2015). Compared to neonates, adults have a higher affinity for Na^+ but a lower maximum capacity of Na^+ transport (Bianchini and Wood, 2008). Na^+ channels may be present and associated with a Na^+/H^+ exchanger in adults, however, in neonates, a proton pump-coupled Na^+ channel at the apical membrane appears to play an important role in the whole-body Na^+ uptake (Bianchini and Wood, 2008). At the basolateral membrane of the salt-transporting epithelia of neonates, Na^+ is pumped from the cells to the extracellular fluid by a Na^+/K^+-ATPase and a Na^+/Cl^- cotransporter whereas K^+ and Cl^- move through specific channels. In adults, a $Na^+/K^+/2Cl^-$ cotransporter replaces the Na^+/Cl^- cotransporter (Bianchini and Wood, 2008). Ca^{2+} is important to both adults and juveniles as their carapace is largely reinforced with Ca^{2+}, but they have different tolerances to low ambient calcium as a result of their different life stage-specific Ca^{2+} requirements (Giardini et al., 2015). Research has described the importance of Ca^{2+} uptake in daphnids but particular transport mechanisms have yet to be suggested (Giardini et al., 2015).

Not only are there variations in the mechanisms in ionoregulation between adult and early life stages of daphnids, there are also sensitivity and structural differences. Toxicological data indicate that the early-life stages of aquatic animals are the most sensitive to ionoregulatory toxicants (Bianchini & Wood, 2002; Bianchini & Wood, 2008; Grosell et al., 2002). The osmoregulatory organ in embryonic and neonate daphnids is the nuchal organ.

The nuchal organ runs along the back of the head and appears as an expanded portion of the developing dorsal ridge and prior to development of the epipodites as ionoregulatory organs (Aladin and Potts, 1995). Early studies of a cervical organ in gymnomeran cladocerans, *Podon leuckarti, P. intermedius* and *Evadne normani,* occupying the same region as the nuchal organ had suggested respiratory function or the ability to attach itself to floating objects (Hootman and Conte, 1975; Potts and Durning, 1980; 1978; Longhurst and Seibert 1971). Subsequent ultrastructure studies revealed that, like the nuchal organ found in *Artemia salina* and *Daphnia magna*, the cervical organ presumably has ion-transporting function (Conte et al., 1972; Halcrow, 1982; Potts and Durning, 1980). The nuchal organ is likely involved in salt excretion in saline water as in *A. salina* and salt uptake in freshwater as in *D. magna* (Aladin and Potts, 1995; Halcrow, 1982; Hootman and Conte, 1975). Ion-transport through the nuchal organ has been inferred in *D. magna* through ultrastructural studies that showed that there is extensive amplification of plasma membranes through apical microvilli and basal infoldings, abundant mitochondria and squamous epidermal cells with greater apical-basal depth at the side of the nuchal organ (Halcrow, 1982).

Daphnids in a parthenogenic cycle incubate eggs in brood chambers, between the carapace, that are open to the external environment (Aladin and Potts, 1995). The egg membrane is presumably impermeable until osmoregulatory organs, the nuchal organ and the water excretory maxillary gland, have developed. However, a study tracing Ca^{2+} from parent to offspring may suggest otherwise but no mechanism has been described (Giardini et al., 2015). The function of the nuchal organ is critical in juveniles when the thoracic appendages move very little or are not yet developed, as in embryos (Halcrow, 1982). The nuchal organ is present until the first embryonic molt when ion transport is taken over by the epipodites.

Thesis Objectives

This thesis examines major ion toxicity of adult *Daphnia magna* and ionoregulation in juvenile *Daphnia magna,* including investigations of the relationship between hemolymph ion homeostasis and ambient ion concentrations (chapter 2), TEP in response to metabolic inhibitors and increased major ion concentrations (chapter 3) and ion transport at the site of the nuchal organ in embryo and neonates (chapter 4). Three hypothesis were tested; increased major ion concentrations will cause disturbances to ionoregulation and alter hemolymph ion concentrations (chapter 2), transepithelial potential responses will be altered upon exposure to increases in major ion concentrations in bathing water (chapter 3) and ion transport occurs at the site of the nuchal organ in embryo and neonate *D. magna* (chapter 4).

In chapter 2, hemolymph ion concentrations (K^+, Na^+, Cl^-) and pH were measured in increased ambient concentrations of K^+, Na^+, Ca^{2+} as single salt solutions or as mixtures (K^+ and Na^+; Na^+ and Ca^{2+}). This chapter was focused on testing if exposure to increased concentrations alters ion regulation and therefore changes the ionic composition of the hemolymph. Hemolymph samples were extracted by inserting a micropipette into the hemocoel of the animal near the heart (figure 1), the sample was then was expelled under oil and ion concentration was measured using ion selective microelectrodes (figure 2).

In chapter 3, TEP was measured in response to metabolic inhibitors, sodium cyanide (NaCN) and 2,4 dinitrophenol (DNP) to determine if ATP-dependent transporters contribute to TEP. The effects of blockers of Na^+/K^+ ATP-ase, (ouabain), K^+ channels (Ba^{2+}), Cl^- channels (diphenylamine-2-carboxylate) and Cl^-/ HCO_3^- exchangers (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid) were assessed to determine the contributions of Na^+, K^+ and Cl^- transporters to TEP. Finally, TEP was measured in *D. magna* exposed to increased concentrations of major ions

as single salt solutions (KCl, K_2SO_4, $KHCO_3$, NaCl, Na_2SO_4, $NaHCO_3$, $CaCl_2$, $CaSO_4$, $MgCl_2$, and $MgSO_4$) and binary salt mixtures (KCl:NaCl and NaCl:$CaSO_4$) to investigate the effect of ambient ion concentration on TEP. TEP was measured by impaling the animal through the cuticle near the heart and referenced to a bath electrode (figure 3).

In chapter 4 transport of Na^+, K^+, Cl^-, H^+, Ca^{2+} and NH_4^+ across the nuchal organ and across regions of the body surface away from the organ in both embryonic and neonate *Daphnia magna* were measured using the scanning-ion selective electrode technique (SIET) (figure 4). Embryos were dissected out of the adult while neonates were collected after natural emergence.

Significance of Research

The hemolymph ion and TEP measurements presented in this thesis are the first to be completed in *D. magna.* The findings have revealed that increasing the ion concentration of ambient water causes hemolymph ion accumulation in *D. magna*. We have identified mechanistic ionoregulatory differences between *D. magna* and other freshwater organisms, notably fish, that will be crucial in developing models for regulating major ion levels and determining toxicity thresholds for freshwater ecosystems. We have highlighted ionoregulatory differences between juveniles and adult *D. magna*. Previous studies have inferred ion transport through a specialized nuchal organ in juvenile daphnids, however direct evidence is lacking. We demonstrated, for the first time, real time, directional ion- flux in the nuchal organ in juvenile *D. magna*. We have furthered the understanding of the physiological mechanisms of major ion toxicity in adult *Daphnia magna*. Results within this thesis have highlighted the importance of understanding ionoregulation mechanisms for developing precautionary regulations for restoring and maintaining freshwater ecosystem health.

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FIGURES

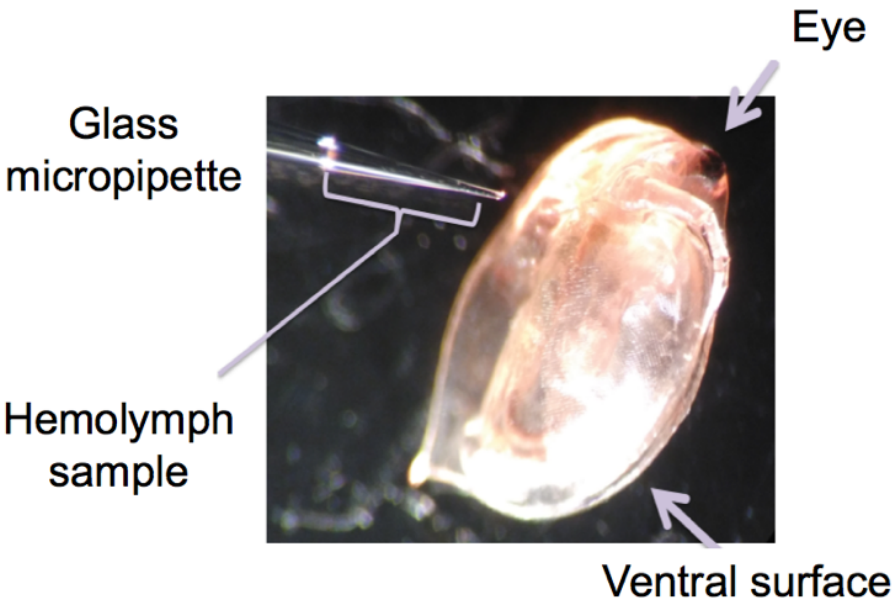

Figure 1-1 Hemolymph collection from *Daphnia magna* in air after blotting dry with filter paper. Micropipette is inserted near the heart (hemocoel sac) and filled with hemolymph via capillary action.

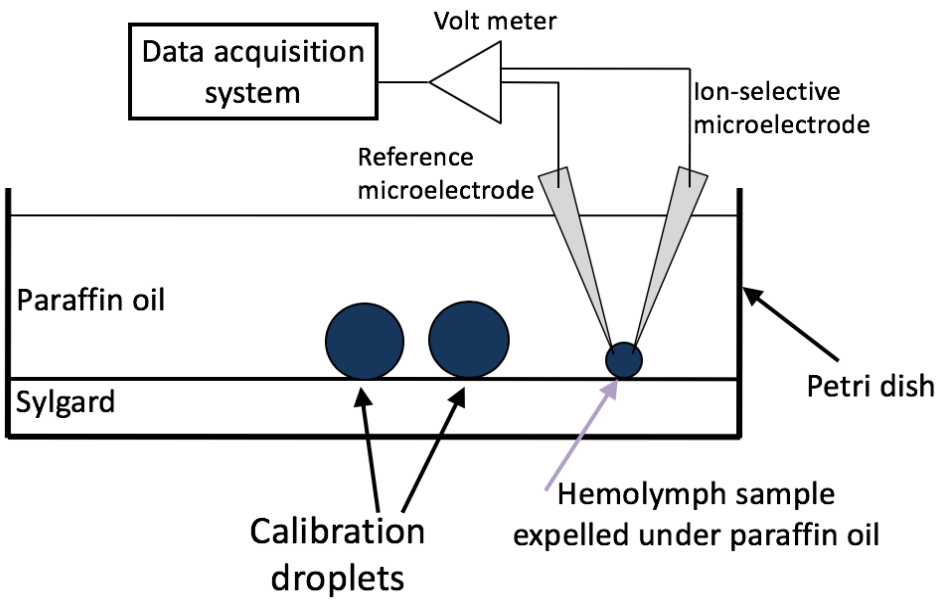
Data acquisition system
Volt meter
Ion-selective microelectrode
Reference microelectrode
Paraffin oil
Sylgard
Petri dish
Calibration droplets
Hemolymph sample expelled under paraffin oil

Figure 1-2 Analysis of hemolymph ion concentrations using ion selective microelectrodes. Set up shows calibration droplets under paraffin oil, hemolymph sample, ion selective electrode and reference electrode and data acquisition system.

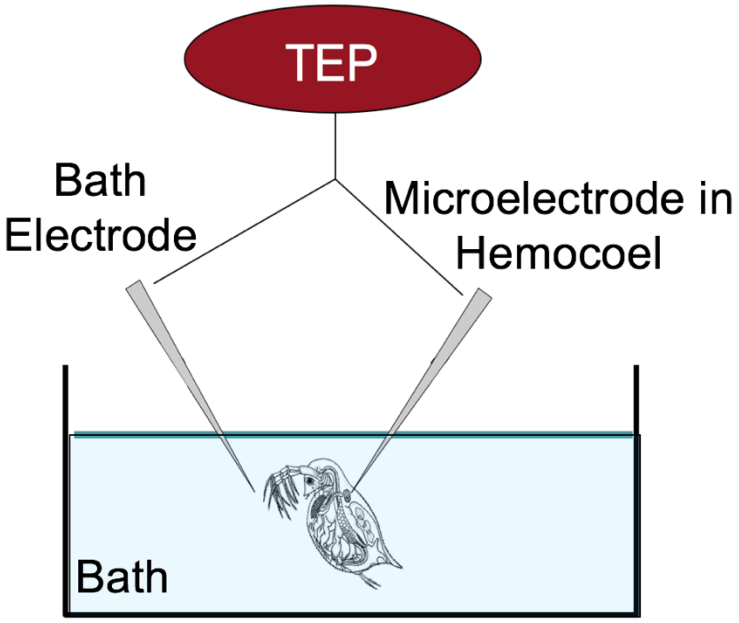
TEP
Bath
Electrode
Microelectrode in
Hemocoel
Bath

Figure 1-3. Transepithelial potential set up. Bath electrode acts as the reference electrode, the impaling electrode is inserted into the hemocoel. The bath water solution is changed through a push-pull perfusion.

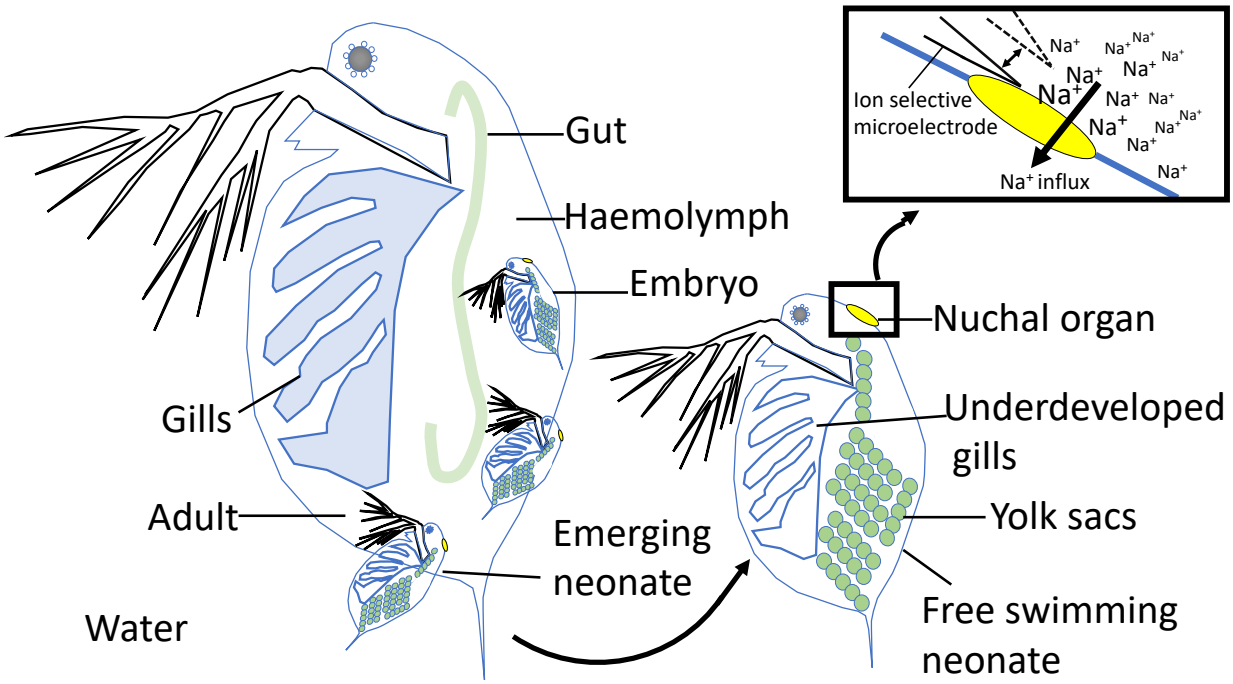
Gut
Haemolymph
Embryo
Gills
Adult
Emerging neonate
Water
Nuchal organ
Underdeveloped gills
Yolk sacs
Free swimming neonate
Ion selective microelectrode
Na+ influx
Na+

Figure 1-4. Life stages of *Daphnia magna* and Na^+ influx at the site of the nuchal organ measured by SIET. Ion concentration gradients are measured in the unstirred layer near the surface of the nuchal organ. The microelectrode is moved from a position within 5µm of the organ surface to a position 50µm further away. Na^+ concentration is measured at the inner and outer limits of microelectrode excursion and the resulting concentration difference is the used to estimate Na^+ flux.

Chapter 2

ALTERATIONS IN HEMOLYMPH ION CONCENTRATIONS AND PH IN ADULT *DAPHNIA MAGNA* IN RESPONSE TO ELEVATIONS IN MAJOR ION CONCENTRATIONS IN FRESHWATER

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ABSTRACT

Increases in the concentrations of major ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-) in freshwater are a growing concern for ecosystem health. These increases may originate from anthropogenic activities such as road de-icing, fracking spills, mining, and fertilizer application and have detrimental effects on freshwater organisms through disturbances in ionoregulation and acid-base balance. The cladoceran *Daphnia magna*, is adapted for active ion uptake and reduction of ion loss to maintain osmotic balance, but alterations in ionic composition of the environmental water are associated with toxicity. In this study, hemolymph ion concentrations were measured using ion selective microelectrode techniques. Increases in the hemolymph concentrations of Na^+ and K^+ correspond to elevations in the concentrations of these ions in ambient water. Water concentrations associated with sustained increases in hemolymph ion concentrations correlate well with LC50 values from previous toxicology studies indicating that Na^+ and K^+ concentrations in hemolymph may predict toxicity. When water K^+ concentration is increased, a simultaneous increase in water Na^+ concentration mitigates the increase in hemolymph K^+ concentration, a finding which is consistent with the reported mitigation of K^+ toxicity by Na^+. When ambient concentrations of K^+, Na^+ and Cl^- are increased, not only is there a rise in hemolymph ion concentration, hemolymph pH is altered and pH regulation appears to be prioritized over inorganic ion regulation in *D. magna*.

Keywords: Major ions, Aquatic toxicology, Major ion toxicity, *Daphnia magna*, Freshwater ionoregulation, Osmoregulation.

INTRODUCTION

There has been increasing concern in recent years about elevations in major ions in surface waters and the possible detrimental effects on aquatic ecosystems (Cañedo-Argüelles et al., 2013; Findlay and Kelly, 2011; Goodfellow et al., 2000; Herbert et al., 2015). The major ions typically present in these freshwater systems include sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations paired with chloride (Cl^-), sulphate (SO_4^{2-}) and (bi)carbonate (HCO_3^-/CO_3^{2-}) anions. These ions are critical for physiological functions, however, fluctuations and elevations of ion concentrations within these environments cause disturbances to hydromineral homeostasis (Mount et al., 2016), resulting in osmoregulatory stress in freshwater animals. These salts originate from diverse sources, many of which are anthropogenic activities such as: irrigation runoff from agriculture (Smedema & Shiati, 2002), saline oil-field discharges (Boelter, Lamming, Farag & Bergman, 1992), road de-icing salt application (Findlay and Kelly, 2011), mountain-top coal mining (Pond et al., 2008), and fracking fluid spills (Blewett et al., 2017). Each of these activities deposit different ion combinations and concentrations in aquatic ecosystems and therefore introduce varying levels of risk (Mount et al., 2016).

The extent of the impact of elevated major ion concentrations in freshwaters has yet to be completely understood. Many countries have not yet judged salinization of freshwater systems to be of concern (Cañedo-Argüelles et al., 2013), and regulatory efforts to monitor pollution by major ions in freshwater systems are compromised by a lack of understanding of the physiological mechanism(s) underlying the toxicity of major ion mixtures. Limited consideration has been given to regulations concerning osmolality, conductivity, total dissolved solids, salinity, and the concentrations of particular cations and anions. All have been suggested as regulatory

endpoints (EPRI, 2016; USEPA, 2011) but no agreement has been reached. Further research on the physiological mechanisms of major ion toxicity is thus required to facilitate freshwater ecosystem management and formulation of appropriate environmental regulations.

Crustaceans living in freshwater environments are adapted for active ion uptake and reduction of passive ion loss to maintain osmotic balance (Bianchini & Wood, 2008). Crayfish, crab and shrimp species have been used extensively in ionoregulatory studies, particularly to model ion transport in salt-transporting epithelia, such as gills (Kirschner et al., 1973). *Daphnia magna* is able to hyper-regulate to counteract the continuous ion loss to the hypo-osmotic medium by actively taking up NaCl (Aladin and Potts, 1995; Bianchini and Wood, 2002; Bianchini and Wood, 2008; Glover et al., 2005;). Whole-body Na^+ uptake is active, saturable and concentration-dependent in daphnids (Aladin and Potts, 1995). The rate of sodium uptake in daphnids is dependent on body size and determines the sensitivity of freshwater species to ionoregulatory toxicants (Bianchini & Wood, 2008). Daphnids have a high ratio of surface area to volume and are extremely sensitive to acute and chronic exposures to toxicants like copper and silver (Bianchini & Wood, 2002; Grosell et al., 2002). It has also been noted that patterns of ionoregulation in freshwater crustaceans differ from those in freshwater fish (Hogstrand and Wood, 1998). Na^+ uptake in freshwater crustaceans is independent of the presence of Cl^- in the external medium and toxicity is associated with an alteration of the whole-body Na^+ concentration (Bianchini & Wood, 2002; Grosell et al., 2002).

There has been a concerted research effort directed towards understanding the toxicological effects of elevations of major ions and their interactions in aquatic ecosystems (Cormier et al., 2013; Erickson et al., 2017; Mount et al., 2016; Pond et al., 2008). Modeling studies provide a selective approach for potential monitoring and regulation of major ions in

aquatic environments (EPRI 2018). Current models are based on the knowledge that major ions disturb electrical gradients across biological membranes differentially, and on the assumption that this disturbance, primarily at the gills of freshwater animals, affects ionoregulation and ultimately leads to toxicity and death.

Recent studies have shown that acute single salt exposure has detrimental effects to many freshwater organisms (Erickson et al., 2017; Mount et al., 2016). Major ion toxicity depends upon the composition of the background water as well as the salt mixtures it contains. There is an additive effect of various ions contributing to overall ion toxicity (Erickson et al., 2017; Mount et al., 2016). The contribution of the anion or cation to salt toxicity has also been evaluated for several single salts, and the cation is now considered to play the larger role in toxicity (Mount et al., 2016). In particular, K^+ is very toxic to daphnids and exposure to high ambient concentrations of K^+ salts causes mortality (Mount et al., 2016). However, altering the ionic composition of the background water, specifically elevating the Na^+ concentration, mitigates K^+ toxicity, resulting in a higher lethal K^+ concentration for 50% mortality (LC50) (Mount et al., 2016).

Given that salinity fluctuations can be detrimental to the function and biodiversity of an aquatic ecosystem (Abbaspour et al., 2012; Balushkina et al., 2009; Carrasco and Perissinotto, 2012;; Velasco et al., 2006), more detailed understanding of the physiological mechanisms of major ion toxicity may contribute to improved environmental regulations. The accumulation of major ions in the hemolymph of daphnids at varying ambient single and binary salt concentrations may be indicative of toxicity and possibly correlate with published LC50 values (Mount et al., 1997; Mount et al., 2016). In this study we have examined the relationship between hemolymph ion homeostasis and ambient ion concentrations. Hemolymph ion

concentrations (K^+, Na^+, Ca^{2+}, Cl^-) and pH have been measured during elevations in ambient concentrations of K^+, Na^+, Ca^{2+} as single salt solutions or as mixtures (K^+ and Na^+; Na^+ and Ca^{2+}).

MATERIALS AND METHODS

Rearing Daphnia magna

A starter culture of *Daphnia magna* Strauss was obtained from commercial suppliers (Boreal Science, St. Catharines, ON and Carolina Biological Supply, Burlington, NC) and maintained at room temperature (23° C ± 1° C) under fluorescent light on a 17h:7h light:dark photoperiod in aerated 20L tanks of dechlorinated Hamilton tap water (DHTW). The water was sourced from Lake Ontario and contained (in mmol l^{-1}: 1 Ca^{2+}, 0.6 Na^+, 0.70 Cl^-, 0.5 SO_4, 0.3 Mg^{2+} and 0.05 K^+, with titration alkalinity of 2.1 mequiv l^{-1}, hardness of ≈140 mg l^{-1}as $CaCO_3$ equivalents, and pH ≈8.0 (Hollis et al., 2001; Leonard et al., 2014). *Daphnia* were fed a 2:2:1 mixture of Spirulina powder: Chlorella powder: yeast 3 times per week.

Test water preparation

Salt additions (KCl, NaCl, $NaHCO_3$, Na_2SO_4, $CaSO_4$) and mannitol were made with a background of DHTW. Experimental concentrations were based on 48h LC50 data reported by Mount et al (2016) for *Ceriodaphnia dubia* (Table 1).

A moderately hard reconstituted water recipe (Table 2) was adapted to make stock solutions with varying concentrations of Na^+ and Ca^{2+} below those in DHTW to evaluate the effect of binary salt solutions on hemolymph ion concentrations (Weber, 1993).

Hemolymph sampling and measurement of ion concentration

Daphnia magna were exposed to test solutions for varying periods (0hr, 0.5hr, 1hr, 1.5hr, 2hr, 3hr, 4hr, 24hr or 48hr) before individuals were collected and blotted dry with a tissue wipe (*i.e.* Kim wipe). Animals were immobilized by placing them on the back of a small petri dish covered with double-sided tape. A glass micropipette was then inserted into the hemocoel near the heart and the sample was drawn up by capillary action. Air pressure applied by a hand-held syringe connected to the glass micropipette through flexible tubing was used to expel the hemolymph into a petri dish containing mineral oil to prevent sample evaporation. Reference and ion-selective microelectrodes were constructed as described previously (Donini et al., 2007; Ruiz-Sanchez et al., 2015). The electrodes were connected through a chlorided silver wire that was connected to a high impedance ($>10^{13}$ Ω) electrometer. Voltages were recorded and analyzed using a data acquisition system (PowerLab, AD Instruments, Sydney, Australia) and LabChart software. Calibration droplets of known ionic composition or pH were selected to bracket the range of concentrations or pH, respectively, of the measured ion in the hemolymph sample. All calibration solutions were prepared from reagent grade salts (MilliporeSigma, Burlington, MA).

The ion concentration of the hemolymph sample ($[ion]_{sample}$), was calculated using the following equation:

$$[ion]_{sample} = C \times 10^{(\Delta V/S)}$$

where C is the known ion concentration of one of the calibration solutions, ΔV is the change in the voltage between the calibration droplet and the hemolymph sample, and S is the slope, or the

difference in voltage between two calibration droplets differing 10-fold in ion concentration (Ruiz-Sanchez et al. 2015). Although ion-selective electrodes measure ion activity and not concentration, data can be expressed in terms of concentrations if it is assumed that the ion activity coefficient is the same in calibration and experimental solutions. Expression of data in terms of concentrations simplifies comparisons with studies in which ion concentrations were measured by techniques such as flame photometry. The pH of the hemolymph sample (pH_{sample}) was calculated using the equation:

$$pH_{sample} = pH_{cal} - (\Delta V/S)$$

where pH_{cal} is the known pH of one of the calibration solutions.

Statistics

Graphing and statistical tests of significance were done using GraphPad Prism 8 (San Diego CA). Differences were considered significant if $p < 0.05$. Data have been expressed as mean ± SEM (N) where N represents the number of animals sampled. Changes in hemolymph [ion] over time, within the same water condition were assessed with one-way ANOVA followed by Tukey's multiple comparisons post hoc test. Changes in hemolymph [ion] over time between two different water conditions were assessed with two-way ANOVA followed by Sidak's multiple comparisons post hoc test (Supplementary Material, Table 1). For experiments where hemolymph ion concentrations were measured after a single 48h exposure, differences between hemolymph [ion] were assessed with an unpaired t-test.

RESULTS

Elevation in hemolymph [K^+] is irreversible when ambient [K^+] exceeds 5mM KCl

Sustained increases in hemolymph [K^+] were seen when bathing solution KCl concentrations were above published LC50 values (Table 1) (Mount et al., 2016). Animals were exposed to five solutions with varying [KCl]. Previous research has shown that the 48h LC50 value of KCl is 5mM for daphnids (Mount et al., 2016). The effects of the LC50 concentration, three concentrations below the LC50, 1mM, 2mM, and 3mM KCl and two concentrations above the LC50, 10mM and 20mM KCl were evaluated. Hemolymph K^+ concentration did not differ significantly from the control value (2.18 ± 0.05, N = 186) when animals were exposed to water containing 1mM KCl and 2mM KCl (Figure 1A, Figure 1B). In 3mM and 5mM KCl, hemolymph [K^+] increased transiently from the control value up to ≈4-5mM K^+ and returned to control levels by 24h (Figure 1, Figure1C, Figure 1D). Animals exposed to 10mM KCl showed a progressive elevation in hemolymph [K^+] up to ≈6mM [K^+] and 100% mortality was observed after 4h (Figure 1E). In 20mM KCl, hemolymph levels were elevated to ≈6mM [K^+] within 0.5h and 100% mortality was observed by 3h (Figure 1F).

Elevation in hemolymph [Na^+] is irreversible when ambient [Na^+] exceeds 20mM NaCl

Sustained increases in hemolymph [Na^+] were seen when bathing solution NaCl concentrations were at or above published LC50 values (Table 1) (Mount et al., 2016). Animals were exposed to four different environmentally relevant concentrations of NaCl, including the previously published 48h LC50 for NaCl of 30mM (Mount et al., 2016) and three concentrations below the LC50 (10mM, 15mM and 20mM NaCl. Hemolymph [Na^+] did not differ significantly

from the control (0 h) value (54.2 ± 0.81, N = 85) in 10mM NaCl and 15mM NaCl (Figure 2A, Figure 2B). In 20mM NaCl and 30mM NaCl there was a sustained elevation of hemolymph [Na^+] up to ≈75mM [Na^+] (Figure 2C, Figure 2D).

Higher freshwater [NaCl] mitigates the impact of elevated freshwater [KCl] on hemolymph [K^+] and lower freshwater [NaCl] exacerbates the impact of elevated freshwater [KCl] on hemolymph [K^+]

Previous studies have shown that the presence of Na^+ mitigates K^+ toxicity for daphnids and therefore increases the LC50 value for water [K^+] (Mount et al. 2016). For measurements of hemolymph [K^+], the control [Na^+] condition was no added [NaCl] (*i.e.* 0.6mM ([Na^+] of DHTW), the high [Na^+] condition was 10mM NaCl and the low [Na^+] condition was 0.1mM NaCl.

High [NaCl]

Previous studies have shown that the 48h LC50 value of KCl was 10mM when [NaCl] was 10mM (Mount et al., 2016). The LC50 concentration, one concentration below the LC50 value (5mM KCl in high [Na^+]) and one concentration above the LC50 value (20mM KCl in high [Na^+]) were evaluated. The addition of 10mM NaCl mitigated the elevation of hemolymph [K^+] in higher [KCl] and animals remained alive when high [NaCl] was present compared to the control condition (*i.e.* 0.6mM [Na^+] in DHTW). In 5mM KCl with high [NaCl], hemolymph [K^+] did not significantly differ from the control level (2.18 ± 0.05, N = 186) (Figure 3A) and were thus significantly lower at 0.5, 1, 1.5, 2, 3 and 4h when compared to 5mM KCl with no added

NaCl (Figure 3A, Figure 1F; Supplementary Table 1). Hemolymph [K^+] in *D. magna* exposed to 5mM KCl with no added NaCl was as high as ≈4mM K^+ (Figure 1D). In 10mM KCl with high [NaCl], hemolymph [K^+] increased from the control level to ≈5mM K^+, then returned to control levels by 24h (Figure 3B) and was significantly lower when compared to hemolymph from animals exposed to 10mM KCl with no added NaCl at 1, 1.5, 2 and 3h (Figure 3B, Figure 1E; Supplementary Table 1). The 24h data were not included in the statistical test because 100% mortality occurred between 5 and 24h in 10mM KCl with control [Na^+] (*i.e.* 0.6 mM). However, in 10mM KCl in high [NaCl], all animals lived to 24h and hemolymph [K^+] was restored to control levels (Figure 3B). In 20mM KCl in high [NaCl], the hemolymph [K^+] increase was sustained, and was significantly higher than 20mM KCl with no added NaCl at 1h and 1.5h (Figure 3C, Figure 1F; Supplementary Table 1). The 3h, 4h, and 24h data were not included in the statistical test because 100% mortality occurred at 3h. By contrast, 100% mortality was observed between 3 and 24h in 20mM KCl with no added NaCl (Figure 1F).

Low [NaCl]

Previous research has shown that the LC50 value of KCl was 2mM when [NaCl] was 0.1mM (Mount et al. 2016). The LC50 concentration, one concentration below the LC50 value (1mM KCl in low Na^+) and two concentrations above the LC50 value (3mM KCl in low [NaCl] and 5mM KCl in low [NaCl]) (*i.e.* 0.6mM [Na^+] in DHTW) were evaluated. In low [NaCl] conditions, hemolymph [K^+] was more sensitive to increases in water [KCl]. In 1mM KCl and 2mM KCl in low [NaCl] there was significant elevation in hemolymph [K^+] from control (2.18 ± 0.05, N = 186) to ≈3.5mM and by 24h hemolymph [K^+] returned to control (0 h) levels (Figure

4A, Figure 4B). In 1mM KCl in low [NaCl], hemolymph [K^+] was significantly higher when compared with 1mM KCl in control [NaCl] at 1.5h (Figure 4A, Figure 1A; Supplementary Table 1). In 2mM KCl in low [NaCl], hemolymph [K^+] was significantly higher when compared with 2mM KCl in control [NaCl] at 1.5, 3, and 4h (Figure 4B, Figure 1B; Supplementary Table 1). In 3mM KCl and 5mM KCl in low [NaCl] there was a sustained increase in hemolymph [K^+] from ≈2mM to ≈3.5mM K^+ (Figure 3C) and ≈5.5mM K^+ (Figure 3D), respectively. In 3mM KCl in low [NaCl], hemolymph [K^+] was significantly lower when compared with 3mM KCl in control [NaCl] at 2h and significantly higher when compared with 3mM KCl in control [NaCl] 24h (Figure 4C, Figure 1C; Supplementary Table 1). In 5mM KCl in low [NaCl], hemolymph [K^+] was significantly higher when compared with 5mM KCl in control [NaCl] at 2 and 4h (Figure 4D, Figure 1D; Supplementary Table 1).

Increase in ambient [KCl] causes elevations in both hemolymph [K^+] and [Na^+]

The experiments described above (Figure 2) evaluated the mitigating effects of NaCl on hemolymph [K^+] elevation and raised the question of the potential effects of elevation of water [KCl] on hemolymph [Na^+]. At 24h in high [NaCl], hemolymph [K^+] was significantly elevated above control levels (*i.e.* [K^+] in DHTW = 2.18 ± 0.05mM, N = 186) in 20mM KCl but not in 10mM KCl or 5mM KCl (Figure 5A). Unexpectedly, in 10mM [NaCl] and increasing [KCl] there was a steady increase in hemolymph [Na^+]. Hemolymph [Na^+] was significantly elevated above control levels (*i.e.* [Na^+] in DHTW = 54.2 ± 0.81mM, N = 85) at 24h in 10mM and 20mM KCl (Figure 5B).

The increase in hemolymph [Na^+] in Daphnia exposed to elevated freshwater [NaCl] is altered by freshwater [Ca^{2+}]

Previous studies have suggested that increased [Ca^{2+}] mitigates Na^+ toxicity (and increases the LC50 value for Na^+) (Mount et al., 2016). Our results show that decreased ambient [Ca^{2+}] causes dysregulation of hemolymph Na^+ concentration compared to control levels of Ca^{2+} (*i.e.* 1mM [Ca^{2+}] in DHTW). Hemolymph Na^+ concentration was measured in animals exposed to the LC50 value, 20mM NaCl + 0.04mM Ca_2SO_4 (Figure 6A) and 30mM NaCl + 0.04mM Ca_2SO_4 (Figure 6B) over a 24h period. Animals exposed to 20mM NaCl + 0.04mM Ca_2SO_4 showed elevated hemolymph Na^+ concentration at 1h relative to control (0h) levels (54.2 ± 0.81, N = 85). However, these elevations were not significantly different at these time points compared to the elevation of hemolymph [Na^+] of animals exposed to 20mM NaCl with control Ca^{2+} levels in DHTW (1mM Ca^{2+}) (Figure 2C, Figure 6A; Supplementary Table 1). In 30mM NaCl + 0.04mM Ca_2SO_4 there was an increase in hemolymph Na^+ concentration from control (0 h) levels at 1h and 24h. In comparison to 30mM NaCl with control Ca^{2+} levels in HDTW (1mM Ca^{2+}), there was a significantly lower hemolymph Na^+ concentration at 3h and 4h in 30mM NaCl + 0.04mM Ca_2SO_4 (Figure 2D, Figure 6B; Supplementary Table 1).

Effects of anions and ambient [$CaSO_4$] on increases in hemolymph [Na^+] elevations in Daphnia exposed to elevated freshwater [NaCl]

For NaCl, the LC50 value was 30mM when [$CaSO_4$] was 0.4mM and 20mM when [$CaSO_4$] was 0.04mM (Mount et al. 2016). The effects of 20mM and 30mM NaCl on hemolymph [Na^+] were therefore evaluated in both high (0.4 mM) and low (0.04 mM) $CaSO_4$ concentrations. When Cl^- was the anion accompanying Na^+, there was no significant difference

in hemolymph [Na^+] when bath [Na^+] was varied from 20mM NaCl to 30mM NaCl and [$CaSO_4$] was varied from 0.04mM Ca^{2+} to 0.4mM Ca^{2+} (Figure 7A). For Na_2SO_4, the LC50 was 20mM in 0.4mM $CaSO_4$ and 10mM in 0.04mM $CaSO_4$ (Mount et al. 2016), therefore both 10mM and 20mM of Na_2SO_4 were evaluated at high and low [$CaSO_4$]. Hemolymph [Na^+] was lower in 0.4mM Ca^{2+} than in 0.04mM Ca^{2+} when SO_4^{2-} was the anion accompanying 10 mM Na^+ in the water (Figure 7B). Also, in 20 mM $NaSO_4$, mortality occurred in 0.04mM Ca^{2+} but not in 0.4mM Ca^{2+} (Figure 7B), For $NaHCO_3$, the LC50 was 15mM in 0.04mM $CaSO_4$ and 20mM in 0.4mM $CaSO_4$ (Mount et al. 2016); animals were therefore exposed to 15mM and 20mM of $NaHCO_3$ in both control and low [$CaSO_4$]. When HCO_3^- was the anion, 0.4 mM $CaSO_4$ mitigated the elevation of hemolymph [Na^+] in 15mM $NaHCO_3$ (Figure 7C). Unexpectedly, in 20mM $NaHCO_3$, hemolymph [Na^+] was lower in 0.04 mM $CaSO_4$ compared to 0.4 mM $CaSO_4$ (Figure 7C). Additionally, when HCO_3^- was the accompanying anion, hemolymph [Na^+] was ≈20mM lower than when Cl^- or SO_4^{2-} was the accompanying anion. Taken together, the results in Figure 7 show that changes in [Ca^{2+}] can alter the change in hemolymph [Na^+] during exposure to increased ambient [Na^+] and that the anion of the Na^+ salt also influences the extent of hemolymph [Na^+] elevation.

Hemolymph [Cl^-] increases with increased ambient [NaCl]

Hemolymph [Cl^-] was measured in animals exposed to water with [Cl^-] far above the level of 0.7mM in DHTW. Given that animals exposed to 10mM KCl and 30mM NaCl showed significant elevations in hemolymph [K^+] and [Na^+], respectively, we measured hemolymph [Cl^-] in these two conditions to determine if there was a corresponding increase in hemolymph [Cl^-]. Animals exposed to 10mM KCl showed a significant decrease in hemolymph [Cl^-] at 3h but

returned to control levels (48.5 ± 1.37, N = 22) by 4h. Although mortality occurred between 5 and 24h, as observed in Figure 1E when [K^+] was measured, there was no corresponding elevation in hemolymph [Cl^-] up to 4h (Figure 8A). By contrast, animals exposed to 30mM NaCl showed significant and progressive increases in hemolymph [Cl^-] over time that did not return to control levels, but there no mortality was observed (Figure 8B).

Effects of increased ambient KCl and NaCl on hemolymph pH

As noted above, significant increases in hemolymph [K^+] and [Na^+] were seen in animals exposed to 10mM KCl and 30mM NaCl, respectively. The extent of changes in hemolymph [K^+] were mitigated by the presence of 10mM NaCl in the water and the changes in hemolymph [Na^+] were affected by changes in water [Ca^{2+}]. Because H^+ regulation is often linked to transport of cations through processes such as Na^+/H^+ exchange, we wished to determine if there were corresponding changes in hemolymph pH in animals exposed to 10mM KCl and 30mM NaCl. Hemolymph pH of animals in DHTW was 8.29 ± 0.02 (N = 46). Hemolymph pH increased at 2h in animals exposed to 10mM KCl and then returned to control levels (Figure 9A). Although mortality occurred between 5-24h, there were no corresponding significant changes in hemolymph pH. In 10mM KCl + 10mM NaCl there was an increase in hemolymph pH at 3h and a decrease at 24h compared to control (0 h) (Figure 9B) and hemolymph pH was significantly lower at 1h and 1.5h relative to hemolymph of animals in 10mM KCl (Figure 9A, 9B; Supplementary Table 1). Animals exposed to 30mM NaCl did not show significant changes in hemolymph pH between 0-4h, but hemolymph pH decreased significantly between 4-24h (Figure 9C). In 30mM NaCl + 0.04mM Ca_2SO_4 there were no changes in hemolymph pH between 0-3h; pH increased at 4h then returned to control (0 h) levels by 24h (Figure 9D). Compared to 30mM

NaCl, animals exposed to 30mM NaCl + 0.04mM Ca_2SO_4 showed a significantly higher pH at 4h and 24h (Figure 9C, Figure 9D; Supplementary Table 1).

Hemolymph [K^+] elevation is due to [KCl] and hemolymph [Na^+] elevation is due to [NaCl] not to an increase in osmolality

Animals were exposed to the LC50 concentration of mannitol (75mM) (Mount et al. 2016), one concentration below the LC50 value (50mM) and one concentration above the LC50 value (100mM). There were no significant changes in hemolymph [K^+] (Figure 10A) or hemolymph [Na^+] (Figure 10B) after 48h in 50 mM, 75mM or 100mM mannitol. There was no mortality observed in any of these conditions.

DISCUSSION

Our results show that hemolymph concentrations of Na^+ and K^+ increase in response to increases in the concentrations of these ions in the water (Figure 1, Figure 2) and that the increase in hemolymph cation concentration is altered by the anion in the Na^+ and K^+ salts in the water (Figure 6, Figure 8). An increase in hemolymph [K^+] or [Na^+] provides a physiological correlate of toxicity and at sublethal exposure levels, hemolymph ion concentrations can be restored over time. Our results also show that increases in hemolymph [K^+] in response to increased water [K^+] can be mitigated by concomitant increases in water [Na^+], and that increases in hemolymph [Na^+] in response to increased water [Na^+] can be altered by concomitant changes in water [Ca^{2+}]. Given that we primarily measured changes in hemolymph cation concentrations in response to changes in ambient salt concentrations, we will focus predominately on discussing the physiological disturbances caused by these cations.

Hemolymph [Na^+] and [K^+] increase in response to increases in water [Na^+] and [K^+]

Our measurements reveal increases in the concentrations of hemolymph cations, $Na^+$and K^+, in response to elevations of these ions in the water bathing *D. magna*. Once ambient ion concentrations approach levels that are associated with toxicity, hemolymph ion concentrations rise as much as 3-fold above control values. At low ambient concentrations of single salts (≤ 5mM K^+, ≤ 15mM Na^+) daphnids are able to recover from these large disturbances in hemolymph ion concentrations and restore the concentrations to control levels over time. The elevation in hemolymph Na^+ and K^+ concentrations in response to water concentrations of these ions at or above previously published LC50 values thus provides a physiological indication of toxicity (Mount et al., 2016).

Animals show a wide variety of physiological adaptations to maintain ionoregulatory homeostasis in a range of salinities while minimizing the expenditure of energy. Crustaceans cope with challenges imposed by variations in environmental ionic composition through structural, biochemical and physiological changes (Aladin and Potts, 1995; Bianchini and Wood, 2008; Halcrow, 1982). As in most invertebrates, the osmolality of crustacean hemolymph is due mostly to inorganic ions, especially Na^+ and Cl^-. Two mechanisms are implicated in the control of the hemolymph Na^+ and Cl^- levels in freshwater crustaceans: (1) reduction in the permeability of ionoregulatory epithelia to limit diffusive losses of ions, and (2) increases in active uptake of NaCl to counterbalance diffusive losses (Pequeux, 1995). At high concentrations of ambient Na^+ and K^+ daphnids are no longer able to cope with the increased salinity and these ions build up in the hemolymph, likely causing toxicity.

The exact physiological mechanism of major ion toxicity is unclear, but it likely differs for Na^+ as opposed to K^+ (Mount et al., 2016). K^+ is highly toxic to daphnids and K^+-toxicity correlates well to K^+ concentration (Mount et al, 2016). Given the depolarizing effects of K^+ on crustacean nerves and muscles (Fatt and Katz, 1953), toxicity in hyperkalemic states likely reflects impaired neuromuscular functioning. Na^+ salts are much less toxic than K^+ salts and no specific Na-related mechanism has been described (Mount et al., 2016). Animals exposed to increased major ion concentrations are unable to osmoregulate and there are consequent effects on higher level processes such as feeding rate, growth, reproduction and survival (Achuthankutty et al., 2000).

Effects of the conjugate anion on the responses to increased cation concentrations in the water

Animals exposed to 10mM KCl showed no increase in hemolymph Cl^- relative to animals in DHTW. However, at the highest dose of NaCl, 30mM, there was an increase in hemolymph Cl^- concentration. The discrepancy is likely due to the higher concentration of Cl^-, not the cation with which it is associated. However, the increases in cation concentrations in hemolymph are much higher than corresponding increases in the anion concentrations. For example, there was a larger increase in hemolymph Na^+ concentration at 30mM NaCl than hemolymph Cl^- concentration; $[Na^+]$ at 24h increased to 1.5 times control $[Na^+]$, whereas the increase in $[Cl^-]$ at 24h was 1.2 times the control concentration. This is consistent with the cation dominating toxicity, but toxicity may not be exclusively cation-driven (Mount et al., 2016). Likewise, when evaluating the protective effect of Ca^{2+}, the anion altered both the magnitude and time course of increases in hemolymph $[Na^+]$. Although previous studies debate the relative contributions of

cations versus anions to toxicity (Mount et al., 1997; Mount et al., 2016), our findings indicate that different anions alter the changes in hemolymph Na^+ and K^+ concentrations in response to increased concentrations of these cations in the water. Likewise, salts of the same cation paired with different anions have different reported LC50 values (Mount et al., 2016).

Some Cations Protect Against Elevations of Other Cations in Hemolymph

Na^+ is protective against elevation of hemolymph [K^+]

When water K^+ concentration is increased to as much as 10mM, a simultaneous increase in water Na^+ concentration mitigates the increase in hemolymph K^+ concentration, a finding which is consistent with mitigation of K^+ toxicity by Na^+ (Mount et al., 2016). In the absence of added NaCl in the water, elevation of hemolymph [K^+] to ≈6mM (Figure 1E, F) was associated with mortality. However, in water containing 10mM NaCl and 20mM KCl, increases in hemolymph [K^+] to ≈8 mM (Figure 3C) were not associated with mortality and hemolymph [K^+] was reduced to ≈5mM by 24h. It thus appears that an elevation in hemolymph [K^+] above ≈6mM is not in and of itself lethal if there is a corresponding increase in water [Na^+] and/or hemolymph [Na^+]. When there is a decrease in [Na^+] below control levels in the water there is enhanced elevation in hemolymph K^+ concentration as water K^+ concentration is increased. Overall, it appears that with the increased Na^+ concentrations in the water, KCl exposure appears to be less of a physiological challenge to hemolymph K^+ homeostasis.

While increased ambient [Na^+] mitigates increases in hemolymph [K^+] when water K^+ concentration is increased to as much as 10mM, in a high and constant concentration of Na^+ and increasing K^+ concentration in the water there is an increase in hemolymph Na^+ concentration

but not in hemolymph K^+ concentration in both 5mM and 10mM KCl. These data suggest there may be a link between regulation of hemolymph [Na^+] and hemolymph [K^+]. The mechanism of response to elevated [K^+] in the water may also result in elevation of hemolymph [Na^+]. Membrane transporters which link the transport of Na^+ and K^+ include the Na^+/K^+ ATP-ase and $Na^+/K^+/2Cl^-$ cotransporters, both of which have been suggested to contribute to ion regulation in adult *D. magna* (Bianchini and Wood, 2008). Additionally, it has been shown that K^+ transport can be uncoupled from Na^+ regulation in crustaceans through K^+/H^+ exchangers, K^+/Cl^- co-transport and K^+ channels (*e.g.* Ca^{2+} -activated K^+ channels and K_{IR} channels) (Harvey, 2014; Thiel and Chang, 2015; Wieczorek et al., 1991).

Ca^{2+} is protective against elevation of hemolymph Na^+ and alters Na^+ hemolymph regulation

Previous findings show that Na^+ uptake is decreased in low [Ca^{2+}] (Glover and Wood, 2005; Havas et al., 1984), which is consistent with our results (Figure 6). Low [Ca^{2+}] is not associated with competitive interactions between Na^+ and H^+, whereas high [Ca^{2+}] is associated with a competitive reaction between Na^+ and H^+ (Glover and Wood, 2005). The use of HCO_3^- as the anion may have an impact on hemolymph pH regulation and therefore the availability of protons, which may affect Na^+ transport and in turn hemolymph [Na^+]. Ca^{2+} and Na^+ reciprocally inhibit each other in a competitive manner as they appear to share the same transport site when environmental [Ca^{2+}] is at high concentration (Glover and Wood, 2005). However, at lower environmental [Ca^{2+}] concentrations, Na^+ uptake appears to be Ca^{2+}-dependent. Mortality in low [Ca^{2+}] may be due to decreased Na^+ influx, insufficient to compensate the high rate of Na^+ depletion through passive ion loss to the water. Increased [Ca^{2+}] facilitates Na^+ uptake, possibly through reductions in the permeability of leakage pathways so as to limit passive Na^+ efflux.

Conversely, when water Na^+ concentration is increased, a simultaneous increase in water Ca^{2+} concentration mitigates the increase in hemolymph Na^+ concentration (Figure 7), a finding which is consistent with mitigation of Na^+ toxicity by Ca^{2+} (Mount et al., 2016).

Acid-base regulation in response to increased ambient KCl and NaCl

Our results do not show a clear association between hemolymph pH and the rise of Na^+, K^+ and Cl^- in the hemolymph that can be explained on the basis of the actions of one particular ion transporter. Rather, there is likely a combination of transporters or other mechanisms that maintain internal acid-base balance. One explanation of our results is that *D. magna* prioritize the regulation of hemolymph pH even if this entails a rise of hemolymph ion concentrations.

In aquatic crustaceans, acid base balance between the animal and its environment is regulated predominately by cation/H^+ exchangers (e.g. K^+/H^+ exchanger and Na^+/H^+ exchangers) and anion exchangers (e.g. Cl^-/HCO_3^-) (Genovese et al., 2005; Lucu, 1990; Onken et al., 1991). Our results in 10mM KCl may reflect the activity of a K^+/H^+ exchanger, resulting in higher K^+ concentration and a lower H^+ concentration inside the animal contributing to the increase in internal pH. A Cl^-/HCO_3^- exchanger is likely contributing to the maintenance of acid base balance in *D. magna* but not contributing to the rise in pH in animals exposed to 10mM KCl as there is no change in hemolymph Cl^- concentrations over time.

Increases in water [Na^+] may have complex effects on *D. magna.* In 30mM NaCl there is a rise in hemolymph concentrations of both Na^+ and Cl^- while pH stays within control levels until 24h when pH decreases significantly. A sustained rise in hemolymph [Na^+] could elevate intracellular Na^+ concentrations, which, in turn, would reduce the activity of Na^+/H^+ exchangers, which transport Na^+ into the cells and H^+ out of the cells. Na^+ uptake is saturable (Glover and

Wood, 2005) and therefore once uptake of Na^+ can no longer increase through this exchanger, H^+ may accumulate inside the animal and cause hemolymph pH to decrease. It is well documented that acid precipitation has caused the disappearance of fish, molluscs and crustaceans from freshwater systems (Leivestad et al., 1976). H^+-linked Na^+ uptake may explain, in part, the high sensitivity of freshwater animals to aquatic acidification and the mechanism behind such mortalities is the breakdown in Na^+ regulation (Vangenechten et al., 1989, Wood, 1989). It has been suggested that an electroneutral Na^+/H^+ exchanger as well as an electrogenic $2Na^+/1H^+$ exchanger may contribute to Na^+ uptake in *D. magna*, as in many other invertebrates (Glover and Wood, 2005). This exchanger can also play a role in divalent cation exchange, such as Ca^{2+}.

CONCLUSION

We set out to determine if animals exposed to elevated ambient ion concentrations showed changes in hemolymph ion concentrations that could be indicative of toxicity. Our results show that hemolymph ion concentrations are altered in response to changes in ambient ion concentrations, particularly as they reach and exceed published LC50 values for daphnids. Future work could examine the effects of putative inhibitors of ion transporters to identify which transporters are involved in responses to the osmoregulatory challenges of increased major ion concentrations. Hemolymph ion regulation is influenced by pumps and leak channels which affect transepithelial potential (TEP). Future measurements of TEP changes in response to increased major ion concentrations could also aid in identification of which ion transporters are affected. Furthering our understanding of the physiological responses to major ion exposure and toxicity could be important in developing predictive models for regulating major ion levels and determining toxicity thresholds for freshwater ecosystems.

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TABLES

Table 2-1. 48h LC_{50} values for *Ceriodaphnia dubia* (Mount et al., 2016).

Salt	**LC50**[1]
KCl	5mM
K_2SO_4	3.5mM
$KHCO_3$	5mM
NaCl	30mM
Na_2SO_4	25mM
$NaHCO_3$	12mM
$CaCl_2$	15mM
$CaSO_4$	16mM[2]
$MgCl_2$	10mM
$MgSO_4$	8.5mM
KCl in 10mM NaCl	10mM
KCl in 0.1mM NaCl	2mM
NaCl in 0.04mM $CaSO_4$	20mM
NaCl in 0.4mM $CaSO_4$	30mM
Na_2SO_4 in 0.04mM $CaSO_4$	10mM
Na_2SO_4 in 0.4mM $CaSO_4$	20mM
$NaHCO_3$ in 0.04mM $CaSO_4$	15mM
$NaHCO_3$ in 0.4mM $CaSO_4$	20mM

[1]Rounded to the nearest whole number

[2]Dissolution limit

Table 2-2 Preparation of Moderately Hard Reconstituted Synthetic Freshwater

Salt (mg/L)[1]				Final Water Quality		
$NaHCO_3$	$CaSO_4 \bullet 2H_2O$	$MgSO_4$	KCl	pH[2]	Hardness[3]	Alkalinity[3]
96.0	60.0	60.0	4.0	7.4-7.8	80-100	60-70

(Weber, 1993)

[1]Reagent grade chemicals added to deionized water

[2]Equilibrium pH after 24h of aeration

[3]Expressed as mg $CaCO_3$/L

FIGURES

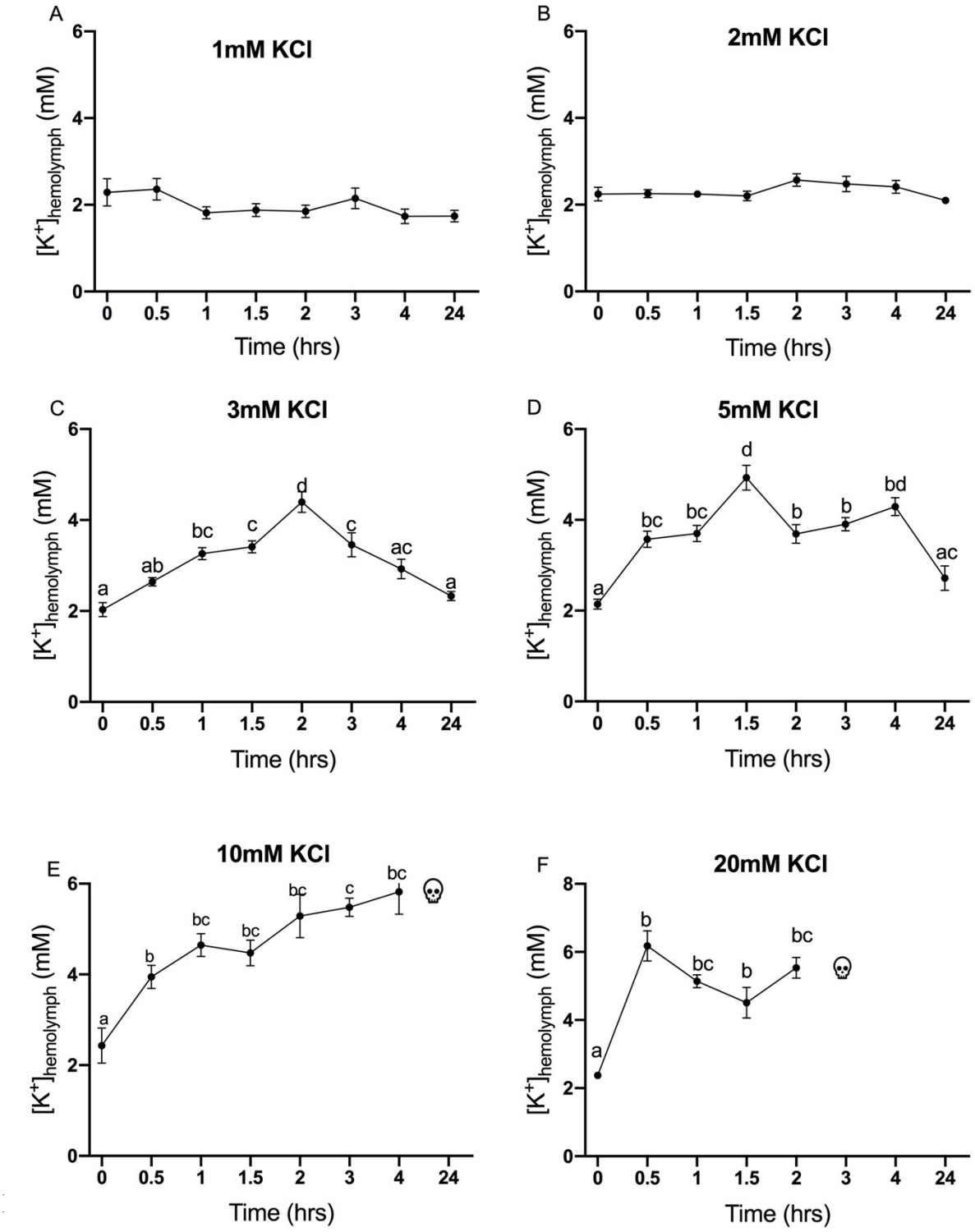

A
1mM KCl
B
2mM KCl
C
3mM KCl
D
5mM KCl
E
10mM KCl
F
20mM KCl
[K+]hemolymph (mM)
Time (hrs)
0
0.5
1
1.5
2
3
4
24

Figure 2-1. Effects of varying KCl concentration in DHTW on hemolymph K^+ concentration ($[K^+]_{hemolymph}$) of *Daphnia magna* (mean ± SEM). Points labelled with the same letter do not differ significantly (ANOVA with Tukey's multiple comparison test). (A) 1mM KCl (N = 9-15 per time) (B) 2mM KCl (N = 6-16) (C) 3mM KCl (N = 6-18) (D) 5mM KCl (N = 6-18) (E) 10mM KCl (N = 10-15). Skull icon indicates mortality of all remaining animals between 4h and 24h. (F) 20mM KCl, (N = 7-12). Skull icon indicates mortality of all remaining animals by 3h.

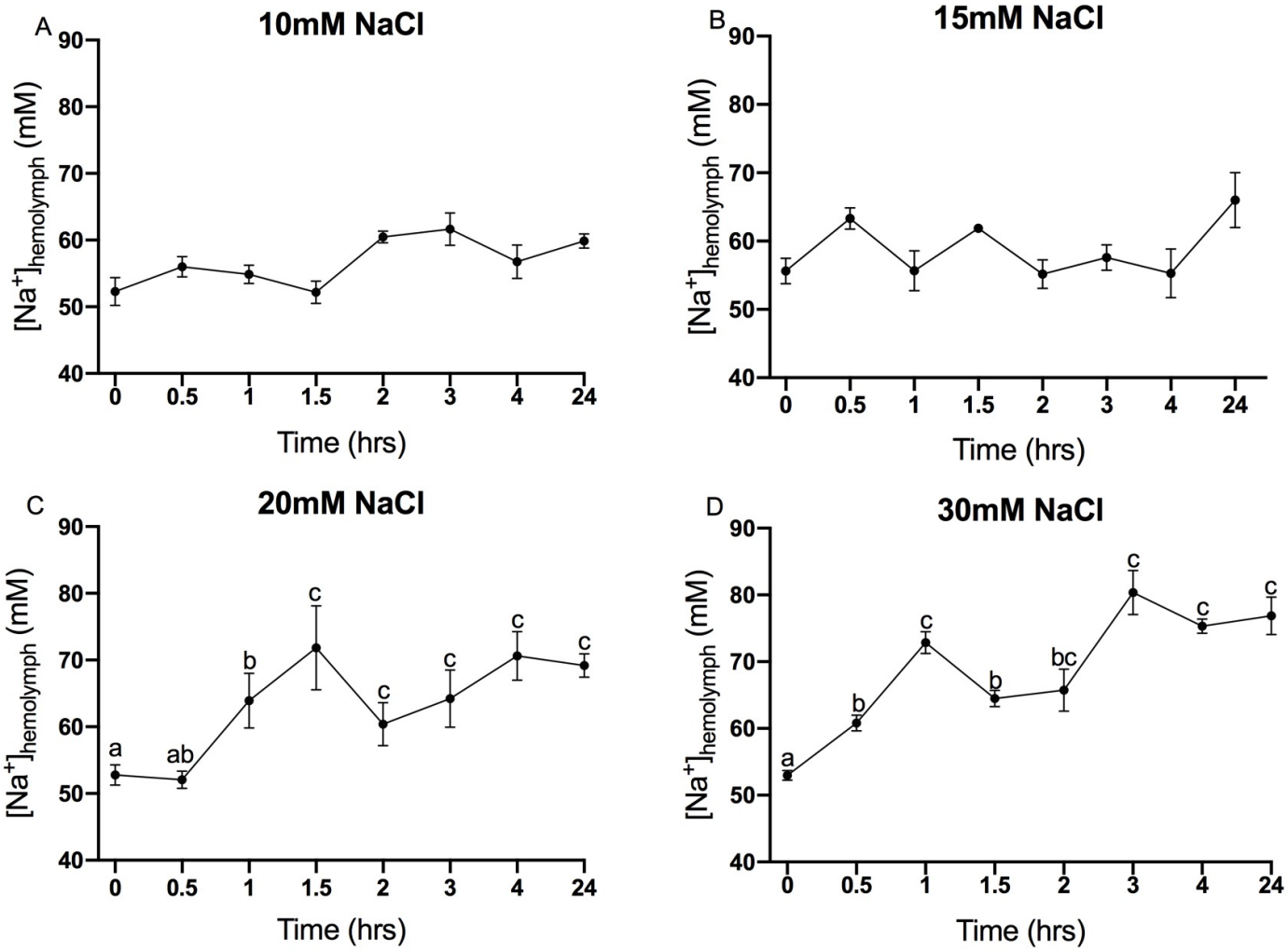

A
10mM NaCl
B
15mM NaCl
C
20mM NaCl
D
30mM NaCl
[Na+]hemolymph (mM)
Time (hrs)
0
0.5
1
1.5
2
3
4
24
40
50
60
70
80
90
a
ab
b
c
bc

Figure 2-2. Effect of varying NaCl concentration in DHTW on hemolymph Na^+ concentration ($[Na^+]_{hemolymph}$) of *Daphnia magna* (mean ± SEM). Points labelled with the same letter do not differ significantly (ANOVA with Tukey's multiple comparison test). (A) 10mM NaCl (N = 6-8 per time) (B) 15mM NaCl (N = 6-8) (C) 20mM NaCl (N = 6-7) (D) 30mM NaCl (N = 6-14).

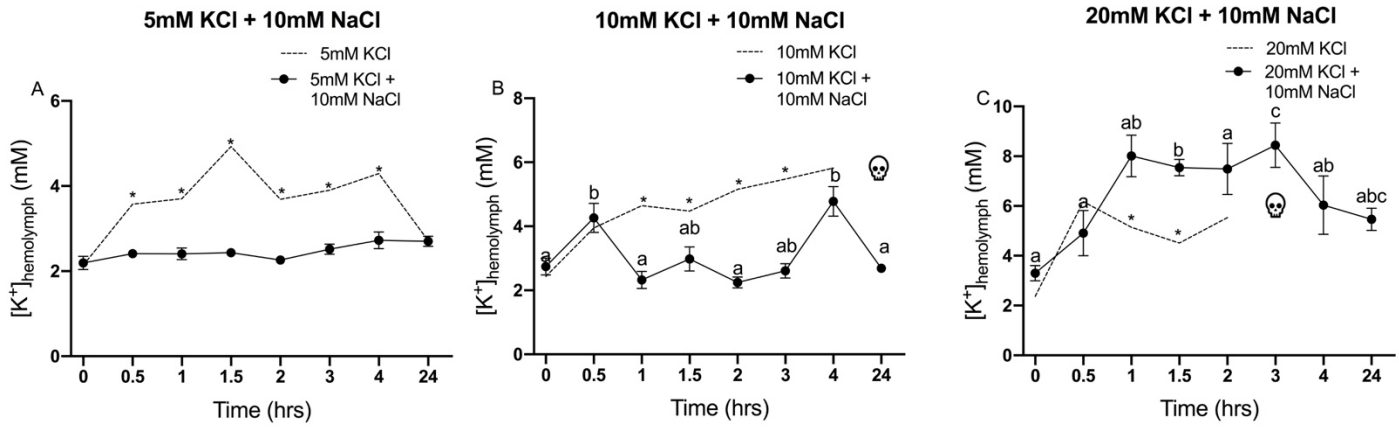

5mM KCl + 10mM NaCl
5mM KCl
5mM KCl + 10mM NaCl
A
[K+]hemolymph (mM)
Time (hrs)
10mM KCl + 10mM NaCl
10mM KCl
10mM KCl + 10mM NaCl
B
[K+]hemolymph (mM)
Time (hrs)
20mM KCl + 10mM NaCl
20mM KCl
20mM KCl + 10mM NaCl
C
[K+]hemolymph (mM)
Time (hrs)

Figure 2-3. Effect of varying KCl concentration in water containing 10mM NaCl on hemolymph K^+ concentration ($[K^+]_{hemolymph}$) of *Daphnia magna* (mean ± SEM). Points labelled with the same letter do not differ significantly (One Way ANOVA with Tukey's multiple comparison test). Dotted line represents data from Figure 1 (D, E, and F) displayed for ease of comparison. Points labelled with an asterisk differ significantly between treatments (Two Way ANOVA with Tukey's multiple comparison test) (A) 5mM KCl + 10mM NaCl (N= 7-10 per time) (B) 10mM KCl + 10mM NaCl (N = 7-10) (C) 20mM KCl + 10mM NaCl (N = 7-10).

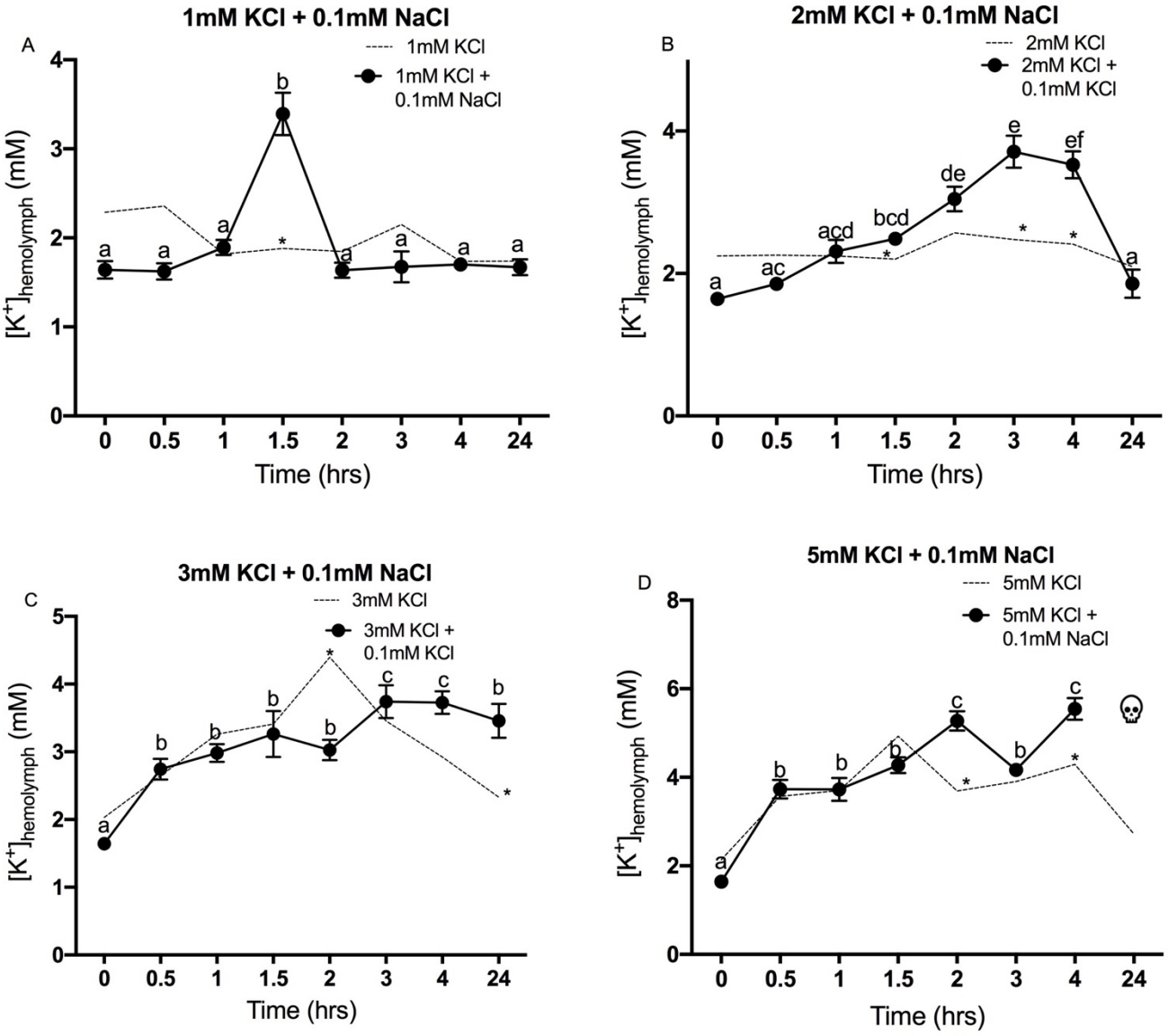

1mM KCl + 0.1mM NaCl
A
1mM KCl
1mM KCl + 0.1mM NaCl
[K+]hemolymph (mM)
Time (hrs)
2mM KCl + 0.1mM NaCl
B
2mM KCl
2mM KCl + 0.1mM KCl
3mM KCl + 0.1mM NaCl
C
3mM KCl
3mM KCl + 0.1mM KCl
5mM KCl + 0.1mM NaCl
D
5mM KCl
5mM KCl + 0.1mM NaCl

Figure 2-4. Effect of varying KCl concentration in water containing 0.1mM NaCl on hemolymph K^+ concentration($[K^+]_{hemolymph}$) in *Daphnia magna* (mean ± SEM). Points labelled with the same letter do not differ significantly (ANOVA with Tukey's multiple comparison test). Dotted line represents data from Figure 1(A, B, C and D) displayed for ease of comparison. Points labelled with an asterisk differ significantly between treatments (Two Way ANOVA with Tukey's multiple comparison test) (A) 1mM KCl + 0.1mM NaCl (N = 7-10 per time) (B) 2mM KCl + 0.1mM NaCl (N = 7-10) (C) 3mM KCl + 0.1mM NaCl (N = 7-10) (D) 5mM KCl + 0.1mM NaCl (N = 6-13).

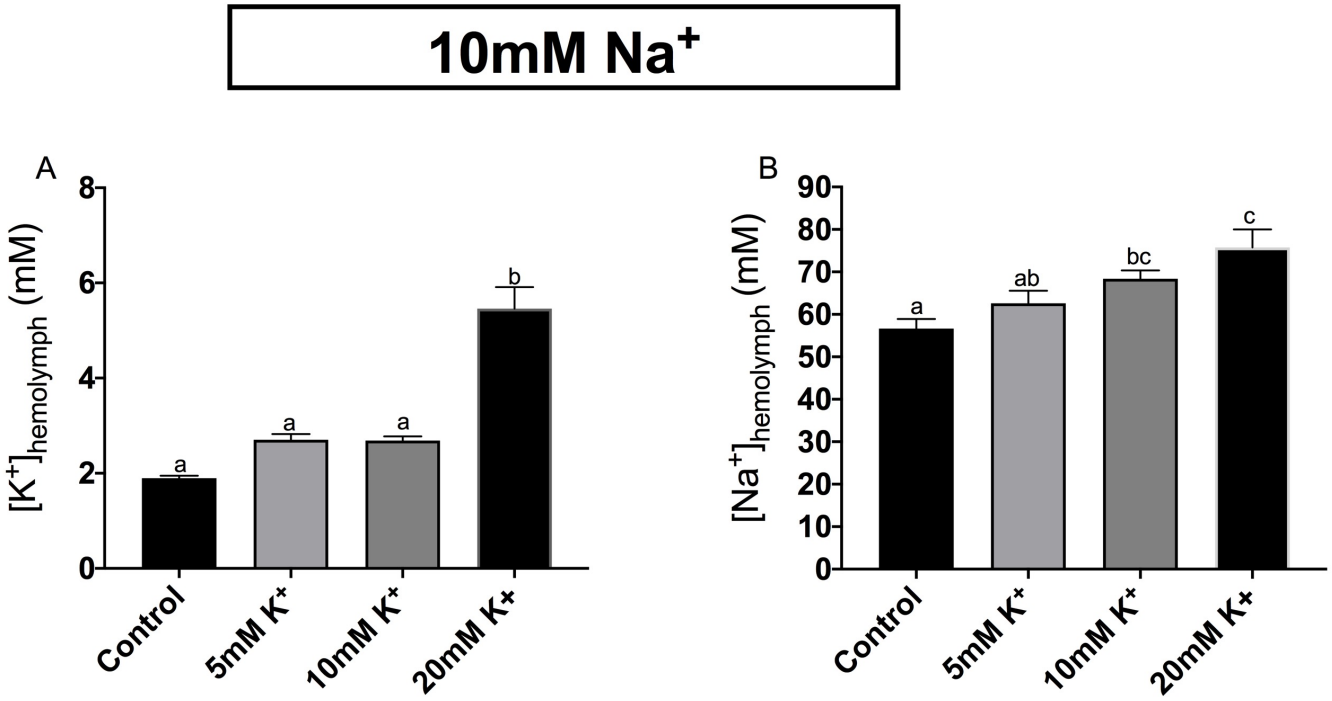
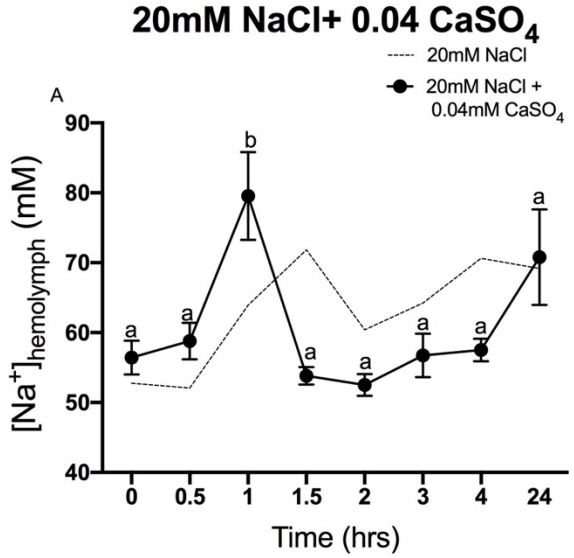

10mM Na+
A
[K+]hemolymph (mM)
0
2
4
6
8
a
a
a
b
Control
5mM K+
10mM K+
20mM K+
B
[Na+]hemolymph (mM)
0
10
20
30
40
50
60
70
80
90
a
ab
bc
c
Control
5mM K+
10mM K+
20mM K+

Figure 2-5. (A) Hemolymph [K^+] in response to control (N = 9), 5mM KCl +10mM NaCl (N = 7), 10mM KCl + 10mM NaCl (N = 7), and 20mM KCl + 10mM NaCl (N = 11) at 24h. (B) Hemolymph [Na^+] in response to control (N = 8), 5mM KCl +10mM NaCl (N = 7), 10mM KCl +10mM NaCl (N = 11), and 20mM KCl + 10mM NaCl (N = 7) at 24h. Points labelled with the same letter do not differ significantly (One-way ANOVA with Tukey's multiple comparison test).


20mM NaCl+ 0.04 CaSO4
20mM NaCl
20mM NaCl + 0.04mM CaSO4
A
[Na+]hemolymph (mM)
Time (hrs)
0
0.5
1
1.5
2
3
4
24
40
50
60
70
80
90
a
a
b
a
a
a
a
a

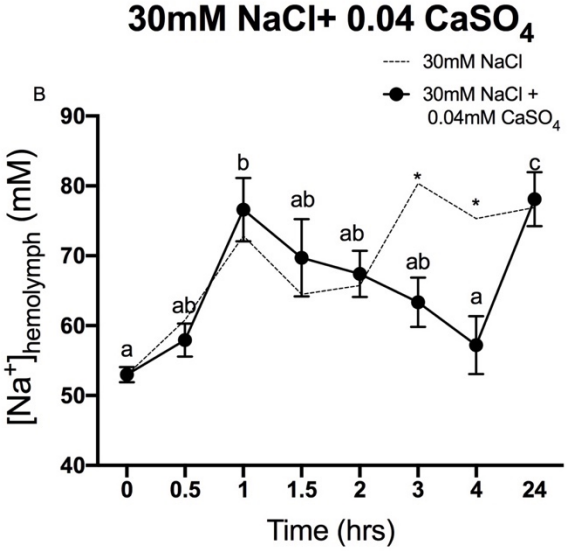

30mM NaCl+ 0.04 CaSO4
30mM NaCl
30mM NaCl + 0.04mM CaSO4
B
[Na+]hemolymph (mM)
Time (hrs)
0
0.5
1
1.5
2
3
4
24
40
50
60
70
80
90
a
ab
b
ab
ab
ab
a
c
*
*

Figure 2-6. Effect of low (0.04mM) Ca^{2+} concentration (as $CaSO_4$) on hemolymph $[Na^+]$ ($[Na^+]_{hemolymph}$) over time. Points labelled with the same letter do not differ significantly (ANOVA with Tukey's multiple comparison test). Dotted lines represent data from Figure 2 (C and D) displayed for ease of comparison. Points labelled with an asterisk differ significantly between treatments (Two Way ANOVA with Tukey's multiple comparison test) (A) 20mM NaCl + 0.04mM Ca^{2+} (N = 6-9 per time point). (B) 30mM NaCl + 0.04mM Ca^{2+} (N = 5-14).

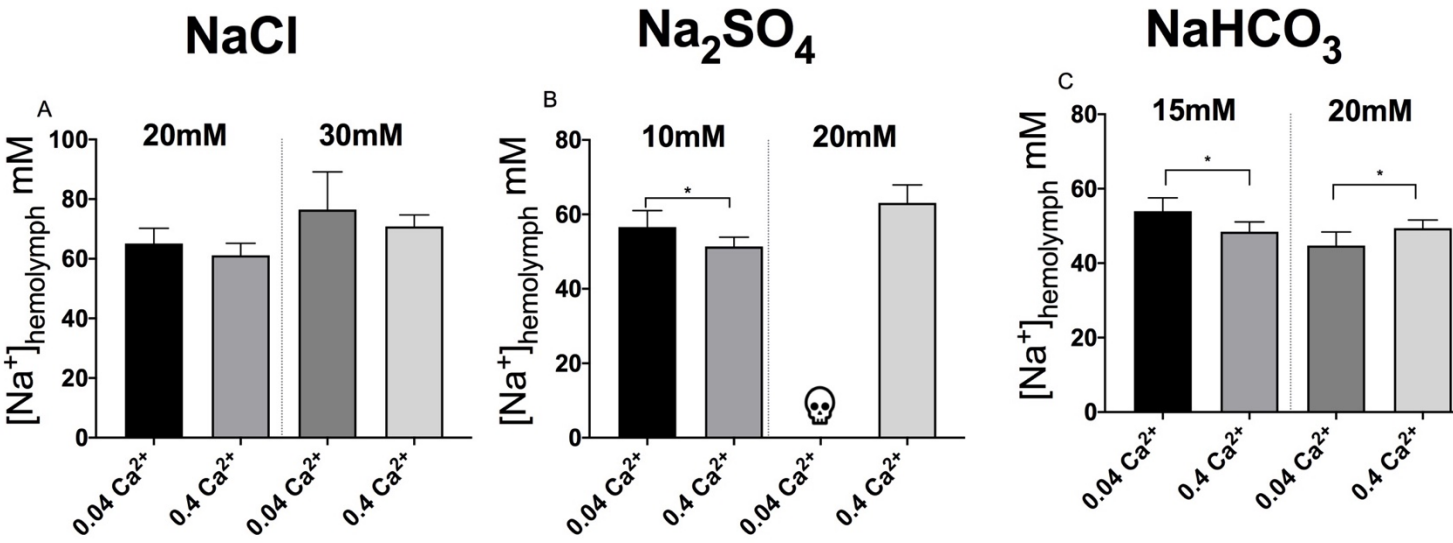

NaCl
A
20mM
30mM
[Na+]hemolymph mM
100
80
60
40
20
0
0.04 Ca2+
0.4 Ca2+
0.04 Ca2+
0.4 Ca2+
Na2SO4
B
10mM
20mM
[Na+]hemolymph mM
80
60
40
20
0
*
0.04 Ca2+
0.4 Ca2+
0.04 Ca2+
0.4 Ca2+
NaHCO3
C
15mM
20mM
[Na+]hemolymph mM
80
60
40
20
0
*
*
0.04 Ca2+
0.4 Ca2+
0.04 Ca2+
0.4 Ca2+

Figure 2-7. Effects of solutions of Na^+ made with Cl^-, HCO_3^- or SO_4^{2-} as the accompanying anion in low (0.04mM) and control (0.4mM) $CaSO_4$ concentrations on hemolymph [Na^+] at 48h. Point labeled with an asterisk differ significantly (Unpaired t-test). (A) 20mM NaCl + 0.04mM [$CaSO_4$] (N = 8), 20mM NaCl + 0.4mM [$CaSO_4$] (N=8), 30mM NaCl + 0.04mM [$CaSO_4$] (N=8), 30mM NaCl + 0.04mM [$CaSO_4$] (N=9). (B) 10mM Na_2SO_4 + 0.04mM [$CaSO_4$] (N = 7), 10mM Na_2SO_4 + 0.4mM [$CaSO_4$] (N = 9), 20 mM Na_2SO_4 + 0.4mM [$CaSO_4$] (N = 8). (C) 15mM $NaHCO_3$ + 0.04mM [$CaSO_4$] (N = 14), 15mM $NaHCO_3$ + 0.4mM [$CaSO_4$] (N = 8), 20mM $NaHCO_3$ + 0.04mM [$CaSO_4$] (N = 15), 20mM $NaHCO_3$ + 0.4mM [$CaSO_4$] (N = 9).

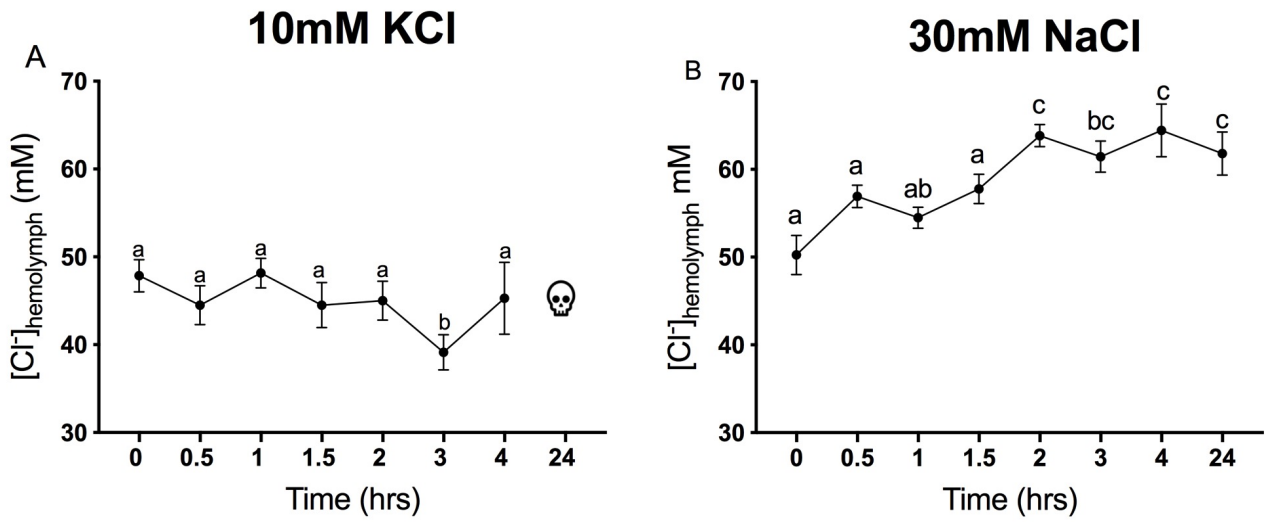

A
10mM KCl
[Cl-]hemolymph (mM)
70
60
50
40
30
a
a
a
a
a
b
a
0
0.5
1
1.5
2
3
4
24
Time (hrs)
B
30mM NaCl
[Cl-]hemolymph mM
a
a
ab
a
c
bc
c
c
Time (hrs)

Figure 2-8. Effects of high ambient KCl and NaCl on hemolymph [Cl^-] ([Cl^-]$_{hemolymph}$) over time (mean ± SEM). Points labelled with the same letter do not differ significantly (ANOVA with Tukey's multiple comparison test). (A) 10mM KCl (N = 7-17). (B) 30mM NaCl (N = 6-15). Skull icon indicates mortality of all remaining animals between 5h - 24h.

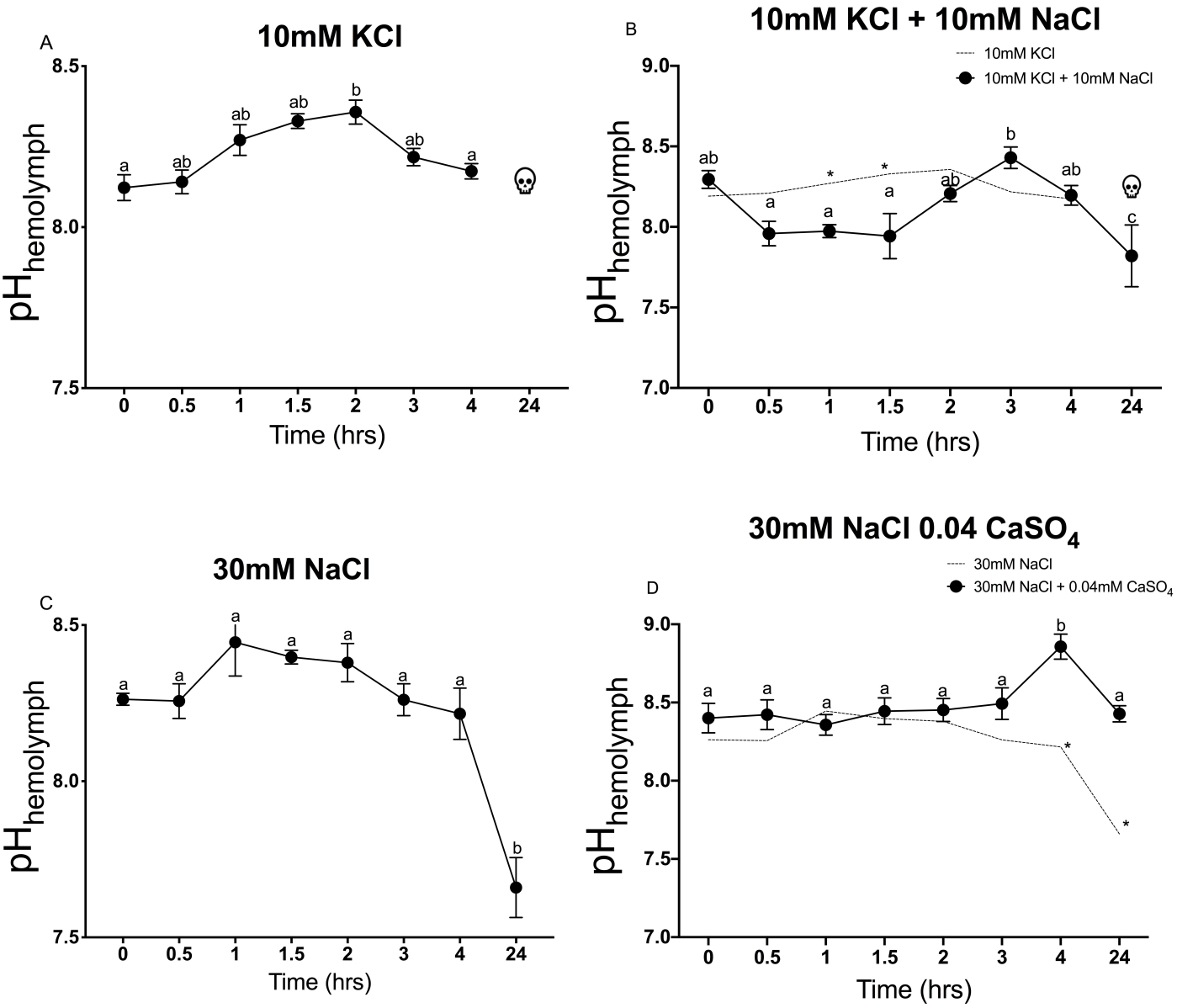
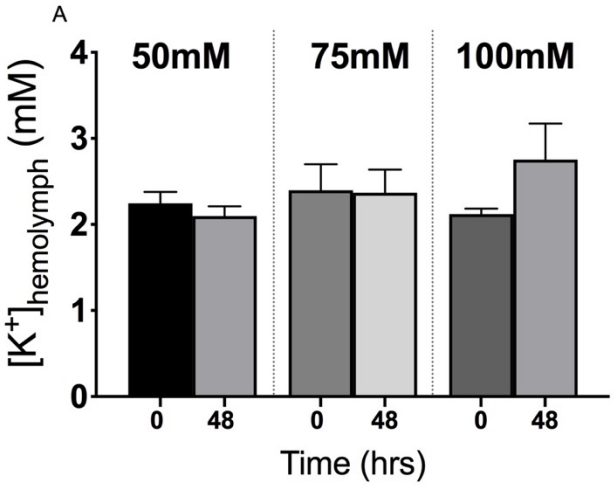

A
10mM KCl
pH hemolymph
Time (hrs)
B
10mM KCl + 10mM NaCl
10mM KCl
10mM KCl + 10mM NaCl
C
30mM NaCl
D
30mM NaCl 0.04 CaSO4
30mM NaCl + 0.04mM CaSO4

Figure 2-9. Hemolymph pH ($pH_{hemolymph}$) in response to increased ambient [KCl] and [NaCl] (mean ± SEM). Points labelled with the same letter do not differ significantly (ANOVA with Tukey's multiple comparison test). Dotted lines in panels C and D represent data from Figure 9 (A and B) displayed for ease of comparison. (A) 10mM KCl (N = 6-10). (B) 10mM KCl + 10mM NaCl (N=7-9). (C) 30mM NaCl (N = 6-13). (D) 30mM NaCl + 0.04mM Ca_2SO_4 (N=9-23). Skull icon indicates mortality of all remaining animals between 4h and 24h.


A
50mM
75mM
100mM
$[K^+]_{hemolymph}$ (mM)
0
1
2
3
4
0
48
0
48
0
48
Time (hrs)

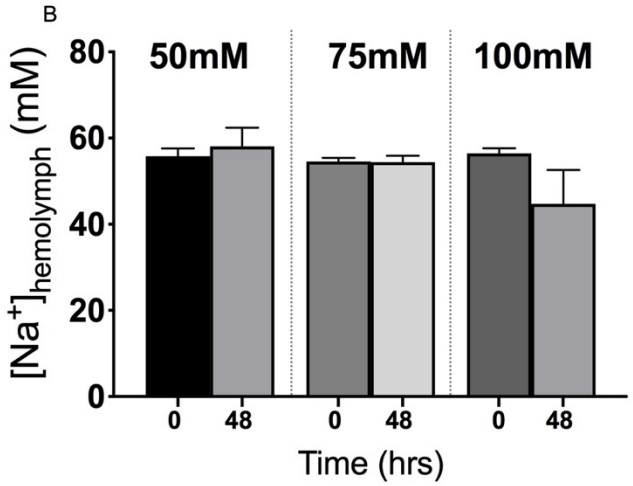

B
50mM
75mM
100mM
$[Na^+]_{hemolymph}$ (mM)
0
20
40
60
80
0
48
0
48
0
48
Time (hrs)

Figure 2-10. Effects of varying [mannitol] on hemolymph [K^+] and hemolymph [Na^+] at 0 and 48h. Differences in [Na^+] or [K^+] between 0 and 48 h for any of the mannitol concentrations tested were not significant (One way ANOVA with Tukey's multiple comparison test). (A) 50mM mannitol 0h (N = 13), 50mM mannitol 48h (N = 8). 75mM mannitol 0h (N = 9), 75mM mannitol 48h (N = 9). 100mM mannitol 0h (N = 11), 100mM mannitol 48h (N = 8). (B) 50mM mannitol 0h (N = 8), 50mM mannitol 48h (N = 6). 75mM mannitol 0h (N = 9) 75mM mannitol 48h (N = 5) 100mM mannitol 0h (N = 10), 100mM mannitol 48h (N = 6).

SUPPLEMENTARY MATERIAL

Table 1.

Two-way ANOVA for Comparing Hemolymph [ion] in Different Ambient Waters Over Time

Measuring hemolymph K^+ 5mM KCl, 5mM KCl + 10 mM NaCl (Figure 3A, Figure 1D)	ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
	Interaction	22.66	7	3.24	F (7, 152) = 10.03	P<0.0001
	Row Factor	32.75	7	4.68	F (7, 152) = 14.50	P<0.0001
	Column Factor	52.12	1	53.12	F (1, 152) = 161.5	P<0.0001
	Residual	49.04	152	0.332		
Measuring hemolymph K^+ 10mM KCl, 10mM KCl + 10 mM NaCl (Figure 3B, Figure 1E)	ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
	Interaction	61.33	6	10.22	F (6, 139) = 8.252	P<0.0001
	Row Factor	82.08	6	13.68	F (6, 139) = 11.05	P<0.0001
	Column Factor	76.86	1	76.86	F (1, 139) = 62.05	P<0.0001
	Residual	172.2	139	1.239		
Measuring hemolymph K^+	ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
	Interaction	42.43	4	10.61	F (4, 66) = 5.291	P=0.0009

20mM KCl, 20mM KCl + 10 mM NaCl (Figure 3C, Figure 1F)	Row Factor	165.3	4	41.32	F (4, 66) = 20.61	P<0.0001
	Column Factor	41.68	1	41.68	F (1, 66) = 20.79	P<0.0001
	Residual	172.2	139	1.239		
Measuring hemolymph K^+ 1mM KCl, 1mM KCl + 0.1mM NaCl (Figure 4A, Figure 1A)	ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
	Interaction	15.38	7	2.197	F (7, 140) = 6.856	P<0.0001
	Row Factor	11.83	7	1.689	F (7, 140) = 5.273	P<0.0001
	Column Factor	0.1938	1	0.1938	F (1, 140) = 0.6050	P=0.4380
	Residual	44.86	140	0.3204		
Measuring hemolymph K^+ 3mM KCl, 3mM KCl + 0.1mM NaCl	ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
	Interaction	12.52	7	1.788	F (7, 124) = 7.232	P<0.0001
	Row Factor	42.70	7	6.099	F (7, 124) = 24.66	P<0.0001
	Column Factor	0.03382	1	0.03382	F (1, 124) = 0.1367	P=0.7122
	Residual	30.67	124	0.2473		

(Figure 4C, Figure 1C)						
Measuring hemolymph K^+ 5mM KCl, 5mM KCl + 0.1mM NaCl (Figure 4D, Figure 1D)	ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
	Interaction	21.03	6	3.505	F (6, 137) = 8.313	P<0.0001
	Row Factor	132.2	6	22.04	F (6, 137) = 52.27	P<0.0001
	Column Factor	3.096	1	3.096	F (1, 137) = 7.342	P=0.0076
	Residual	57.76	137	0.4216		
Measuring hemolymph Na^+ 20mM NaCl, 20mM NaCl 0.04mM $CaSO_4$ (Figure 2C, Figure 5A)	ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
	Interaction	3183	7	454.7	F (7, 104) = 2.585	P=0.0169
	Row Factor	4383	7	626.1	F (7, 104) = 3.558	P=0.0018
	Column Factor	163.3	1	163.3	F (1, 104) = 0.9284	P=0.3375
	Residual	1829	104	175.9		

Measuring hemolymph Na^+ 30mM NaCl, 30mM NaCl + 0.04mM $CaSO_4$ (Figure 2D, Figure 5B)	ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
	Interaction	2914	7	416.3	F (7, 139) = 5.106	P<0.0001
	Row Factor	7261	7	1037	F (7, 139) = 12.72	P<0.0001
	Column Factor	343.3	1	343.3	F (1, 139) = 4.211	P=0.0420
	Residual	11334	139	81.54		
Measuring hemolymph pH 10mM KCl+10mM NaCl, 10mM KCl (Figure 9A, Figure 9B)	ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
	Interaction	27.59	6	4.598	F (6, 116) = 7.494	P<0.0001
	Row Factor	22.49	6	3.749	F (6, 116) = 6.111	P<0.0001
	Column Factor	811.5	1	811.5	F (1, 116) = 1323	P<0.0001
	Residual	71.17	116	0.6135		
Measuring hemolymph pH	ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
	Interaction	4.120	7	0.5885	F (7, 186) = 6.135	P<0.0001

30mM NaCl, 30mM NaCl +0.04mM Ca_2SO_4 Figure 9C, Figure 9D)	Row Factor	3.854	7	0.5506	F (7, 186) = 5.740	P<0.0001
	Column Factor	2.653	1	2.653	F (1, 186) = 27.66	P<0.0001
	Residual	17.84	186	0.09593		

Chapter 3

TRANSEPITHELIAL PONENTIAL (TEP) RESPONSES TO INCREASED AMBIENT CONCENTRATIONS OF MAJOR IONS IN ADULT *DAPHNIA MAGNA*

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ABSTRACT

Anthropogenic activities such as road de-icing, mountaintop coal mining, fertilizer application and hydraulic fracturing spills deposit excess major ions including Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, and HCO_3^- into freshwater ecosystems. This is cause for concern, as elevations in major ions can have detrimental effects on freshwater animals, including osmoregulatory stress. While some jurisdictions monitor Cl^-, SO_4^{2-}, conductivity and total dissolved solids, pollution from major ions is not widely covered by environmental regulations because there are gaps in our knowledge of the physiological mechanisms of major ion toxicity and our ability to predict it. The Multi-ion Toxicity (MIT) model, developed by the Electric Power Research Institute (EPRI), aims to model the differential toxicity of ions to predict overall major ion toxicity using transepithelial potential (TEP). TEP measurements evaluating the effects of metabolic inhibitors including ouabain, sodium cyanide (NaCN), iodoacetic acid (IAA), and 2,4-dinitrophenol (DNP), revealed that ATP-dependent pumps contribute directly to the maintenance of TEP in *D. magna.* Depolarization in response to changes in concentrations of Na^+, K^+, $Ca^{2+}$and Mg^{2+} in the bath water indicate that diffusion of these ions also contributes to the measured TEP. The accompanying anion of the salt also influences the TEP response. Increased ambient Cl^- concentration yielded an unexpected result of depolarization which may indicative of electrogenic transport of Cl^- in *D. magna*. These physiological responses to elevated levels of major ions in the water will be important for the development of predictive models for regulating major ion levels in freshwater environments and for determining toxicity thresholds for freshwater ecosystems. The MIT model has yet to be experimentally supported and our data on *Daphnia magna* from this study will aid in the further development of EPRI's MIT model.

INTRODUCTION

Freshwater salinization through anthropogenic activities is causing detrimental effects to freshwater organisms (Schuler et al., 2019). Major ions; sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) cations paired with chloride (Cl^-), sulphate (SO_4^{2-}) and (bi)carbonate (HCO_3^-/CO_3^{2-}) anions are being introduced into freshwater ecosystems through irrigation runoff from agriculture (Smedema & Shiati, 2002), saline oil-field discharges (Boelter et al., 1992), road de-icing salt application (Findlay and Kelly, 2011), mountain-top coal mining (Pond et al., 2008), hydraulic fracturing fluid spills (Blewett et al., 2017) and general urbanization (Estévez et al., 2019). Elevated concentrations of major ions in freshwater environments cause osmoregulatory stress for freshwater species. Freshwater animals actively take up ions to counter passive ion loss to their environment and increased ambient ion concentrations disturb this homeostasis. Regulatory efforts to protect freshwater ecosystems have yet to include comprehensive monitoring of pollution by major ions. There has been no agreement on regulatory endpoints, although osmolarity, conductivity, total dissolved solids, salinity and the concentrations of particular cations and anions have all been suggested (EPRI 2016; USEPA 2011). Pollution by major ions is not widely monitored, in part, because of the lack of knowledge of the physiological mechanisms of major ion toxicity.

In an effort to improve environmental regulations, the toxicology of major ions has been studied (Cormier and Suter, 2013; Cormier et al., 2013; Erickson et al., 2017; Mount et al., 2016; Pond et al., 2008). Additionally, the Electric Power Research Institute (EPRI) has developed a predictive toxicity model, the Multi-ion Toxicity (MIT) model, that aims to model the differential toxicity of ions (EPRI, 2018). The model is based on the knowledge that the electrical gradients across biological membranes, such as the gills, are disturbed by increased

concentrations of major ions. This disturbance is thought to lead to toxicity and death. The model is based on the notion that a change in transepithelial potential (TEP) is predictive of mortality. TEP can be predicted by the Goldman-Hodgkin Katz Equation.

Equation:

$$TEP = \frac{RT}{F}\ln\left(\frac{p_k[K^+]_o + p_{Na}[Na^+]_o + p_{Cl}[Cl^-]_i}{p_k[K^+]_i + p_{Na}[Na^+]_i + p_{Cl}[Cl^-]_o}\right)$$

where:

R: universal gas constant, which is 8.314 joules/mole/ºK

T: absolute temperature, in ºK (*i.e.* Temperature in ºC + 273

F: Faraday constant, which is 9.649×10^4 Coulombs per mol

TEP: transepithelial potential, in volts (equivalent to joules per Coulomb)

p_{ion}: permeability for subscripted ion, in meters per second

$[ion]_o$: ion activity in the external water in mM

$[ion]_i$: ion activity in the extracellular fluid of the organism (blood plasma or hemolymph) in mmol per L

This equation takes into account both monovalent and divalent ions and was originally developed to predict cellular membrane potential (Goldman, 1943; Hodgkin and Katz, 1949) but has been modified by EPRI (EPRI 2018) to predict TEP across the biological membranes. The Goldman, Hodgkin-Katz equation also requires knowledge of the ionic composition of the hemolymph (Morris, Sakarya, Koh and O'Donnell, unpublished observations). To date, the MIT model has been built on electrochemical theory and toxicity data but has had no experimental or

physiological validation.

Daphnia magna, a branchiopod crustacean in the order Cladocera, is a commonly studied model species for toxicology (Ebert, 2005). Sensitivity of *D. magna* to changes in water chemistry, ionic composition and pollutants make it an ideal organism to study major ion toxicity and physiological measurements in daphnids may provide direction to EPRI's MIT model. Daphnids have been the focus of model efforts and toxicity studies (Erickson et al., 2018; Mount et al., 2016, EPRI 2018) and are more sensitive to changes in ambient ion concentrations than fish (Mount et al., 1997., Tietge et al., 1997). Daphnids have different ion transporters (Bianchini and Wood, 2008; Glover and Wood, 2005) and ionoregulatory mechanisms (Hogstrand and Wood, 1998) than fish and therefore may regulate their TEP differently. In freshwater fish, TEP has been found to be entirely a diffusion potential, reflecting the differential permeabilities of Na^+ and Cl^- at the gill (Kerstetterr et al., 1970; Potts and Eddy, 1973; Eddy, 1975; McWilliams and Potts, 1978; Potts, 1984). In saltwater fish, there is both a diffusive and electrogenic component to TEP (Eddy, 1975, Potts, 1984). Other crustaceans, such as crayfish, crab and shrimp species have been used extensively in ionoregulatory studies, and TEP in these species has been evaluated (Cameron, 1978; Harris and Coley, 1991; Kirschner et al., 1973; Zare and Greenaway, 1998).

This study describes the first experimental measurements of TEP in *Daphnia magna* and provides baseline data for the EPRI MIT model. Microelectrode measurements of TEP were performed in response to metabolic inhibitors to determine possible electrogenic components of TEP in daphnids. Additionally, TEP was evaluated in increased ambient concentrations of major ions to investigate the impact of excess major ions in freshwater systems. Single salt solutions (KCl, K_2SO_4, $KHCO_3$, NaCl, Na_2SO_4, $NaHCO_3$, $CaCl_2$, $CaSO_4$, $MgCl_2$, and $MgSO_4$) were

evaluated. The contributions of Na^+, K^+ and Cl^- transporters to TEP were assessed through measurements of the effects of blockers of Na^+/K^+ ATP-ase (ouabain), K^+ channels (Ba^{2+}), Cl^- channels (diphenylamine-2-carboxylate) and Cl^-/HCO_3^- exchangers (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid).

METHODS

Rearing Daphnia magna

A starter culture of *Daphnia magna* Strauss was obtained from commercial suppliers (Boreal Science, St. Catharines, ON and Carolina Biological Supply, Burlington, NC) and maintained at room temperature (23° C ± 1° C), under fluorescent light on a 17:7-h light:dark photoperiod in aerated 20 L tanks of dechlorinated Hamilton tap water (DHTW). The water was sourced from Lake Ontario and contained (in mmol l^{-1}: 1 Ca^{2+}, 0.6 Na^+, 0.70 Cl^-, 0.5 SO_4, 0.3 Mg^{2+} and 0.05 K^+, with titration alkalinity of 2.1 mequiv l^{-1}, hardness of ≈140 mg l^{-1}as $CaCO_3$ equivalents, and pH ≈8.0 (Hollis et al., 2001; Leonard et al., 2014). *Daphnia* were fed a 2:2:1 mixture of Spirulina powder: Chlorella powder: yeast 3 times per week.

Transepithelial potential measurements

TEP was measured after impalement of adult *D. magna* using double-barrelled theta-glass microelectrodes (TST150, WPI, New Haven, CT, USA). After pulling, both barrels were filled with 150mM KCl and one barrel was connected through a chlorided Ag wire to the headstage of a high impedance electrometer (HiZ 223, Warner Instruments, Hamden CT). The natural bevel resulting from the prominent spear-like projection of the central septum of theta

glass microelectrodes facilitates impalement. Potentials were measured with respect to an AgCl-pellet connected to the bath through a salt bridge filled with 3% agar in 150 mM KCl. An animal was blotted dry with a Kim wipe and secured in a petri dish with petroleum jelly. This dish was connected to two 20-mL syringes through tubing, creating a perfusion chamber. The dish was filled with dechlorinated Hamilton tap water or a solution of known ionic concentration as the bath solution. Using a micromanipulator, the microelectrode was guided to pierce the cuticle near the heart, into the hemocoel. Only one measurement was recorded per animal. The bath solution was changed through push-pull perfusion using the two 20-mL syringes and TEP was recorded to determine the effect of varying salt concentration solutions on TEP. TEP was usually stable within 2 minutes and was recorded 5-10 minutes after perfusions.

The criteria used for acceptable impalements were as follows: Microelectrode voltage was stable (± 1mV) for ≥ 1 minute prior to impaling the test animal; rapid voltage deflection on advancing the microelectrode into the hemocoel; microelectrode voltage was stable (± 1mV) for ≥ 30 seconds after impalement; microelectrode voltage returned within 3mV of the pre-impalement electrode voltage after being withdrawn from the hemocoel.

Experimental solutions

All stock solutions were made with reagents in dechlorinated Hamilton tap-water (DHTW), salt additions were made with a background of DHTW. The salts tested were KCl, K_2SO_4, $KHCO_3$, NaCl, Na_2SO_4, $NaHCO_3$, $CaCl_2$, $CaSO_4$, $MgCl_2$ and $MgSO_4$. Experimental concentrations were based on 48hr LC50 data reported by Mount et al (2016) for *Ceriodaphnia dubia*. Five concentrations for each salt were tested: two concentrations below the LC50 concentration, the LC50 concentration and two concentrations above the LC50 concentration.

Concentrations were tested in a geometric series, 25%, 50%, 100%, 200% and 400% of the LC50 value. This was followed for all salts except for $CaSO_4$, for which experimental concentrations were determined by the dissolution limit (DL) of $CaSO_4$ in water. Mount et al. (2006) reported 220 mg $CaSO_4$/L with a saturated solution at 16.2mM to be the dissolution limit. This concentration was not found to be acutely toxic to *Ceriodaphnia dubia* (Mount et al., 2016). TEP was therefore measured in 1mM, 2mM, 4mM, 8mM and 16mM $CaSO_4$.

TEP was also measured for animals bathed in a physiological saline that approximated hemolymph ionic composition, as determined by measuring concentrations of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and osmolality in the hemolymph (36mM NaCl, 13mM $NaHCO_3$, 1mM Na isethionate, 0.7mM $CaCl_2$, 0.3mM $MgCl_2$, 4mM mannitol). Osmolality was measured using a Wescor Vapor Pressure Osmometer, Vapro, Model 5520 (Logan, UT). HCO_3^- was estimated based on our measurements of hemolymph ions (Table 1) (Morris, Sakarya, Koh and O'Donnell, unpublished observations) and the requirement for cation and anion balance as well as on the concentration that was previously reported for daphnid hemolymph of >13mM (Weber and Pirow, 2009). The role of Na isethionate was to ensure that cation-anion balance was maintained for saline containing the measured [Na^+]. Mannitol was used to make up the remaining osmolality not accounted for by ions in our solution. In hemolymph, this discrepancy is likely due to other constitutes including, amino acids, proteins, and ammonia.

To evaluate the effect of a particular cation independent from anion, in response to CN^-, an impermeable cation, N-methyl-D-glucamine (NMDG) was used to keep Cl^- constant. NMDG was added to each of the test solutions and titrated with HCl to achieve a pH of 7.5 and equal concentration of Cl^- between each set of test solutions, so that the only variable was the concentration of the cation of interest.

TEP was measured in response to 7 specific inhibitors; ouabain, sodium cyanide (NaCN), iodoacetic acid (IAA), barium chloride ($BaCl_2$), 2,4-dinitrophenol (DNP), 4,4' diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) and diphenylamine-2-carboxylic acid (DPC).

1mM NaCN inhibits aerobic metabolism through blockade of mitochondrial electron transport (Pettersen and Cohen, 1993), whereas the combination of NaCN + IAA inhibits ATP generation through both aerobic and glycolytic pathways (Dickens, 1933). DNP uncouples electron transport from oxidative phosphorylation and 1mM DNP was found to be sufficient to alter TEP in the gills of the fiddler crab (Drews and Graszynski, 1987). 1mM ouabain has been reported to be sufficient to inhibit Na^+/K^+ ATPase activity in daphnids (Bianchini and Wood, 2002). 1mM $BaCl_2$, a K^+ channel inhibitor, was selected as 0.4mM $BaCl_2$ was described to be sufficient to immobilize daphnids (Anderson, 1944). Concentrations of 1mM DIDS, a Cl^-/HCO_3^- exchanger inhibitor and 1mM DPC, a Cl^- channel inhibitor, were selected based on previous studies of Na^+ transport in *D. magna* (Bianchini and Wood, 2008).

Ouabain, NaCN, IAA, $BaCl_2$ and DNP (all at 1mM) were dissolved in DHTW. The working concentration of 1mM DIDS was made by diluting a stock solution (100mM) of DIDS in 0.1M $KHCO_3$ with DHTW. TEP in the final concentration of 1mM $KHCO_3$ (100-fold dilution from working stock solution) did not differ from that in DHTW (Supplementary Material Table 1).

DPC was dissolved in dimethylsulfoxide (DMSO) so that the final solution was 1mM DPC and 0.1% DMSO. Bianchini and Wood (2008) found there was no effect of 1% DMSO on Na^+ influx. We evaluated TEP response to 0.1% DMSO and found that TEP in 0.1% DMSO did not differ from that in DHTW (Supplementary Table 1). Ouabain, sodium cyanide (NaCN),

iodoacetic acid (IAA), $BaCl_2$ and 2,4-dinitrophenol (DNP) (all at 1mM) were dissolved in DHTW.

Statistics

Graphing and statistical tests of significance were done using GraphPad Prism 8 (San Diego CA). Differences were considered significant if $P \leq 0.05$. Effects of drugs or metabolic inhibitors on TEP with one variable compared to DHTW (control) were assessed with a paired t-test and experiments with multiple variables were assessed with repeated measures one-way ANOVA followed by Tukey's multiple comparisons post hoc test. The effects of water ion concentration on TEP were analysed by linear regression of TEP versus the log of the ion concentration. ANCOVA was used to test if the slopes of the linear regression were significantly different within each salt category, grouped by cation (Supplementary Table 2).

RESULTS

The effects of physiological saline and metabolic inhibitors; NaCN, NaCN + Iodoacetate, and DNP on TEP revealed that ATP-dependent pumps contribute directly to the maintenance of the TEP (Figure 1). Changes in TEP in response to changes in concentrations of K^+, Na^+, Ca^{2+}, Mg^{2+} and Cl^- (Figures 2-5) in the water indicate that diffusion of these ions also contribute to the measured transepithelial potential, whereas there was no evidence for contributions from NH_4^+ (Supplementary Material Figure 1B).

Effects of Metabolic Inhibitors and Physiological Saline on TEP

Control TEP in DHTW was -8.1 ± 0.3mV (N=155), inside negative. Measured TEP became more negative, compared to the control water (DHTW), in 1mM NaCN, 1mM NaCN + IAA and 1mM 2,4 Dinitrophenol (DNP), all of which are metabolic poisons (Figures 1A, 1B and 1C). We evaluated 1mM NaCN + 9mM Na^+ isethionate and 10mM NaCN to maintain constant [Na^+] without introducing an additional anion *(e.g.* Cl^-). Na^+ isethionate was used to eliminate the potentially confounding factor of changes in [Cl^-] (if NaCl was used) by replacing Cl^- with the impermeable anion, isethionate. We found there were no significant changes in TEP between the two concentrations of NaCN (Supplementary Material Figure 1A). Physiological saline was tested to minimize the ion concentration gradient between outside and inside the animal by bathing the animal in a solution that was approximately the same composition as the hemolymph. TEP became more positive in physiological saline (36mM NaCl, 13mM $NaHCO_3$, 1mM $C_2H_5NaO_4S$, 0.7mM $CaCl_2$, 0.3mM $MgCl_2$, 4mM mannitol). Saline composition was determined by using measured Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and osmolality in the hemolymph (Table 1).

Effects of K^+ on TEP

TEP was measured in animals exposed to 5 concentrations of KCl, K_2SO_4 and $KHCO_3$. The concentrations of each K^+ salt were determined by previous published LC50 values (Mount et al., 2016). For KCl, concentrations of 1.25mM, 2.5mM, 5mM (LC50), 10mM and 20mM KCl were used. Increased [KCl] caused depolarization and there was a significant positive correlation between TEP and [KCl]. The slope of the linear regression line was 3.0mV/decade (Figure 1A). TEP was measured in 0.875mM, 1.75mM, 3.5mM (LC50), 7mM and 14mM K_2SO_4. Increased

[K_2SO_4] depolarized TEP (Figure 1B). [K_2SO_4] and TEP were highly correlated and the slope was 2.24mV/decade. Increased [$KHCO_3$] caused TEP to become more negative, rather than less negative as seen with the other K^+ salts (Figure 2C). 1.25mM, 2.5mM, 5mM (LC50), 10mM and 20mM $KHCO_3$ were evaluated. TEP and [$KHCO_3$] were highly correlated and the slope was -2.92mV/decade. The slopes of the linear regression between KCl, K_2SO_4 and $KHCO_3$ were significantly different (Supplementary Table 2).

Effects of Na^+ on TEP

TEP was measured in response to 5 concentrations of NaCl, Na_2SO_4 and $NaHCO_3$. Na^+ concentrations were based on previously published LC50 values (Mount et al., 2016). TEP in response to 7.5mM, 15mM, 30mM (LC50), 60mM and 120mM NaCl was evaluated and TEP became more positive as [NaCl] increased (Figure 3A). TEP was highly correlated with [NaCl] and the slope of the linear regression line was 11.1mV/decade (Figure 3A). TEP in response to 6.25mM, 12.5mM, 25mM (LC50) 50mM and 100mM of Na_2SO_4 was evaluated. TEP and [Na_2SO_4] were highly correlated and TEP became more positive as [Na_2SO_4] increased (Figure 3B). The slope was 3.43mV/decade. TEP was measured in 3mM, 6mM, 12mM, 24mM and 48mM $NaHCO_3$. Increased [$NaHCO_3$] caused depolarization (Figure 3C). TEP and [$NaHCO_3$] were highly correlated and the slope was 3.13mV/decade. The slopes of the linear regression between NaCl, Na_2SO_4 and $NaHCO_3$ were significantly different (Supplementary Table 2).

Effects of Ca^{2+} on TEP

TEP was measured in response to 5 concentrations of $CaCl_2$ and $CaSO_4$. The concentrations evaluated for $CaCl_2$ were guided by previously published LC50 data (Mount et

al., 2016), 3.75mM, 7.5mM, 15mM (LC50), 30mM and 50mM. TEP became more positive as [$CaCl_2$] increased and [$CaCl_2$] was well correlated to measured TEP (Figure 4A). The slope was 10.4mV/decade. The 5 concentrations of $CaSO_4$ were determined by the dissolution limit (DL) of $CaSO_4$ in water was found to be 220 mg $CaSO_4$/L with saturation at 16.2 mM (Mount et. al., 2016). This concentration was not found to be acutely toxic to *Ceriodaphnia dubia* (Mount et. al., 2016). 1mM, 2mM, 4mM, 8mM, 16mM were evaluated and TEP became more positive in increased [$CaSO_4$] (Figure 4B). The slope was 6.27mV/decade. The slopes of the linear regression between $CaCl_2$ and $CaSO_4$ were not significantly different (Supplementary Table 2).

Effects of Mg^{2+} on TEP

TEP was measured in animals exposed to 5 concentrations of $MgCl_2$ and $MgSO_4$ selected on the basis of previously published LC50 values (Mount et al., 2016). For $MgCl_2$, concentrations of 2.5mM, 5mM, 10mM (LC50), 20mM and 40mM were evaluated. TEP became more positive in increased [$MgCl_2$] (Figure 5A). TEP and [$MgCl_2$] were highly correlated and the slope was 8.65mV/decade. 4.25mM, 8.5mM, 17mM (LC50), 34mM and 68mM of $MgSO_4$ were evaluated. TEP become more positive in increased [$MgSO_4$]. [$MgSO_4$] and TEP were well correlated and the slope was 4.13mV/decade (Figure 5B). The slopes of the linear regression between $MgCl_2$ and $MgSO_4$ were significantly different (Supplementary Table 2).

Effects of Cl^- on TEP

TEP was measured in 1mM and 10mM solutions of choline Cl^-, N-methyl-D-glucamine chloride (NMDG-Cl^-) and NMDG- SO_4. In 10mM choline Cl^- and 10mM NMDG-Cl^-, TEP

became more positive than in DHTW (0.7mM Cl^-) (Figure 6A, Figure 6B). NMDG-SO_4 had no effect on TEP compared to DHTW (Figure 6C).

The Effect of K^+, Na^+ and Cl^- in the presence of a metabolic inhibitor on TEP

In an attempt to separate electrogenic and diffusional contributions to TEP, the effects of K^+, Na^+ and Cl^- in the presence of a metabolic blocker (CN^- + K^+, CN^- + Na^+, CN^- + Cl^-) on TEP were evaluated. [Cl^-] and pH were kept constant using N-Methyl-D-glucamine titrated with HCl. N-Methyl-D-glucamine is generally considered to be an impermeant cation in biological systems. TEP became more negative in 1mM NaCN compared with DHTW and became more positive in response to 1mM NaCN + 1mM KCl and 1mM $NaCN^-$ +10mM KCl (Figure 7A), 1mM $NaCN^-$ + 9mM NaCl. (Figure 7B), 1mM NaCN + 1mM NMDG-Cl^- and 1mM NaCN + 10mM NMDG-Cl^- (Figure 7C).

Ion transport inhibitors

Measured TEP became more negative in 1mM ouabain, a specific inhibitor of Na^+ / K^+ ATPase, compared to the control bath water of DHTW (Figure 8A). In response to 1mM $BaCl_2$, a K^+ channel inhibitor, TEP became more positive (Figure 8B). TEP became more negative in both 1mM DPC (diphenylamine-2-carboxylic acid), a Cl^- channel blocker and 1mM DIDS (4,4' diisothiocyano-2,2'-stilbenedisulfonic acid), a Cl^-/HCO_3^- exchanger inhibitor (Figure 8C, Figure 8D).

DISCUSSION

Overall, our results show that both ATP dependent pumps and ion diffusion (K^+, Na^+, Ca^{2+} and Mg^{2+}) contribute to TEP. We suggest below that our results are also consistent with the contribution of an electrogenic Cl^-/ $2HCO_3^-$ exchanger to TEP. Additionally, anions contribute to changes in TEP.

ATP-Dependent Pumps Contribute to TEP

CN^- inhibits aerobic ATP production by blocking mitochondrial electron transport. Our TEP measurements reveal that in response to exposure to 1mM (Na)CN^-, TEP becomes more negative compared to control TEP in DHTW by ~8mV (Figure 1A). This suggests that an ATP-dependent pump drives TEP ~8mV more positive than would be the case if ionic diffusion alone were responsible for maintaining the TEP in *D. magna*. It is possible that an outwardly directed ATP-dependent anion pump contributes to TEP and tends to drive TEP to inside positive values, so its inhibition results in hyperpolarization. Active transport contributes ~5-10mV to the TEP in isolated perfused *Uca tangeri*; TEP decreased significantly when both (K)CN^- and DNP were perfused through the gills (Drews and Graszynski, 1987). Alternatively, an increase in cation permeability and outward cation diffusion may explain the hyperpolarization of TEP in response to CN^-. Active transport contributes to TEP in *Necturus maculosus* gallbladder epithelia (Bello-Reuss et al., 1981), and the hyperpolarization seen in *Necturus maculosus* gallbladder epithelia upon exposure to NaCN has been attributed to an increase in K^+ permeability of cell membranes (Bello-Reuss et al., 1981).

Given that daphnids show considerable plasticity in coping with environmental changes in oxygen concentration and temperature, including adaptive changes in glycolytic enzymes

(Zeis et al., 2009), we also measured the effects of inhibition of ATP production through glycolysis. Glycolysis can be inhibited by compounds such as iodoacetate (IAA), which inhibit the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The extent of hyperpolarization of TEP in Daphnia exposed to 1mM CN^- alone or in combination with 1mM IAA were similar, (Figure 1B) indicating that glycolysis likely does not contribute to TEP maintenance in *D. magna*.

DNP inhibits ATP by uncoupling electron transport from oxidative phosphorylation, which acts as a protonophore, allowing protons to leak across the inner mitochondrial membrane and thus bypass ATP synthase. TEP in response to 1mM DNP was evaluated to confirm that ATP dependent pumps contribute to TEP in *D. magna.* Unlike CN^-, DNP is uncharged and therefore is unlikely to contribute to TEP through its own diffusion. TEP also became more negative in response to DNP, confirming the results with CN^-, and supporting a role for active transport in the maintenance of TEP (Figure 1C).

To ensure active transport is contributing to TEP in *D. magna*, we superfused animals with physiological saline to eliminate diffusion gradients (Potts, 1984; C.M. Wood and B. Po, personal communication) and TEP became more positive but far exceeded 0mV (Figure 1D). If there was no active transport component, TEP would collapse to 0mV as the diffusional gradients would no longer be present and there would be no other component to TEP. It has been well documented that seawater fish also have an electrogenic component to TEP as the active efflux of Cl^- drives TEP more positive (Potts and Hedges, 1991). However, this result was not seen in freshwater fish. Both fathead minnow and rainbow trout yielded TEP of 0mV when transferred to physiological saline (C.M. Wood and B. PO, personal communication). Additionally, 1mM ouabain, was tested to determine the contribution of the Na^+/K^+ ATP-ase to

active transport of Na^+ and K^+. TEP became more negative in response to ouabain (Figure 10A), as seen in perfused, isolated *Uca tangeri* gills (Drews and Graszynski, 1987) showing that the active transport of Na^+ and K^+ contributes directly to TEP in *D. magna.*

Diffusional gradients

K^+

Our results indicate that there is likely a K^+ diffusion gradient contributing to TEP in *D. magna.* In response to a series of concentrations of KCl, K_2SO_2 and $KHCO_3$ there were significant changes in TEP (Figure 2A, Figure 2B, Figure 2C). When ATP-dependent processes were inhibited, the addition of K^+ shifted TEP to less negative values, consistent with diffusive influx of K^+ when water [K^+] is increased (Figure 7A). Additionally, TEP was altered when animals were exposed to $BaCl_2$, a K^+ channel inhibitor (Figure 9B).

Na^+

In addition to active Na^+ transport via Na^+/K^+ ATP-ase (Figure 9A) contributing to TEP, our TEP measurements reveal that Na^+ diffusion likely also contributes to TEP. There was significant depolarization of TEP in response to 5 increasing concentrations of NaCl, Na_2SO_2 and $NaHCO_3$ (Figure 3A, Figure 3B, Figure 3C). When ATP dependent processes were inhibited by CN^-, increased [Na^+] shifted TEP to less negative values, consistent with diffusive influx of Na^+ independent of active transport (Figure 7B). Testing the effects of phenamil and isopropyl amiloride on TEP would confirm that the diffusive transport of Na^+ through Na^+ channels and Na^+/ H^+ exchangers, respectively, contribute to TEP directly (Chen et al., 2017; Parks et al.,

2007). Additionally, the epithelial Na^+ channel blocker amiloride inhibits Na^+ uptake in adult *D. magna*, indicating that Na^+ channels are likely involved in Na^+ uptake (Glover and Wood, 2005).

Ca^{2+} and Mg^{2+}

TEP became more positive in response to increased $CaCl_2$, $CaSO_4$ and $MgCl_2$ and $MgSO_4$ (Figure 4A, 4B, 5A, 5B). These results suggest that diffusional gradients of Ca^{2+} and Mg^{2+} contribute to TEP in *D. magna*. Increases in the concentrations of Ca^{2+} and Mg^{2+} bathing crayfish gills (*Actacus astacus* and *Pacifastacus leniusculus*) have also been found to cause depolarization of TEP which have been attributed to electrogenic, active influx of Ca^{2+} (Kirschner, 1994). In freshwater fish, Ca^{2+} decreases diffusive permeability and differentially decreases Na^+ permeability relative to Cl^- permeability, and previous studies in *D. magna* have also shown that Mg^{2+} may be more effective than Ca^{2+} in this capacity (Pane et al., 2003). This is consistent with our findings that TEP becomes more positive in both increased Ca^{2+} and Mg^{2+}

Cl^-

The direction of the change in TEP in response to changes in $[Cl^-]$ is opposite to that predicted for diffusive movement of Cl^- through channels. TEP became less negative in 10mM solutions of choline-Cl^- or NMDG-Cl^- compared to 1mM solutions or DHTW (Figure 6). These effects remained when metabolism was inhibited with CN- (Figure 7C). Although the gills of killifish are permeable to NMDG (Wood and Grosell, 2008), we found no change in TEP compared to DHTW in 1mM or 10mM NMDG-SO_4 (Figure 6C) and we also observed that 10mM choline-Cl^- and NMDG-Cl^- showed the same TEP response. It is more likely that, in contrast to fish, NMDG is impermeable in *D. magna* and that the TEP response in NMDG

solutions is due to the changes in [Cl^-]. The observed changes in TEP in response to changes in [Cl^-] are consistent with the contribution of an electrogenic $Cl^-/2HCO_3^-$ exchanger (Genovese et al., 2005). In response to 1mM of DIDs, a Cl^-/HCO_3^- exchanger inhibitor, TEP also became more negative (Figure 8D). TEP became more negative in 1mM DPC, DPC is a Cl^- channel blocker that was used to evaluate the contributions of Cl^- channel activity to TEP (Figure 8C). Hyperpolarization in response to 1mM DPC was also seen in perfused, isolated *Uca tangeri* gills (Drews and Graszynski, 1987). It is possible that DPC is also blocking the suggested $Cl^-/2HCO_3^-$ exchanger, which is consistent with the hyperpolarization we see in response to both DPC and DIDS (Reuss et al., 1987).

Anion Contribution to TEP

It has been suggested that major ion toxicity in daphnids is largely dependent on the cation and independent of the anion (Mount et al., 2016). However, our TEP measurements indicate that the anion does contribute to TEP in *D. magna.* A 10-fold concentration change for any cation resulted in changes in TEP that were dependent upon the anion (Figure 2, Figure 3, Figure 4, Figure 5). Conversely, TEP becomes more negative with increasing concentrations of $KHCO_3$, but more positive with increasing concentrations of $NaHCO_3$. The difference between changes in TEP in response to $KHCO_3$ and $NaHCO_3$ may reflect the contributions of multiple transporters to TEP and pH regulation. It has been suggested that internal pH regulation in aquatic crustaceans is dominated by cation/H^+ exchangers (e.g. K^+/H^+, Na^+/H^+, $2Na^+/H^+$) and anion exchangers (e.g. $Cl^-/2HCO_3^-$) (Genovese et al., 2005; Lucu, 1990; Onken et al., 1991; Onken et al., 2000; Glover and Wood, 2005). The change in TEP may thus reflect the contribution of electrogenic ($Cl^-/2HCO_3^-$, $2Na^+/H^+$) versus electroneutral (K^+/H^+, Na^+/H^+)

exchangers and also the effects of concomitant changes in intracellular and hemolymph pH on ion channel or paracellular conductance which may be different for Na^+ versus K^+.

The discrepancies between ΔTEP for salts with the same cation but different anions could reflect the contribution of that anion, assuming the cation has the same contribution to TEP independent of the anion present. Specifically, we suggest that Cl^- is contributing to TEP through mechanisms other than diffusion through channels (e.g. $Cl^-/2HCO_3^-$ exchanger) as we saw larger changes in TEP when Cl^- was the associated anion. Previous research has shown that salts of the same cation but different anion, as such KCl, K_2SO_4 and $KHCO_3$ all have different LC50 values (Mount et al., 2016). If perturbation of TEP is a correlate of toxicity, the contribution of anions to TEP in *D. magna* would be expected.

CONCLUSION

We set out to evaluate TEP in response to increased ambient ion concentrations of single salt solutions. Our results of changes in TEP in response to ion concentrations approaching or exceeding previously published LC50 data are consistent with a link between TEP and toxicity (Mount et al., 2016). Additionally, our results reveal that the mechanism of TEP regulation in daphnids is different than that of fish in that there is a substantial contribution of an ATP-dependent electrogenic mechanism, and that Cl^- contributes to TEP in a manner opposite to that expected on the basis of Cl^- diffusion but consistent with the operation of an electrogenic $Cl^-/2HCO_3^-$ exchanger. These findings will need to be taken into account in future iterations of the EPRI MIT model and in creating environmental regulations related to major ion toxicity. Our results offer the first experimental TEP measurements in daphnids and will contribute to further development of the EPRI MIT model. Understanding physiological responses to increased

ambient ion concentrations and mechanisms of major ion toxicity will allow for monitoring of pollution by major ions and the development for predictive models such as the EPRI MIT model as well as establishing environmental regulations for major ions within aquatic systems.

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TABLES

Table 3-1. Measured Hemolymph Ion Concentration and Osmolality

Ion	Concentration/Osmolality
K^+	2.2mM
Na^+	51mM
Ca^{2+}	0.7mM
Mg^{2+}	0.5mM
Cl^-	40mM
Osmolality	110mmol/kg

[1](Morris, Sakarya, Koh and O'Donnell, *unpublished observations*)

FIGURES

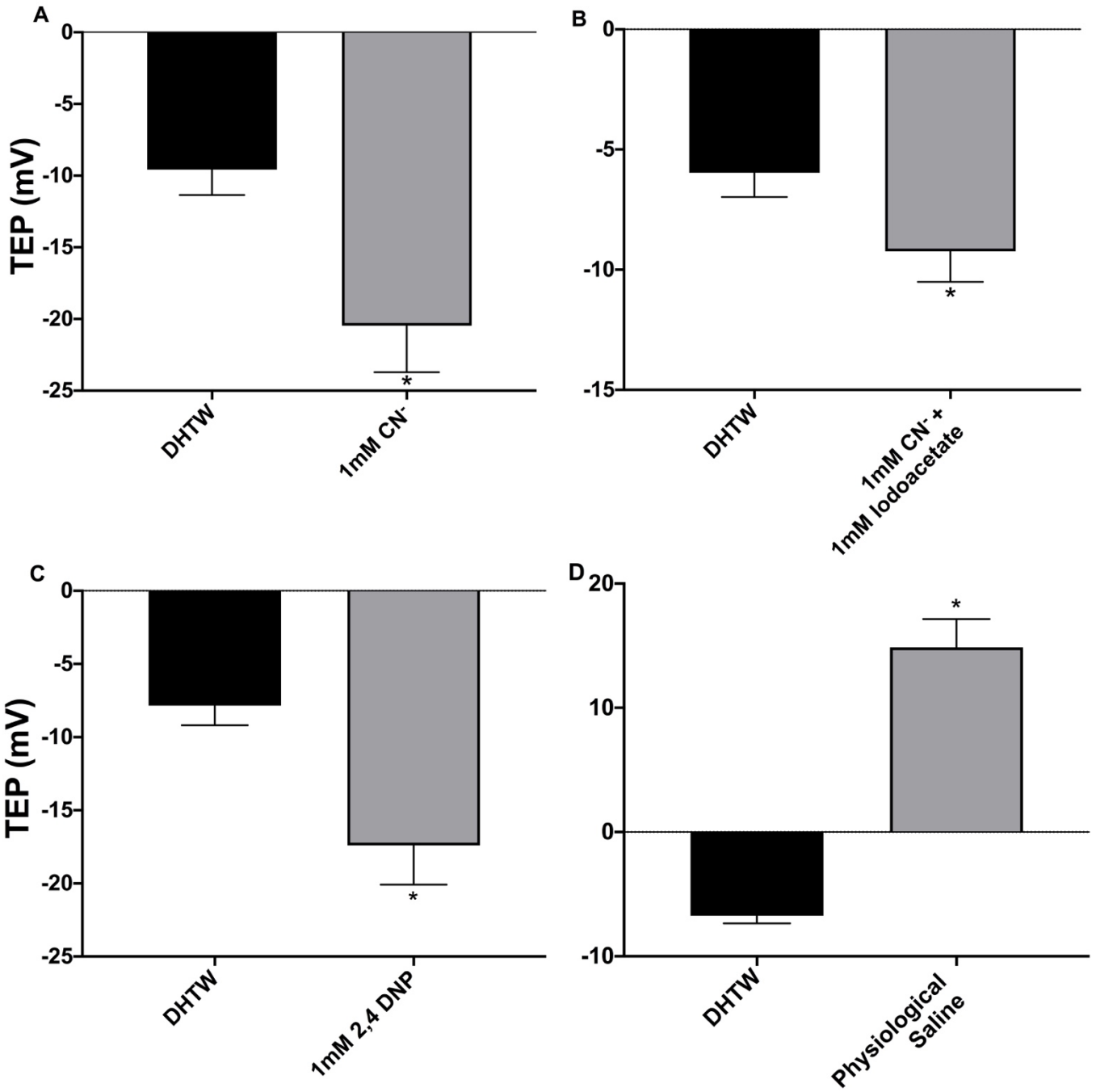
A
TEP (mV)
0
-5
-10
-15
-20
-25
*
DHTW
1mM CN-
B
0
-5
-10
-15
*
DHTW
1mM CN- +
1mM Iodoacetate
C
TEP (mV)
0
-5
-10
-15
-20
-25
*
DHTW
1mM 2,4 DNP
D
20
10
0
-10
*
DHTW
Physiological
Saline

Figure 3-1. Effects of metabolic inhibitors and physiological saline on TEP. (A) TEP in Dechlorinated Hamilton Tap Water (DHTW) before (as control) and after perfusion with 1mM NaCN in DHTW. (B) TEP in DHTW before and after perfusion with 1mM NaCN + 1mM Iodoacetate in DHTW. (C) TEP in DHTW before and after perfusion with 1mM 2,4, dinitrophenol in DHTW. (D) TEP in DHTW before and after perfusion with physiological saline (36mM NaCl, 13mM $NaHCO_3$, 1mM $C_2H_5NaO_4S$, 0.7mM $CaCl_2$, 0.3mM $MgCl_2$, 4mM mannitol). N = 8 (A, B); N= 6 (C, D). Asterisks (*) denote significant differences ($P < 0.05$).

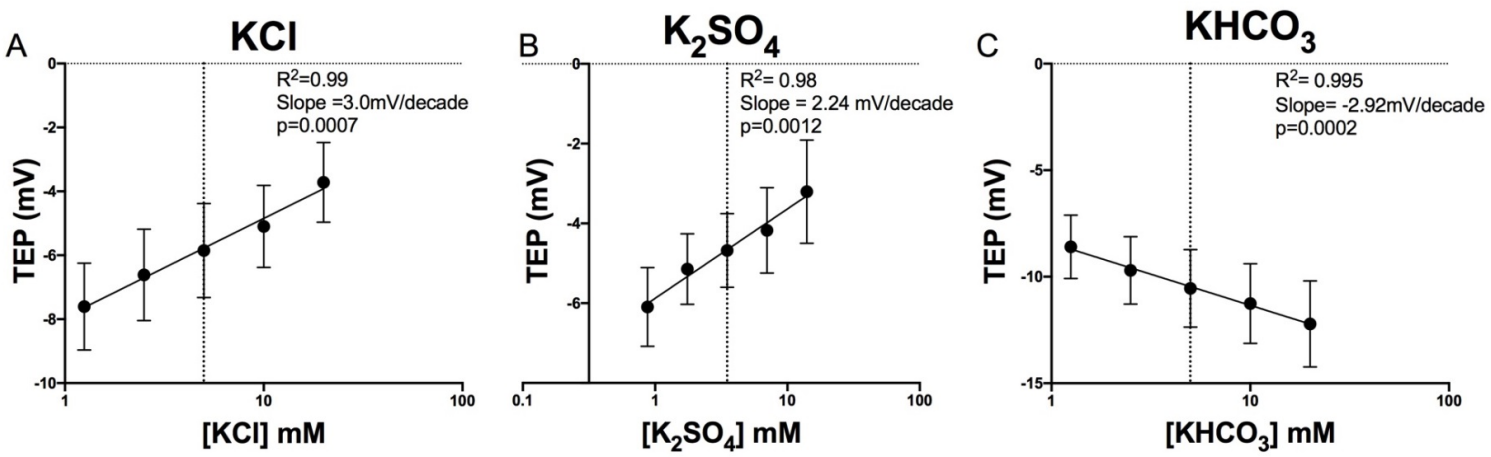
A
KCl
R2=0.99
Slope =3.0mV/decade
p=0.0007
TEP (mV)
[KCl] mM
B
K2SO4
R2= 0.98
Slope = 2.24 mV/decade
p=0.0012
TEP (mV)
[K2SO4] mM
C
KHCO3
R2= 0.995
Slope= -2.92mV/decade
p=0.0002
TEP (mV)
[KHCO3] mM

Figure 3-2. Changes in TEP in response to 5 concentrations (25%, 50%, 100%, 200% and 400% of the LC50) of KCl, K_2SO_4 and $KHCO_3$. Dotted lines indicate corresponding LC50 values. (A) KCl (1.25mM, 2.5mM, 5mM, 10mM, 20mM) N=6. (B) K_2SO (0.875mM, 1.75mM, 3.5mM, 7mM, 14mM) N=5. (C) $KHCO_3$ (1.25mM, 2.5mM, 5mM, 10mM, 20mM) N=6.

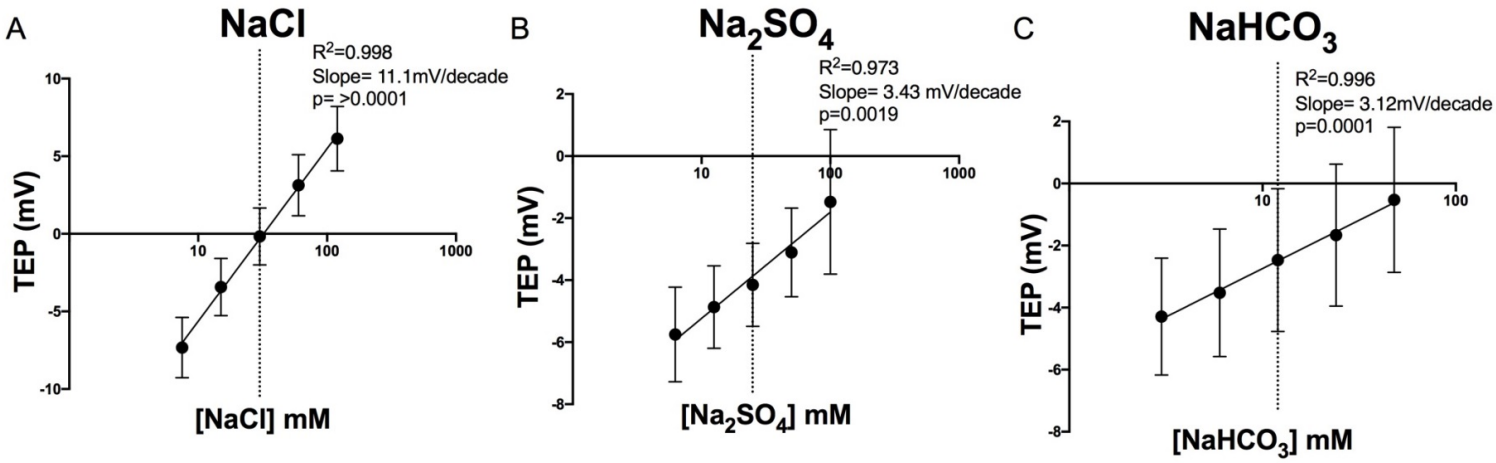
A
NaCl
R2=0.998
Slope= 11.1mV/decade
p=_>0.0001
TEP (mV)
[NaCl] mM
B
Na2SO4
R2=0.973
Slope= 3.43 mV/decade
p=0.0019
TEP (mV)
[Na2SO4] mM
C
NaHCO3
R2=0.996
Slope= 3.12mV/decade
p=0.0001
TEP (mV)
[NaHCO3] mM

Figure 3-3. Changes in TEP in response to 5 concentrations (25%, 50%, 100%, 200% and 400% of the LC50) of NaCl, Na_2SO_4 and $NaHCO_3$. Dotted lines indicate corresponding LC50 values. (A) NaCl (7.5mM, 15mM, 30mM, 60mM, 120mM) N=6. (B) Na_2SO_4 (6.25mM, 12.5mM, 25mM, 50mM, 100mM) N=6. (C) $NaHCO_3$ (3mM, 6mM, 12mM, 24mM, 48mM) N=6.

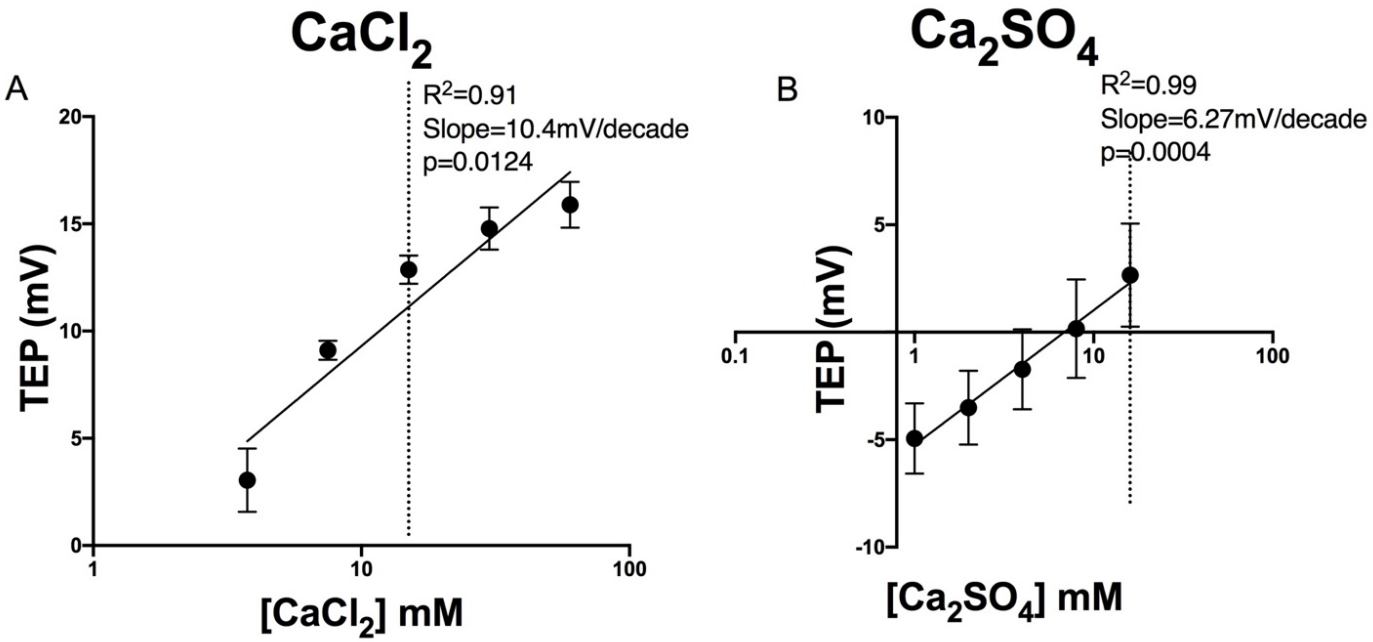
A
CaCl2
R2=0.91
Slope=10.4mV/decade
p=0.0124
TEP (mV)
20
15
10
5
0
1
10
100
[CaCl2] mM
B
Ca2SO4
R2=0.99
Slope=6.27mV/decade
p=0.0004
TEP (mV)
10
5
-5
-10
0.1
1
10
100
[Ca2SO4] mM

Figure 3-4. Changes in TEP in response to 5 concentrations of $CaCl_2$ and $CaSO_4$. Concentrations of 25%, 50%, 100%, 200% and 400% of the LC50 were used for $CaCl_2$; dotted line indicates LC50 value for $CaCl_2$. 5 concentrations of $CaSO_4$ at or below the dissolution limit (dotted line) were used for $CaSO_4$. (A) $CaCl_2$ (3.75mM, 7.5mM, 15mM, 30mM, 60mM) N=6. (B) $CaSO_4$ (1mM, 2mM, 4mM, 8mM, 16mM) N=6.

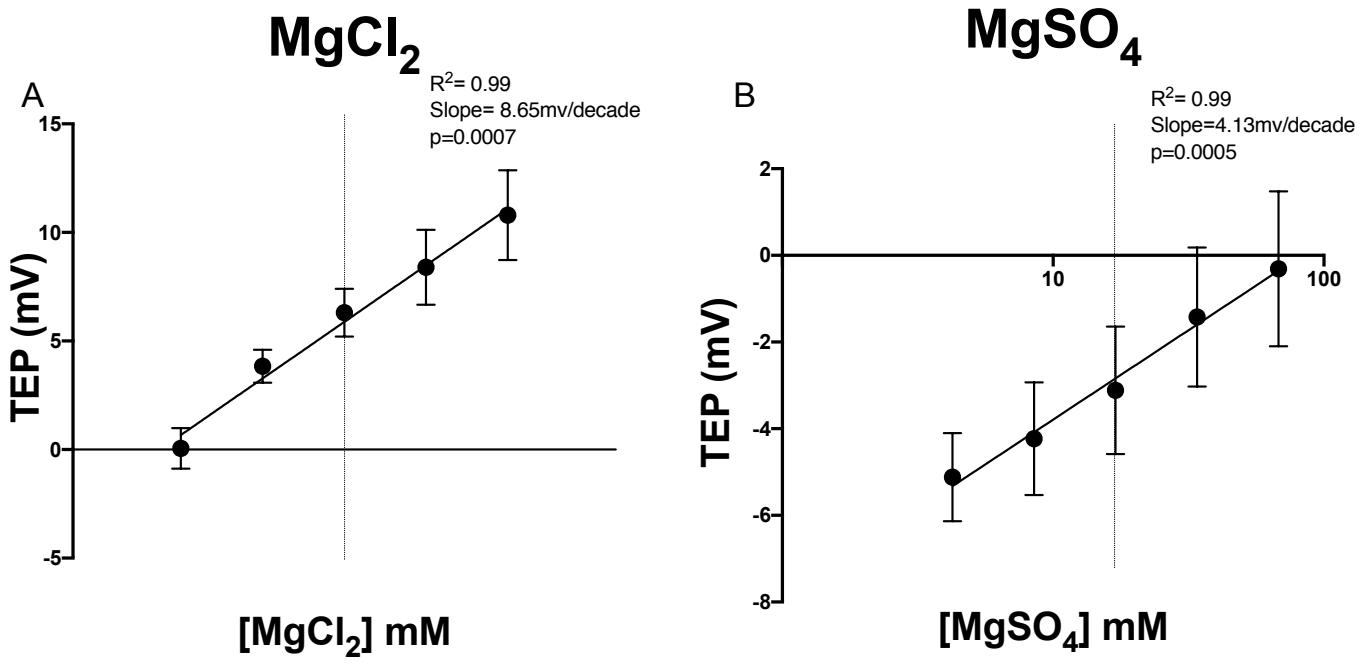
MgCl2
A
R2= 0.99
Slope= 8.65mv/decade
p=0.0007
TEP (mV)
15
10
5
0
-5
[MgCl2] mM
MgSO4
B
R2= 0.99
Slope=4.13mv/decade
p=0.0005
TEP (mV)
2
0
-2
-4
-6
-8
10
100
[MgSO4] mM

Figure 3-5. Changes in TEP in response to 5 (25%, 50%, 100%, 200% and 400% of the LC50) concentrations of $MgCl_2$ and $MgSO_4$. Dotted vertical lines indicate corresponding LC50 values. (A) $MgCl_2$ (2.5mM, 5mM, 10mM, 20mM, 40mM) N=6. (B) $CaSO_4$ (4.25mM, 8.5mM, 17mM, 34mM, 68mM) N=6.

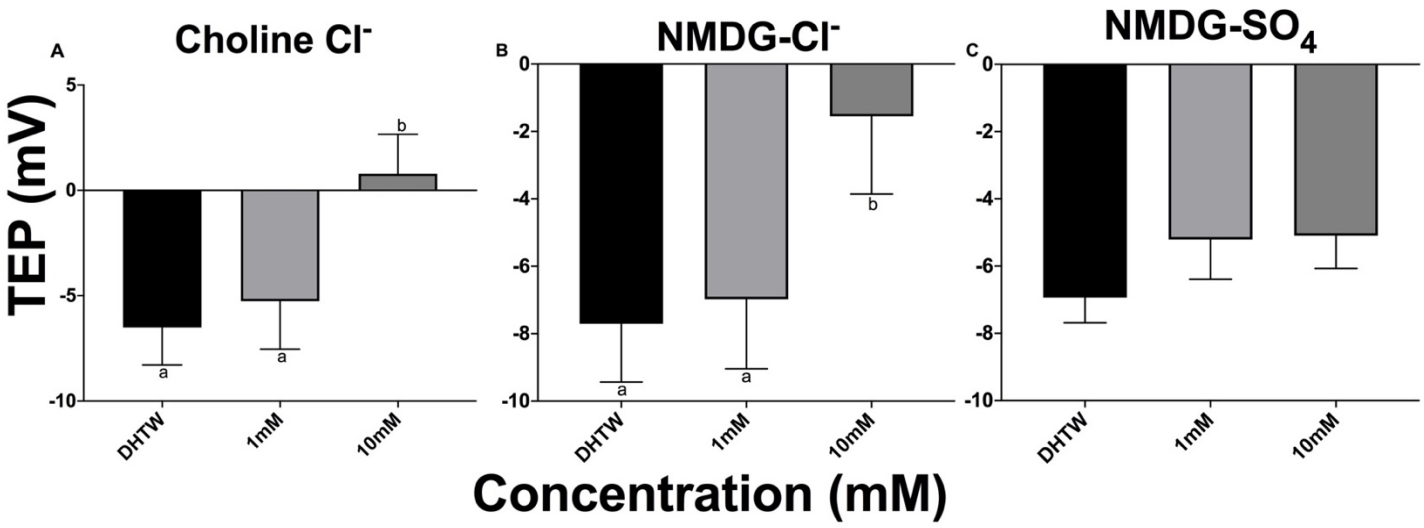
A
Choline Cl-
B
NMDG-Cl-
C
NMDG-SO4
TEP (mV)
5
0
-5
-10
-2
-4
-6
-8
a
a
b
a
a
b
DHTW
1mM
10mM
Concentration (mM)

Figure 3-6. Changes in TEP in response to 1mM and 10mM of choline Cl^-, NMDG-Cl^- and NMDG-SO_4. (A) choline Cl^- N=6. (B) NMDG-Cl^- N=6. (C) NMDG-SO_4 N=8. Bars labeled with the same letter do not differ significantly.

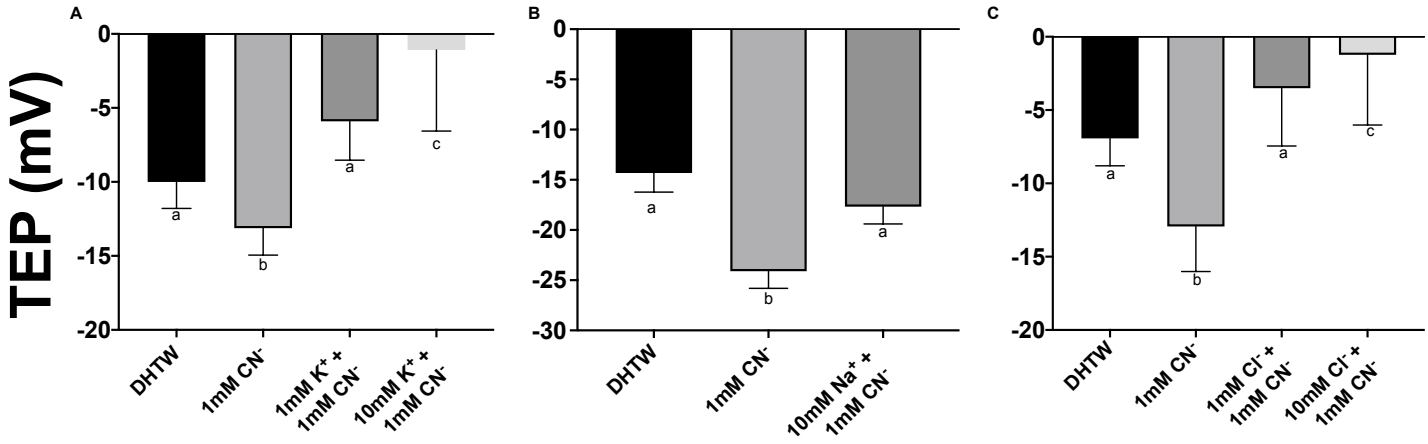
A
B
C
TEP (mV)
0
-5
-10
-15
-20
-25
-30
a
b
c
DHTW
1mM CN-
1mM K+ + 1mM CN-
10mM K+ + 1mM CN-
10mM Na+ + 1mM CN-
1mM Cl- + 1mM CN-
10mM Cl- + 1mM CN-

Figure 3-7. The effect of CN^- and K^+, Na^+ or Cl^- on TEP in *Daphnia magna.* Bars labeled with the same letter do not differ significantly. (A) 1mM NaCN, 1mM NaCN + 1mM KCl, 1mM NaCN + 10mM KCl. [Cl^-] and pH were kept constant using N-Methyl-D- Glucamine titrated with HCl. N=7. (B) 1mM NaCN, 1mM NaCN + 9mM Na^+. X axis labels reflect added [ion]. [Cl^-] and pH were kept constant using N-Methyl-D- Glucamine titrated with HCl. N=7. (C) 1mM NaCN, 1mM NaCN + 1mM NMDG-Cl^-, 1mM NaCN + 10mM NMDG-Cl^-. N=6.

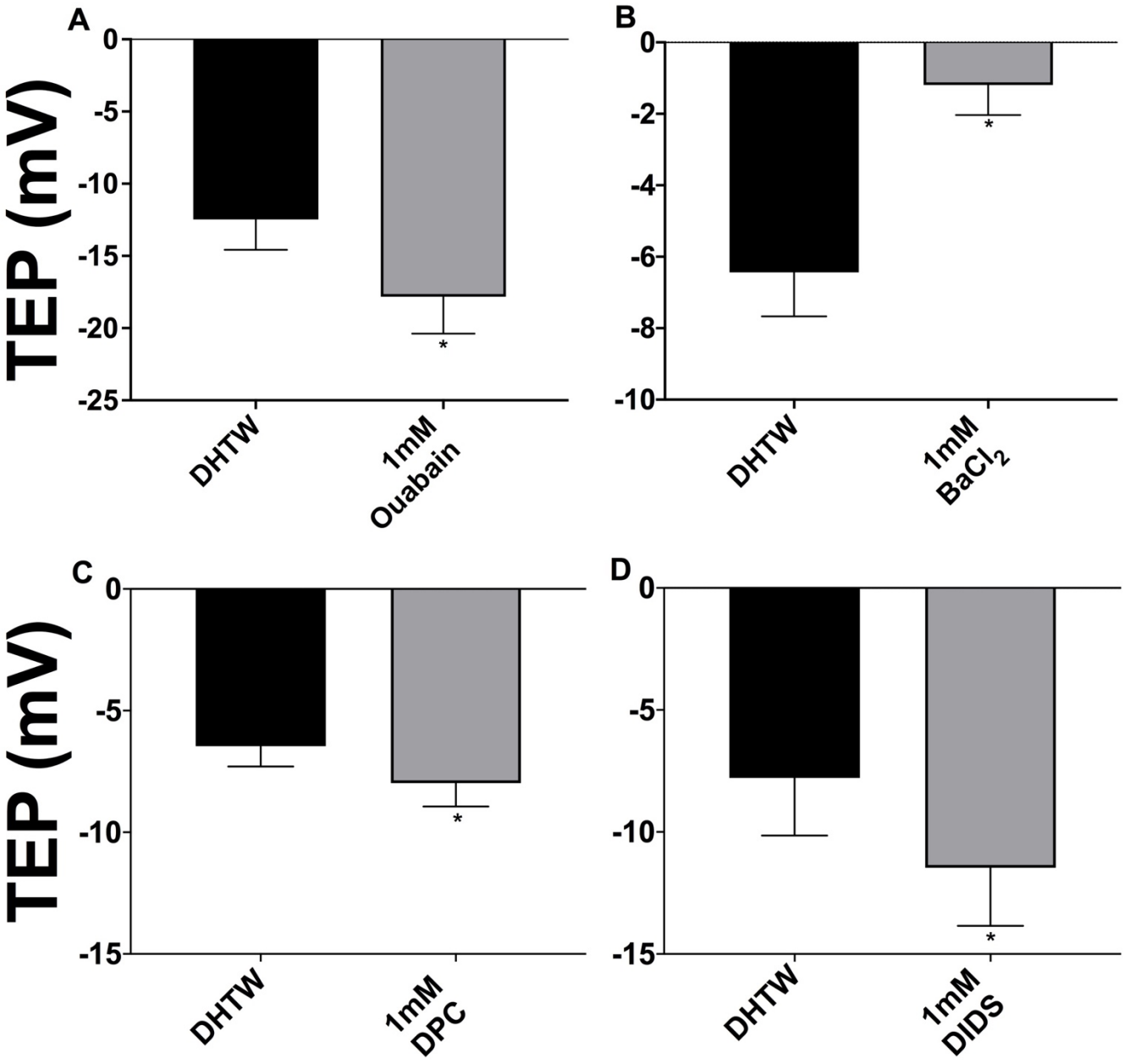
A
TEP (mV)
0
-5
-10
-15
-20
-25
*
DHTW
1mM Ouabain
B
0
-2
-4
-6
-8
-10
*
DHTW
1mM $BaCl_2$
C
TEP (mV)
0
-5
-10
-15
*
DHTW
1mM DPC
D
0
-5
-10
-15
*
DHTW
1mM DIDS

Figure 3-8. The effects of ion transporter inhibitors on TEP in *D. magna.* Bars labeled with the same letter do not differ significantly. (A) 1mM Ouabain. N=7. (B) 1mM $BaCl_2$. N=7. (C) 1mM DPC. N=7. (D) 1mM DIDS. N=6.

SUPPLEMENTARY MATERIAL

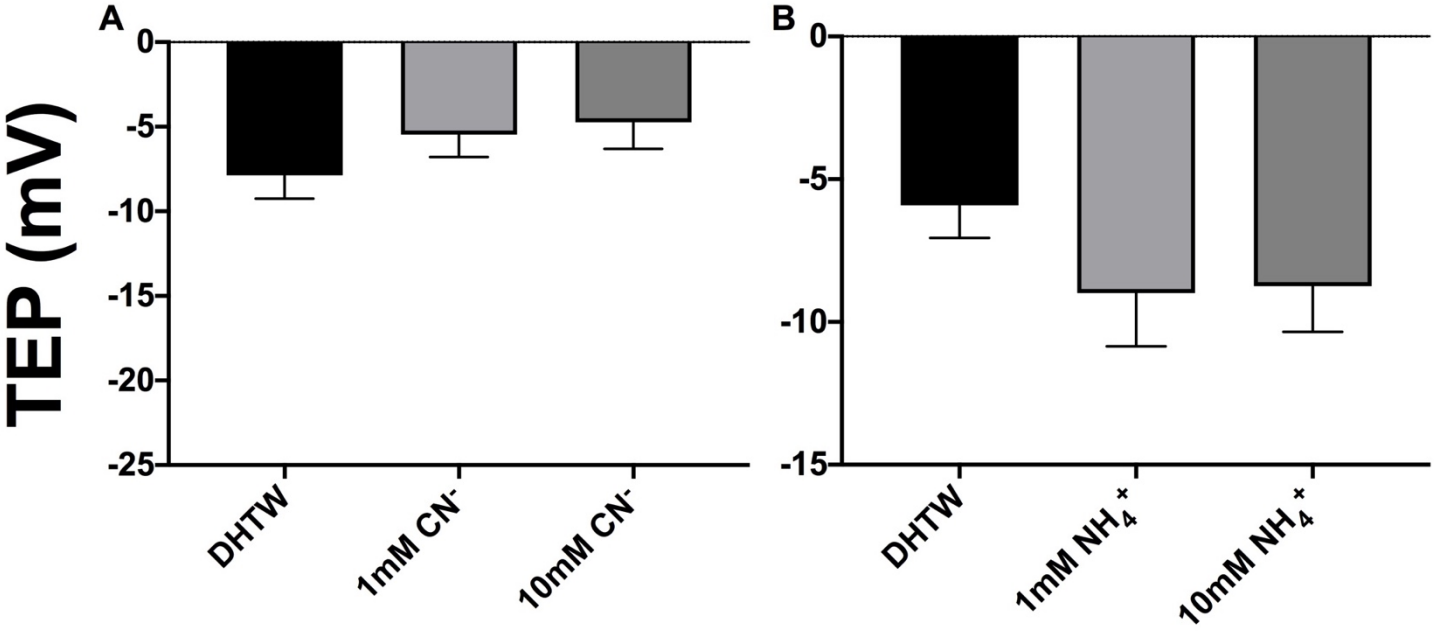

Figure 1

The effect of CN^- and NH_4^+ on TEP in *D. magna.* Repeated Measures ANOVA with Tukey's multiple comparison test $p \leq 0.05$. (A) 1mM NaCN + 9mM Na^+ isethionate and 10mM NaCN. Control is DHTW. N=6. (B) 1mM NH_4^+ and 10mM NH_4^+. [Cl^-] and pH were maintained constant by NMDG titrated with HCl.

Table 1. Change in TEP between DHTW and 0.1% DMSO and 1mM $KHCO_3$

Solution	Δ TEP ± SEM	P-Value*
0.1% DMSO	0.484 ± 0.984	0.559
1mM $KHCO_3$	0.053 ± 0.695	0.929

***p<0.05 denotes statistical significance from paired t-test. N=3**
Δ TEP=change in TEP from one solution to another

Table 2. ANCOVA for comparing slopes of linear regression grouped by salts with the same cation.

Salt	F	DFn	DFd	P*
KCl, K_2SO_4, $KHCO_3$	13.28	2	12	0.0009
NaCl, Na_2SO_4 $NaHCO_3$	291.5	2	9	0.0001
$CaCl_2$, $CaSO_4$	4.480	1	6	0.0787
$MgCl_2$, $MgSO_4$	48.63	1	6	0.0004

***p<0.05 denotes statistical significance**

Chapter 4

MULTIPLE FUNCTIONS OF ION TRANSPORT BY THE NUCHAL ORGAN IN EMBRYOS AND NEONATES OF THE FRESHWATER BRANCHIOPOD CRUSTACEAN, *DAPHNIA MAGNA*

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Keywords: ion transport, nitrogen excretion, acid/base balance, freshwater ionoregulation, calcification

ABSTRACT

The nuchal organ, also referred to as the dorsal organ or neck organ, is a dorsal structure located posteriorly to the compound eye, between the bases of the second antennae of embryonic and neonate branchiopod crustaceans such as the water flea, *Daphnia magna*. The ultrastructure of the nuchal organ is similar to ion-transporting tissues in other crustaceans, including abundant mitochondria and extensive amplification of apical and basal plasma membranes through microvilli and infoldings, but direct evidence for ion transport is lacking. We used the scanning ion- selective electrode technique to measure transport of Na^+, K^+, H^+, Cl^-, NH_4^+ and Ca^{2+} across the nuchal organ and body surface of embryos and neonates bathed in dechlorinated Hamilton tap water. Influx of Na^+ and efflux of H^+ and NH_4^+ was found to occur across the nuchal organ of both embryos and neonates. We propose that the efflux of K^+ and Cl^- across the nuchal organ in embryos is related to the expansion of the haemocoel and release of intracellular solutes into the extracellular space during development. K^+ is taken up across the nuchal organ later during development, coincident with expansion of the intracellular compartment through the development of gills and other organs. Ca^{2+} influx across the nuchal organ and body surface of neonates but not embryos is presumably related to calcification of the exoskeleton. Increases in the levels of Na^+ and Ca^{2+} in the water within the brood chamber suggest maternal provisioning of ions for uptake by the embryos. Our data thus support roles for the nuchal organ in ionoregulation, pH regulation and nitrogenous waste excretion.

INTRODUCTION

The gill is the predominant organ for ionoregulation in euryhaline crustaceans, and the structures of the transporting cells and mechanisms involved have been well-characterized in large species such as the blue crab *Callinectes sapidus* and the Chinese mitten crab *Eriochier sinensis* (Henry et al., 2012; Larsen et al., 2014). Smaller crustaceans such as branchiopods pose technical challenges and have been less well studied, but in adult brine shrimp, the branchiae are thought to be the main sites of ion uptake. Classic work involving staining with $AgNO_3$ and use of $KMnO_4$ to oxidize the transporting cells indicates that the first 10 pairs of branchiae are the sites of ion excretion in adult *Artemia salina* in hypertonic conditions, and probably the site of ion uptake in hypotonic media (Conte, 1984; Croghan, 1958). Hyperosmotic regulation in embryonic ostracods (seed shrimp) is proposed to reflect both salt reserves in the yolk of the egg as well as the absorption of salts by special cells located in the non-calcified zone of the inner shell layer (Aladin and Potts, 1996). In the freshwater copepod, *Eurytemora affinis,* structures termed Crusalis organs are thought to function as osmoregulatory organs (Johnson et al., 2014).

Another ionoregulatory structure, termed the nuchal organ (also referred to as the neck organ or dorsal gland), is present in a wide variety of crustaceans, both larval and adult, including branchiopods, copepods and malacostracans (Martin and Laverack, 1992). In branchiopods, the nuchal organ is the preferred term to describe structures that contain mitochondria-rich ion transporting cells that are probably involved in salt uptake in freshwater and salt excretion in saline water (Aladin and Potts, 1995). In the nauplius larva of the brine shrimp *Artemia salina*, there is direct evidence for salt excretion by the nuchal organ in larvae preloaded with ^{22}Na (Russler and Mangos, 1978).

Although a role for the nuchal organ in ion uptake by freshwater species has been inferred from ultrastructural studies, direct evidence for uptake is lacking. In the freshwater cladoceran *Daphnia magna*, the nuchal organ appears as an expanded portion of a dorsal ridge which runs from the front to the back of the head in first instar juveniles. In electron micrographs, the perimeter of the nuchal organ is delineated by a densely staining portion of cuticle which separates the thin cuticle covering of the nuchal organ from the thicker and less densely staining surrounding cuticle. The cells that form the nuchal organ fill much of the haemocoelic space between the cuticle and the gut, and are differentiated from surrounding squamous epidermal cells by greater apical–basal depth, extensive amplification of plasma membranes through apical microvilli and basal infoldings, and abundant mitochondria (Halcrow, 1982).

It has been suggested that the nuchal organ is most useful whilst juveniles remain in the brood chamber, particularly in the earlier stages of embryonic development when the thoracic appendages move very little (Halcrow, 1982). Cladocerans incubate their eggs in brood chambers formed by the carapace, except when laying resting eggs (Aladin and Potts, 1995), and the brood chamber remains open to the environment in most brackish and freshwater genera, including *Daphnia*. The egg membrane must therefore be impermeable until the larval organs of osmoregulation have developed. These include both the nuchal organ and the maxillary gland for water excretion. In the free-swimming neonate, the nuchal organ probably functions for about 12 h only (Halcrow, 1982). The nuchal organ disappears at the first embryonic moult when the animal begins to feed and the epipodites of the thoracic appendages probably assume a role in ion uptake (Aladin and Potts, 1995).

In this study, we used the scanning ion-selective electrode technique (SIET) to provide the first direct measurements of the transport of Na^+, K^+, Cl^-, H^+, Ca^{2+} and NH_4^+ across the nuchal organ and across regions of the body surface away from the organ in both embryonic and neonate *Daphnia magna*. Because development of the embryo is associated with formation of the heart and the haemocoel, changes in ion transport may be related not just to the need to replenish ions lost to the environment in freshwater but also to the conversion of the intracellular volume, typically with a Na^+/ K^+ ratio much less than 1, into extracellular space with a Na^+/ K^+ ratio much greater than 1. There may also be changes in ion transport associated with metabolism of yolk proteins into amino acids and subsequent synthesis of new proteins during the formation of tissues in the neonate such as the thoracic appendages and cuticle. Metabolism during development may thus lead to the formation of organic ions that may alter total cation and anion levels in the newly formed extracellular compartment. Lastly, in view of evidence that isolated embryos do not equilibrate with calcium in the environment and that calcium is transferred to the embryo from the mother (Giardini et al., 2015), we used ion-selective microelectrodes to determine whether embryos are exposed to concentrations of Ca^{2+} and other ions in the brood chamber that differ from the concentrations in the surrounding water.

MATERIALS AND METHODS

Daphnia culture

A starter culture of *Daphnia magna* Straus were obtained from a commercial supplier and maintained at room temperature (23° C) in aerated 20 l tanks of dechlorinated Hamilton tap water (DHTW). The water was sourced from Lake Ontario water, containing (in mmol l^{-1}): 1 Ca, 0.6 Na, 0.70 Cl, 0.3 Mg and 0.05 K, with titration alkalinity of 2.1 mequiv l^{-1}, hardness of ~ 140

mg l^{-1} as $CaCO_3$ equivalents, and pH ~ 8.0 (Hollis et al., 2001; Leonard et al., 2014). *Daphnia* were fed a 2:2:1 mixture of *Spirulina* powder: *Chlorella* powder: yeast 3 times per week.

SIET measurements

SIET measurements were made with hardware from Applicable Electronics (Forestdale, MA, USA) and Automated Scanning Electrode Technique (ASET) software (ASET-LV4, Science Wares, Falmouth, MA, USA). Micropipettes were pulled from 1.5mm borosilicate glass (World Precision Instruments Inc., Sarasota, FL, USA) to tip diameters of ~3 μm on a P-97 Flaming- Brown pipette puller (Sutter Instruments Co., Novato, CA, USA). Na^+-selective microelectrodes were backfilled with 150 mmol l^{-1} NaCl and tip filled with a cocktail consisting of 3.5% Na ionophore X, 0.6 % potassium tetrakis (4-chlorophenyl) borate and 95.9% 2-nitrophenyl octyl ether (Jayakannan et al., 2011). Na^+ ionophore X has a high selectivity for Na^+ over Ca^{2+} (>3000-fold) and for Na^+ over K^+ (~400-fold). Ion-selective microelectrodes for the other ions were constructed with the following ionophores (Sigma-Aldrich, St. Louis, USA), with backfill and calibration solutions (in mmol l^{-1}) indicated in parenthesis: K^+ ionophore I, cocktail B (150 KCl backfill, 0.5/5 KCl calibration); Ca^{2+} ionophore I, cocktail A (100 $CaCl_2$ backfill, 0.1/1/10 $CaCl_2$ calibration); H^+ ionophore I, cocktail B (100 NaCl/100 Na citrate at pH=6 backfill, 1 HEPES, 0.6 $NaHCO_3$, 1 $CaCl_2$ at pH=6.5, pH=8.3 calibration); NH_4^+ ionophore I, cocktail A (100 NH_4Cl backfill, 0.1/1 NH_4Cl calibration); Cl^- ionophore I, cocktail A (150 KCl backfill, 0.5/5 NaCl calibration). Because Cl^--selective microelectrodes based on chloride ionophores are known to be sensitive to organic anions that may be released from tissues (Chao and Armstrong, 1987; Del Duca et al., 2011; Kondo et al., 1989; Messerli et al., 2008), SIET measurements of Cl^- flux were also made with a solid-state Cl^- microelectrode (Donini and

O'Donnell, 2005) that is insensitive to organic anions such as bicarbonate and acetate (Saunders and Brown, 1977). The solid-state Cl^- microelectrode consisted of the fine tip (~10 μm diameter) of a chlorided silver wire glued into the barrel of a glass micropipette with hot melt glue so that a length of wire approximately 50 μm long and 10 μm in diameter protruded from the micropipette tip. To further reduce the exposed surface area of silver at the tip, the solid-state microelectrode was coated with a layer of petroleum jelly (~5 μm thick) which was then partially removed at the tip by wiping with a small piece of tissue paper so that the exposed chlorided silver wire was reduced to approximately 10 μm in diameter and 5–10μm in length, thus allowing finer spatial resolution for measurement of Cl^- concentration.

Measurements of Na^+, K^+, Cl^- and Ca^{2+} flux were made in DHTW. Measurements of K^+ flux were also made in DHTW containing 1 mmol l^{-1} KCl. NH_4^+ flux was measured in DHTW 0.1 mmol l^{-1} NH_4Cl. Preliminary measurements of H^+ flux were also made in DHTW. However, because protons may diffuse freely or in association with buffers in the saline, proton transport rates must be corrected for buffering using equations described in Messerli et al. (2006). For these experiments, a synthetic Hamilton tap water containing similar levels of Na^+, Cl^- and Ca^{2+} and known buffer concentrations was made using (in mmol l^{-1}) 0.6 $NaHCO_3$, 1 $CaCl_2$ and 1 HEPES, adjusted to pH 8. Measurements of Na^+ transport kinetics were done in water containing six concentrations of NaCl from 0.07 to 2.62 mmol l^{-1}, and 0.5 mmol l^{-1} $CaCl_2$, and Michaelis–Menten curves were fitted to the mean flux at each concentration. Measurements of Ca^{2+} transport kinetics were done in water containing 0.04–1.56 mmol l^{-1} $CaCl_2$ and 1 mmol l^{-1} NaCl. We began with 0.04 mmol l^{-1} and added $CaCl_2$ from a stock solution to approximately double the concentration for each step increase. Five concentration steps were sufficient to reach plateau values for the flux for some animals, whereas six or seven concentration steps were required for

others. We therefore determined the Michaelis– Menten parameters for each animal and the mean values presented in the Results are thus the means of the K_m and V_{max} values for each animal.

Ion flux was measured in embryos and neonates, corresponding to developmental stages 5 and 6, respectively (Kast-Hutcheson et al., 2001). Stage 5 is late in embryonic maturation; the second embryonic membrane has ruptured, and the second antennae are partially extended. The antennal setae are poorly developed and the tail spine is folded against the carapace. This stage occurs 45–50 h after deposition into the brood chamber. Stage 6 corresponds to a fully developed neonate, >48 h after deposition into the brood chamber. The organism is free swimming (*i.e.* emerged from the brood chamber), the setae on the second antennae setae are developed and the tail spine is fully extended from the carapace.

Embryos were collected under water with the aid of a stereomicroscope. Two pairs of forceps were used to pry apart the carapace of the adult so that the embryos spilled out of the brood chamber. Neonates were collected from a Petri dish containing 10–20 gravid adults. The dish was examined at 15 min intervals and the neonates were collected by suction using a plastic transfer pipette. Embryos and neonates were placed on their sides in a small depression made in petroleum jelly on the bottom of a 2 cm Petri dish. The cuticle of neonates adhered to the petroleum jelly and the lateral surface was thus uppermost, with the nuchal organ visible along the dorsal edge. For the embryos, bands of petroleum jelly were manipulated with fine forceps or a pin to form a small chamber enclosing the embryo positioned on its side so that approximately half the body surface, including the nuchal organ, was visible from above through a stereomicroscope. SIET measurements were made at the centre of the nuchal organ and at locations 20 μm anterior and posterior to the centre. At each measurement site, computer-

controlled stepper motors moved the ion-selective microelectrode between an inner position within 3–5 μm of the nuchal organ and an outer position 30 or 50 μm further away along a line perpendicular to the tissue surface. Three replicate measurements were made at each site, and the mean voltage difference between the two limits of excursion was converted into a concentration difference using Eqn 1:

$$\Delta C = C_B \cdot 10^{(\Delta V/S)} - C_B \qquad (1)$$

where ΔC is the concentration difference between the two points (in μmol cm^{-3}); C_B is the background ion concentration (in μmol cm^{-3}), calculated as the average of the concentrations at each point measured; ΔV is the voltage difference between the two limits of excursion obtained from ASET-LV4 (in mV); and S is the slope of the electrode (in mV) for a 10-fold change in ion concentration.

Flux was estimated from the measured concentration gradients using Fick's law:

$$J_I = D_I \, \Delta C / \Delta x \qquad (2)$$

where J_I is the net flux of the ion (in pmol cm^{-2} s^{-1}); D_I is the diffusion coefficient (Robinson and Stokes, 1968) of the ion (1.55×10^{-5} cm^2 s^{-1} for Na^+ and Cl^-; 1.92×10^{-5} cm^2 s^{-1} for K^+; 1.19×10^{-5} cm^2 s^{-1} for Ca^{2+}; 9.31×10^{-5} cm^2 s^{-1} for H^+; and 2.09×10^{-5} cm^2 s^{-1} for $NH4^+$); ΔC is the concentration gradient (in μmol cm^{-3}); and Δx is the distance between the inner and outer excursion limits (in cm). Positive values of J_I denote efflux, from the tissue surface to the water,

and negative values denote influx into the embryo or neonate.

Flux at each of the three sites was averaged, and then a mean value for the three sites was calculated. The typical interval between removal of the embryo from the brood chamber and the first flux measurement was 2–3 min. To determine whether there were changes over time associated with the securing of the embryos and neonates in the Petri dish, five sets of measurements at 3 min intervals were made for each preparation. To determine whether ion flux occurred at sites other than the nuchal organ, control measurements were made along the postero-lateral surface of the carapace, >100 μm from the nuchal organ.

Measurements of brood chamber ion concentrations

Ion-selective microelectrodes fabricated as described above were used to measure the concentrations of Na^+, K^+, NH_4^+, Ca^{2+}, H^+ and Cl^- in the brood chamber of *Daphnia* in three different states: without eggs in the brood chamber, with eggs, and with embryos. Adult *Daphnia* were secured with petroleum jelly to the bottom of a petri dish filled with DHTW and a micromanipulator was used to position the microelectrode tip in the brood chamber. A second type of liquid membrane Cl^- selective microelectrode based on 2% Cl^- ionophore II, 0.03% tridodecylmethylammonium chloride and 97.97% % 2-nitrophenyl octyl ether (Messerli and Smith, 2008) was used in some of the measurements of the brood chamber water, as discussed below. Potential differences between the ion-selective microelectrode and a reference electrode consisting of an Ag/AgCl pellet connected to the bath through an agar bridge containing 150 mmoll l^{-1} KCl in 4% agar were measured using a high impedance electrometer (pH AMP, AD Instruments, Australia) connected to a data acquisition system (Powerlab) running LabChart software.

Statistics

Graphing and statistical tests of significance were done in GraphPad Prism 6 (San Diego, CA, USA). Changes in ion flux at the nuchal organ over time and between embryos and neonates at the same time points were assessed with two-way ANOVA followed by Šidák's multiple comparisons test. Differences between the magnitude of ion flux at sites away from the nuchal organ and zero were assessed with a one-sample t-test. Differences were considered significant if $P<0.05$.

RESULTS

Na^+ influx at the nuchal organ of embryos and neonates

The nuchal organ in embryos and neonates of *D. magna* is located near the base of the second antennae, opposite the rostrum and overlapping the anterior portion of the heart (Fig. 1A, B). Neonates were readily distinguishable from embryos by the polygonal patterning of the cuticle, well- developed setae on the second antennae, a more pronounced dorsal ridge and a prominent tail spine, which extended away from the carapace (Fig. 1). Na^+ influx was localized to the nuchal organ, declining to near-zero values at the junction of the nuchal organ and the surrounding cuticle (Fig. 1C). The influx of Na^+ at the nuchal organ was sustained for five sets of measurements made at 3 min intervals in both embryos and neonates (Fig. 2). There were no significant differences in magnitude of the flux between embryos and neonates at each time point, and there were no significant changes over time (two-way repeated measures ANOVA followed by Šidák's multiple comparisons test). One-sample t-tests indicated that Na^+ flux at

sites away from the nuchal organ was not significantly different from zero in embryos (4.4 ± 4.0 pmol cm^{-2} s^{-1}, N=6) or neonates (-22.4 ± 13.8 pmol cm^{-2} s^{-1}, N=6).

Analysis of transport kinetics revealed that the maximum rate of Na^+ influx (V_{max}) across the nuchal organ of embryos was 518.1± 25 pmol c cm^{-2} s^{-1} and the bath Na^+ concentration at which transport was half-maximal (K_m) was 0.433±0.06 mmol l^{-1} (Fig. 3). The latter value is below the measured Na^+ concentration in the DHTW used in these experiments (0.74 mmol l^{-1}). Comparison of Figs 2 and 3 indicated that flux in neonates measured with microelectrodes based on Na^+ ionophore X in DHTW water containing 0.74 mmol l^{-1} Na^+ was ∼−320 pmol cm^{-2} s^{-1}, similar to the value of -315 pmol cm^{-2} s^{-1}predicted using 0.74 mmol l^{-1} and the Michaelis–Menten parameters derived from measurements in water containing 0.07–2.62 mmol l^{-1} NaCl and 0.5 mmol l^{-1} $CaCl_2$ (Fig. 3).

K^+ fluxes at the nuchal organ

There were pronounced changes in K^+ flux at the nuchal organ during development. In DHTW containing 1 mmol l^{-1} KCl, K^+ influx at the nuchal organ of neonates (∼-50 pmol cm^{-2} s^{-1}; Fig. 4A) was approximately one-quarter of the magnitude of Na^+ influx. By contrast, there was an efflux of K^+ from the nuchal organ of embryos of ∼80 pmol cm^{-2} s^{-1}. One-sample *t*-tests indicated that K^+ fluxes at sites away from the nuchal organ were not significantly different from zero in embryos (4.1 ± 2.3 pmol cm^{-2} s^{-1}, N=6) or neonates (-0.5 ± 1.5 pmol cm^{-2} s^{-1}, N=6). K^+ flux was also measured at the nuchal organ of neonates bathed in DHTW without any added K^+. This water contained 0.04 mmol K^+ and there was an influx of K^+ of -10 ± 2.2 pmol cm^{-2} s^{-1} (N=5), approximately 20% of the influx seen in water containing 1 mmol l^{-1} K^+. For five embryos bathed in DHTW without any added K^+, the water near the nuchal organ contained

0.078 mmol K^+ and there was a K^+ efflux of 59.4 ± 10.2 pmol cm^{-2} s^{-1} across the nuchal organ, approximately 75% of the efflux seen in water containing 1 mmol l^{-1} K^+.

Cl^- efflux at the nuchal organ of embryos and neonates

Measurements of Cl^- flux at the nuchal organ with microelectrodes based on Cl^- ionophore I, cocktail A, indicated a sustained efflux of Cl^- in both embryos and neonates (Fig. 4B). There were no significant differences in the magnitudes of Cl^- flux between embryos and neonates at each time point, and there were no significant changes over time (two-way repeated measures ANOVA followed by Šidák's multiple comparisons test). Cl^--selective microelectrodes based on Cl^- ionophore I are known to be sensitive to organic anions, and we therefore measured Cl^- fluxes at the nuchal organ with solid-state Cl^- microelectrodes (Fig. 4C). These measurements also revealed a sustained efflux of Cl^- at the nuchal organ; there were no significant differences in the magnitudes of Cl^- flux between embryos and neonates at each time point, and there were no significant changes over time (two-way repeated measures ANOVA followed by Šidák's multiple comparisons test). Moreover, there were no significant differences between the magnitudes of Cl^- fluxes measured with solid state Cl^- microelectrodes relative to those based on Cl^- ionophore I in embryos or neonates (2-way repeated measures ANOVA followed by Sidak's multiple comparisons test). The latter result indicates that Cl^- fluxes at the nuchal organ are not due to interference by organic anions on the Cl^--selective microelectrodes based on Cl^- ionophore I. One-sample *t*-tests indicated that Cl^- flux at sites away from the nuchal organ was not significantly different from zero in embryos (3.2 ± 5.7 pmol cm^{-2} s^{-1}, N=6) or neonates (-2.0 ± 9.4 pmol cm^{-2} s^{-1}, N=6).

H^+ efflux at the nuchal organ

There was an efflux of H^+ from the nuchal organ of embryos of 3.2 ± 1.1 pmol cm^{-2} s^{-1} [1] (N=7) after 15 min in DHTW. One-sample t-tests indicated that H^+ flux at sites away from the nuchal organ was ~3% of that at the nuchal organ, but was significantly different from zero (0.08 ± 0.02 pmol cm^{-2} s^{-1}, N=6). Although most protons diffuse in association with a buffer in relatively hard water such as DHTW, it was not possible to correct for buffer effects because of uncertainties regarding the precise concentrations of carbonate, bicarbonate and dissolved organic matter.

H^+ flux was therefore measured in a synthetic Hamilton tap water known ionic and buffer composition and the raw flux was corrected for buffering using the equations of Messerli et al. (2006). There were no significant changes in corrected H^+ flux over time (Fig. 4D), but the larger mean H^+ efflux in neonates relative to embryos was close to significance (P=0.06; two-way repeated measures ANOVA followed by Šidák's multiple comparisons test). One-sample t-tests indicated that H^+ flux at sites away from the nuchal organ of embryos in synthetic Hamilton tap water was less than 1% of that at the nuchal organ, but was significantly different from zero (8.1 ± 2.0 pmol cm^{-2} s^{-1}, N=6). H^+ flux at sites away from the nuchal organ of neonates was not significantly different from zero (-4.7 ± 6.3 pmol cm^{-2} s^{-1}, N=6).

NH_4^+ efflux at the nuchal organ

Preliminary measurements indicated that there were dramatic changes in the magnitude of NH_4^+ efflux from the nuchal organ during development. Measurements were therefore made in neonates within 1 h of emergence from the adult and at >2 h after emergence.

There were no significant changes in NH_4^+ efflux from the nuchal organ of embryos or >2 h neonates over time. However, NH_4^+ efflux from neonates within 1 h of emergence from the brood chamber was 4- to 5-fold greater than that in embryos or in neonates >2 h after emergence (Fig. 4E) and there was a significant increase in the efflux at 15 min relative to that at 3 min in neonates <1 h after emergence (two-way repeated measures ANOVA followed by Šidák's multiple comparisons test). One-sample t-tests indicated that $NH4^+$ efflux at sites away from the nuchal organ of neonates <1 h after emergence was less than 1% of that at the nuchal organ, but was significantly different from zero (1.0 ± 0.4 pmol cm^{-2} s^{-1}, N=6). NH_4^+ flux at sites away from the nuchal organ of embryos was not significantly different from zero (1.5 ± 0.9 pmol cm^{-2} s^{-1}, N=6).

Ca^{2+} transport across the of body surface and nuchal organ of embryos and neonates

In contrast to transport of other ions, Ca^{2+} transport was not confined to the nuchal organ. In embryos, there was a small influx of Ca^{2+} at the nuchal organ at 3 min (Fig. 4F), but the value at the nuchal organ (-2.4 ± 1.3 pmol cm^{-2} s^{-1}, N=7) was not significantly different from that away from the nuchal organ (-5.2 ± 1.5 pmol cm^{-2} s^{-1}, N=7). Ca^{2+} influx across the nuchal organ in embryos was not sustained, and was not significantly different from zero after the first measurement at 3 minutes (Fig. 4F). Ca^{2+} influx increased dramatically in neonates relative to

embryos. However, the influx over the nuchal organ at 3 min (-43.6 ± 5.2 pmol cm^{-2} s^{-1}, N=8) was not significantly larger than that over the body surface at sites away from the nuchal organ (-27.7 ± 8.7 pmol cm^{-2} s^{-1}, N=6), consistent with Ca^{2+} uptake over the entire exoskeleton. Flux at sites away from the nuchal organ for all ions measured is summarized in Table S1.

Neonates were exposed to five to seven Ca^{2+} concentrations between 0.04 and 1.56 mmol l^{-1} for analysis of transport kinetics (Table S2). The Michaelis-Menten parameters calculated by non-linear regression for each neonate (N = 6) were: K_m = 0.146 ± 0.040 mmol l^{-1}, V_{max} = -68.5 ± 15.3 pmol cm^{-2} s^{-1}. The mean R^2 value for the nonlinear regression equations was 0.93 ± 0.02 (range 0.84 to 1.00).

Ion concentrations in the brood chamber

The concentrations of K^+, Na^+, NH_4^+ and Ca^{2+} in the brood chamber were 2-fold to 4-fold higher than those in the bathing water for *Daphnia* without eggs (Fig. 5A–D). For *Daphnia* with embryos in the brood chamber, concentrations of K^+ and NH_4^+ were 24% and 126%, respectively, above those in the water, whereas the concentrations of Na^+ and Ca^{2+} were within 5% of those in the bath water. Chloride concentrations in the brood chamber were 3-fold to 4-fold higher than those in the bathing water for *Daphnia* with or without eggs (Fig. 5E). The larger size of the tip of the solid-state Cl^- electrode relative to the liquid membrane ion-selective microelectrodes precluded measurements of Cl^- concentration within the brood chamber of *Daphnia* containing embryos. Attempts to measure brood chamber Cl- concentration with a liquid membrane Cl^- microelectrode based on Cl^- ionophore I, cocktail A were discontinued because there was evidence that some component within the brood chamber interfered with the microelectrode. The time required to respond to a change in Cl^- concentration increased from a

few seconds to > 15 minutes after the microelectrode tip had been positioned within the brood chamber (N = 17; data not shown). Measurements with microelectrodes based on chloride Ionophore II (N = 8, data not shown) were also unsuccessful; the response time increased and the slope of the microelectrode decreased after sampling of the brood chamber. The pH within the brood chamber did not differ significantly from the bath in *Daphnia* with or without eggs or embryos (Fig. 5F).

DISCUSSION

Our results provide direct evidence for a role of the nuchal organ in Na^+ uptake, pH regulation and ammonia excretion (Fig. 6). We suggest below that transport of K^+ and Cl^- across the nuchal organ may be related to the formation and expansion of the haemocoel as the circulatory system develops. Our results also show influx of Ca^{2+} across the cuticle of neonates but not embryos, consistent with calcification of the exoskeleton through deposition of calcium salts.

Contribution of Na^+ influx at the nuchal organ to ionoregulation

Our results indicate a sustained influx of Na^+ at the nuchal organ of both embryos and neonates. Kinetic analysis of the influx suggests that the K_m (0.433 mmol l^{-1}) was slightly below the level of Na^+ in the water in which the animals were reared. The significance of this influx to ionoregulation in the embryos and neonates can be appreciated through estimates of haemolymph volume and nuchal organ surface area.

Approximating the embryo shape as an ellipsoid with major and minor axes of 0.700 mm and 0.430 mm (Fig.1), respectively, gives a volume of $4/3\pi(0.350)(0.215)(0.215) = 0.068$ mm^3.

It has been estimated that haemolymph volume in adult *Daphnia magna* corresponds to 61% of animal volume (Kobayashi and Nezu, 1986). An upper limit of haemolymph volume in the embryo is thus (0.61)(0.068) =0.041 μl. Based on a diameter of the nuchal organ (Fig. 1) of 80 μm, its area is 5 x 10^{-5} cm^2. Transport across this area at rate of ~ -320 pmol cm^{-2} s^{-1} (Fig. 2) is equivalent to 0.057 nmol h^{-1}. Our measurements of Na^+ concentration in adult *Daphnia magna* (Morris & O'Donnell, unpublished) indicate a value of 51 mmol l^{-1} for animals reared in dechlorinated Hamilton tap water; the estimated haemocoel Na^+ content is thus: (0.051)(0.041) = 2.1 nmol. Complete replacement of Na^+ in the haemocoel of the embryo could be achieved by ~37 h of transport (2.1/0.057) across the nuchal organ, of similar magnitude to the time required (~24 h) for development from the time of appearance of the nuchal organ (coincident with appearance of the antennae (Kast-Hutcheson et al., 2001; Mittmann et al., 2014) through to the emergence of a free swimming neonate. A smaller haemolymph volume as a proportion of animal volume in embryos and neonates relative to adults will decrease the time required for transport of the entire haemocoel content of Na^+. Clearly, haemolymph volume is negligible in the early embryo before the haemocoel is formed, and there may be significant Na^+ content of the egg, in which case the nuchal organ's ionoregulatory role may be partly to replenish passive losses of Na^+ across other body surfaces. Later in development, there may be additional uptake of Na^+ across the epipodites (gills) of the thoracopods (thoracic appendages), which are present in embryos by the time the second antennae have elongated (Mittmann et al., 2014). It has been suggested that the nuchal organ is most useful during the juvenile's stay in the brood chamber, and that it functions for <<12 h in the neonate, before the nuchal organ switches from ion transport to cuticle secretion (Halcrow, 1982).

A previous study of Na^+ uptake by *Daphnia* investigated ion exchange across the whole

animal (Bianchini and Wood, 2008) and therefore multiple sites (gut, gill) and mechanisms may have contributed. These authors proposed that a vacuolar-type H^+- ATPase sensitive to bafilomycin in the apical membrane of cells involved in uptake from the water by neonates creates an electrical gradient favouring Na^+ uptake through channels sensitive to the drug phenamil. Such a proposal is consistent with our findings of outwardly directed H^+ flux and inwardly directed Na^+ flux at the nuchal organ. The K_m for Na^+ uptake by whole neonates in that study (0.351 mmol l^{-1}; Bianchini and Wood, 2008) is similar to the K_m for Na^+ uptake at the nuchal organ (0.433 mmol l^{-1}). Further studies of the nuchal organ using SIET will allow the role of specific transporters in Na^+ influx across a single epithelium to be assessed through the application of transport inhibitors and toxins. Silver, for example, causes mortality at extremely low concentrations through inhibition of sodium uptake pathways (Bianchini and Wood, 2003), and it will be of interest in future studies to examine the influence of silver on Na^+ influx across the nuchal organ. Whole-animal studies have also shown that both the epithelial Na^+ channel blocker phenamil and the vacuolar H^+-ATPase inhibitor bafilomycin A1 inhibit Na^+ uptake in Daphnia neonates (Bianchini and Wood, 2008), and that the Na^+ channel and Na^+:H^+ exchange inhibitor amiloride blocks Na^+ uptake in adults (Glover and Wood, 2005). The latter study also revealed complex relationships between ambient Ca^{2+} levels and Na^+ uptake, with Ca^{2+} inhibiting Na^+ uptake at low Na^+ levels, but stimulating Na^+ uptake at high Na^+ levels. Acidic pH severely inhibits sodium influx in adults when calcium concentration is high (Glover and Wood, 2005). Additional studies using SIET will allow analysis of the interrelationships of water pH and hardness on Na^+ uptake by the nuchal organ.

Roles of the nuchal organ in acid-base balance and nitrogen excretion

The magnitude of H^+ efflux from the nuchal organ of embryos and neonates bathed in

synthetic Hamilton tap water was approximately 300-fold larger than the uncorrected flux calculated from the measured H^+ concentration values at two points within the unstirred layer. This difference between corrected and uncorrected H^+ flux is a common finding in media containing significant levels of buffers. In a study of mammalian gastric oxyntic cells, buffers enhanced the diffusion of protons by a factor of 2249 (*i.e.*, 1374 by 1 mM HEPES and 875 by 5 mM HCO_3^- ; Demarest and Morgan, 1995). The large flux of H^+ across the nuchal organ suggests a significant role in acid–base balance, particularly as H^+ flux across the body surface was only 1% of that at the nuchal organ. An efflux of H^+ could be used to drive Na^+ uptake through a Na^+- H^+ exchanger. Alternatively, efflux of H^+ could indicate the activity of a vacuolar H^+-ATPase known to be implicated in Na^+ uptake, or hydration of metabolic CO_2 passing out through the nuchal organ, followed by hydration of CO_2 and dissociation of carbonic acid into H^+ and HCO_3^- through the actions of carbonic anhydrase.

The efflux of NH_4^+ across the nuchal organ of both embryos and neonates may be a consequence of catabolism of protein from yolk granules into amino acids for energy production in embryos and neonates. Given the large H^+ efflux across the nuchal organ, the NH_4^+ gradient measured with SIET could be a consequence of diffusion trapping of NH_3 that has diffused across the nuchal organ. It is worth noting in this context that ammonia excretion in adult Daphnia is enhanced at low environmental pH relative to the rate of excretion at circumneutral pH (Al-Reasi et al., 2013).

Although our measurements indicated efflux of ammonia across the nuchal organ, it must be noted that ammonium ionophore I, cocktail A is only 4 times more selective for NH_4^+ than for K^+. Efflux of K^+ from the nuchal organ of embryos may thus lead to an overestimate of apparent NH_4^+ efflux, and influx of K^+ across the nuchal organ of neonates will lead to an underestimate

of apparent NH_4^+ efflux. A corrected flux can be estimated by accounting for the effects of K^+ on the NH_4^+-selective microelectrode during SIET measurements. For embryos, the corrected NH_4^+flux is 55% of the uncorrected value, whereas interference from K^+ at the nuchal organ of neonates results in a small underestimate (2%) of the NH_4^+ flux (see Appendix).

Transport of K^+ and Cl^- across the nuchal organ

Most freshwater animals require uptake of both Na^+ and Cl^- to replace passive loss of these ions. The efflux of Cl^- across the nuchal organ was, therefore, an unexpected finding. We suggest that Cl^- efflux reflects displacement of extracellular Cl^- by production of other anions such as bicarbonate. *Daphnia pulex* is known to have both elevated levels of bicarbonate in the haemolymph (20.9 mmol l^{-1}) and an elevated extracellular pH of 8.33 (Weber and Pirow, 2009). If similar conditions apply to *D. magna*, then both bicarbonate and negative charges on circulating amino acids, peptides and proteins could lead to an anion surplus, favouring efflux of Cl^- across the nuchal organ. Efflux of K^+ from embryos bathed in water containing 1 mm K^+ may also be a consequence of developmental processes. Development of an egg into an embryo requires the formation of extracellular space (typically with $Na^+/K^+>>1$). If there were no change in the volume of cytoplasm (with $Na^+/K^+<<1$), formation of extracellular fluid would require uptake of both Na^+ and K^+. We suggest that intracellular volume is converted into extracellular volume, and that release of cytoplasmic K^+ into the extracellular environment may thus lead to excess K^+ in the extracellular space during early development and expansion of the haemocoel. Later in development, there is an influx of K^+ across the nuchal organ of neonates. This influx is coincident with tissue development (e.g. gills, gut, epidermal cells) that re-expands total intracellular volume, necessitating uptake of K^+. Although we have no data indicating changes in

total intracellular volume during development, the smaller number of yolk granules in neonates relative to embryos is consistent with breakdown of the yolk and release of ions.

Influx of Ca^{2+} across the body surface in neonates

Our SIET measurements indicating negligible Ca^{2+} transport across the nuchal organ or body surface of embryos is consistent with an earlier study which used radioactively labelled calcium (^{45}Ca) to trace calcium from mothers to embryos (Giardini et al., 2015). That study demonstrated that calcium is transferred to the embryo from the mother, and that isolated embryos do not equilibrate with calcium in the environment.

SIET measurements indicated Ca^{2+} influx in neonates, but there was no difference in the magnitude of the Ca^{2+} flux across the nuchal organ relative to sites away from the nuchal organ, when the Ca^{2+}-selective microelectrode tip was positioned over the posterior regions of the carapace. Daphnia require dissolved calcium to harden the new carapace post-moult, and previous studies have shown that the necessary Ca^{2+} is acquired by uptake from the environment (Tan and Wang, 2009). Our measurements of Ca^{2+} kinetics derived a K_m of 0.146 mmol l^{-1}, considerably below the levels in the relatively hard water (dechlorinated Hamilton tap water) used for rearing *D. magna* in this study. The efficiency of Ca^{2+} uptake presumably aids rapid calcification of the cuticle.

Further studies using SIET will aid analysis of Ca^{2+} transport across the body surface of Daphnia in low- Ca^{2+} waters, particularly in neonates, as juveniles are more sensitive to calcium deficiency than adults (Hessen et al., 2000). Such studies could examine the influence of water

chemistry (pH, HCO_3^-, hardness) and temperature on Ca^{2+} uptake, and the possible impacts of freshwater acidification from anthropogenic increases in atmospheric CO_2.

Maternal provisioning of ions

Increases in the concentrations of K^+, Na^+ and Cl^- in the empty brood chamber of *D. magna* above the corresponding values in the surrounding water indicate that these ions are released from the female. Care was taken to avoid touching the surface of the brood chamber when positioning the microelectrode tip, and the finding of lower Ca^{2+} concentrations in the brood chamber of *D. magna* without eggs suggests that elevated concentrations of the other ions are not simply the result of leakage following damage to the wall of the brood chamber. One caveat is that for measurements with the solid-state Cl^- microelectrode, the larger tip size relative to that of the liquid membrane ion-selective microelectrodes made it more likely that the surface of the brood chamber was contacted by the microelectrode tip during measurements of Cl^- concentration. The concentration of each ion species within the brood chamber will reflect the rate of ion release from the female, uptake or release by the egg or embryo, and convective and/or diffusive exchange of the brood chamber water with the water outside the female. When eggs are present in the brood chamber, the increases in concentration of Na^+, K^+ and Cl^- in the brood chamber could result from release from either the female or the egg, but irrespective of the source of ions in the brood chamber, these increases would tend to reduce any passive loss of ions from the developing eggs. The increased concentration of NH_4^+ in the brood chamber above that in the bathing water, by contrast, creates a larger gradient opposing efflux of NH_4^+out of the

egg or embryo if ammonia is transported as the ion (but not if excretion is occurring as the gas NH_3, which is then trapped as the ion by combining with H^+ to form NH_4^+). Measurements of Ca^{2+} concentration in the brood chamber revealed a complex pattern of changes. Although the concentration of Ca^{2+} in the brood chamber of *D. magna* was slightly lower than the bathing water around *D. magna* with no eggs in the brood chamber, the increase in Ca^{2+} concentration above that in the bathing water when eggs were present is consistent with a previous suggestion of maternal provisioning that was based on the flux of Ca^{45} (Giardini et al., 2015). However, this raises the question of how Ca^{2+} is taken up through the egg membranes, which are assumed to be impermeable to ions to minimize ion loss by the eggs before development of ion-transporting organs. Although we saw only transient uptake of Ca^{2+} by isolated embryos, it is conceivable that uptake is sustained in the ionic and hormonal milieu within the brood chamber. It will be of interest in future studies to determine whether maternal provisioning of Ca^{2+} through release of Ca^{2+} into the brood chamber is of greater significance for *Daphnia* reared in soft water, given that effects of low Ca on growth rate are most apparent during the first days after hatching, reflecting the higher Ca demands of the early juveniles (Hessen et al., 2000). It is worth noting that eggs of land isopods (suborder Oniscidea) are brooded in a fluid-filled maternal marsupium until a few days following the second embryonic moult and that there is evidence for maternal control of the marsupial environment (Surbida and Wright, 2001). Eggs of *Armadillidium vulgare* possess a well-developed dorsal organ underlying a broad silver-staining saddle on the vitelline membrane. Like the nuchal organ of *Daphnia*, the dorsal organ has been implicated in ion regulation and acid excretion, but it also plays a role in calcium provisioning (Wright and O'Donnell, 2010).

APPENDIX

Correcting NH_4^+ fluxes for interference by K^+ on NH_4^+-selective microelectrodes.

Correction for this interference requires estimation of the concentration of K^+ at the inner and outer limits of microelectrode excursion. By re-arranging Fick's equation (see Eqn 2 in Materials and Mehods) to solve for ΔC, a K^+ efflux in embryos in DHTW of 59.4 pmol cm^{-2} s^{-1} corresponds to ΔC of 0.0155 mmol l^{-1}. The mean K^+ concentration in the unstirred layer near the nuchal organ in DHTW was 0.078 mmol l^{-1}, so the K^+ concentration at the inner and outer limits of microelectrode excursion can thus be estimated as 0.078 + (0.0155 /2) = 0.86 mmol l^{-1} and 0.078 - (0.0155 /2) = 0.70 mmol l^{-1}, respectively. For embryos in K^+ in DHTW containing 0.1 mmol l^{-1} NH_4^+, the concentration of NH_4^+ near the nuchal organ was 0.14 mmol l-1 and the uncorrected NH_4^+ efflux was 36 pmol cm^{-2} s^{-1}, corresponding to ΔC of 0.0086 mmol l^{-1} NH_4^+, and uncorrected NH_4^+ concentrations at the inner and outer excursion limits of 0.144 and 0.136 mmol l^{-1}. The selectivity coefficient for NH_4^+ microelectrodes based on ammonium ionophore I is 0.25. The corrected NH_4^+ concentration at the inner and outer limits of electrode excursion are thus 0.144 – (0.25 * 0.086) = 0.123 mmol l^{-1} and 0.136 – (0.25 * 0.070) = 0.118 mmol l^{-1}. The corrected ΔC is thus 0.0047 mmol l^{-1} and the corrected NH_4^+ efflux is 19.8 pmol cm^{-2} s^{-1}, approximately 55% of the uncorrected value. Corresponding calculations for neonates within 1 h of emergence indicate that interference from K^+ produces only a small underestimate (2%) of the NH_4^+ efflux.

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Figure 1

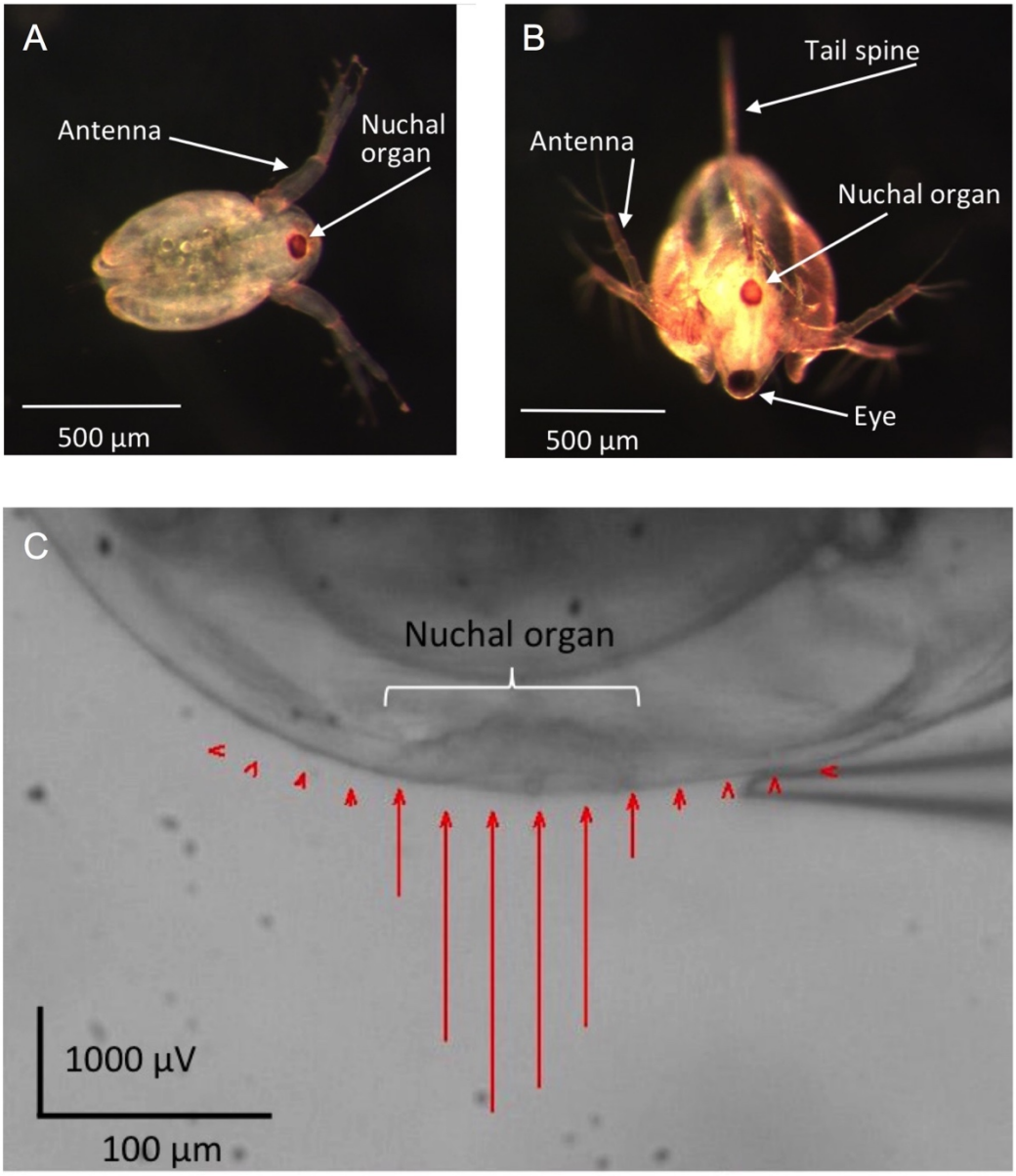

A
Antenna
Nuchal organ
500 µm
B
Tail spine
Antenna
Nuchal organ
Eye
500 µm
C
Nuchal organ
1000 µV
100 µm

Figure 4-1. The nuchal organ in *Daphnia magna* (A) embryos and (B) neonates. The preparations were stained with 1% silver nitrate solution to enhance visibility of the nuchal organ. (C) Voltage differences measured with a Na^+-selective microelectrode positioned at 14 locations over or near the nuchal organ. The tip of each arrow indicates the location of the microelectrode tip at the inner excursion limit during measurements by SIET. The length of each arrow corresponds to the voltage difference between inner and outer excursion limits when the microelectrode was moved orthogonal to the tissue surface from the inner excursion limit to a position 50 μm further away. The outline of the nuchal organ is indicated by the white horizontal bracket.

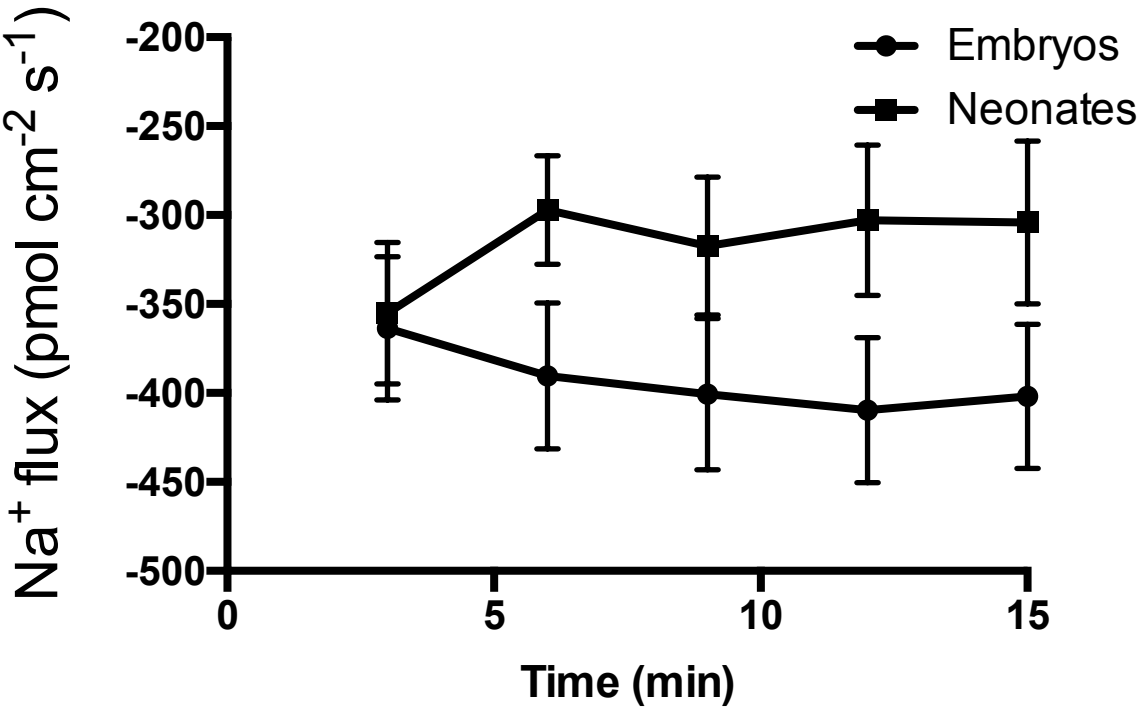

Embryos
Neonates
Na^+ flux (pmol cm^{-2} s^{-1})
-200
-250
-300
-350
-400
-450
-500
0
5
10
15
Time (min)

Figure 4-2. Na^+ fluxes (mean ± s.e.m.) at the nuchal organ of embryos (*N*=10) and neonates (*N*=7) measured at 3 minute intervals. In this and all subsequent figures, negative values correspond to influx and positive values correspond to efflux (*i.e.* from nuchal organ to water).

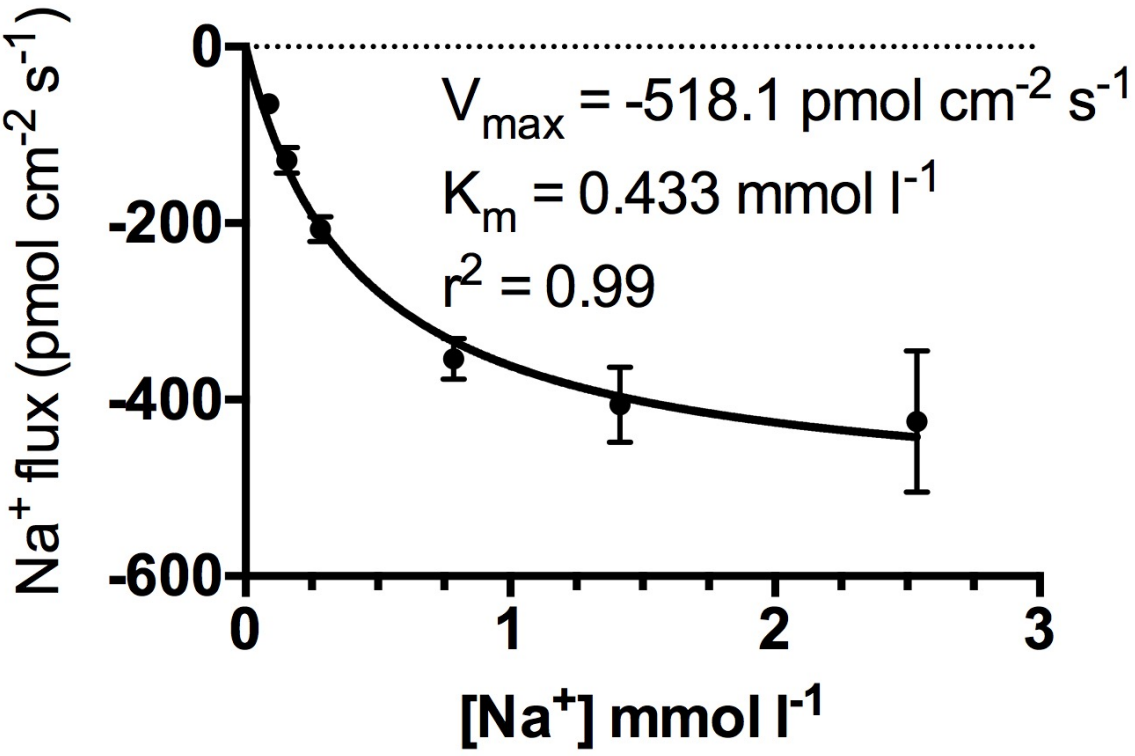

0
-200
-400
-600
0
1
2
3
Na^+ flux (pmol cm^{-2} s^{-1})
$[Na^+]$ mmol l^{-1}
V_{max} = -518.1 pmol cm^{-2} s^{-1}
K_m = 0.433 mmol l^{-1}
r^2 = 0.99

Figure 4-3. Na^+ flux at the nuchal organ of embryos as a function of water Na^+ concentration. Each point shows the mean ± s.e.m. for *N*=6 embryos. The water contained NaCl at the indicated concentration plus 0.5 mmol l^{-1} $CaCl_2$. The solid line represents the fit to the Michaelis–Menten equation by non-linear regression analysis.

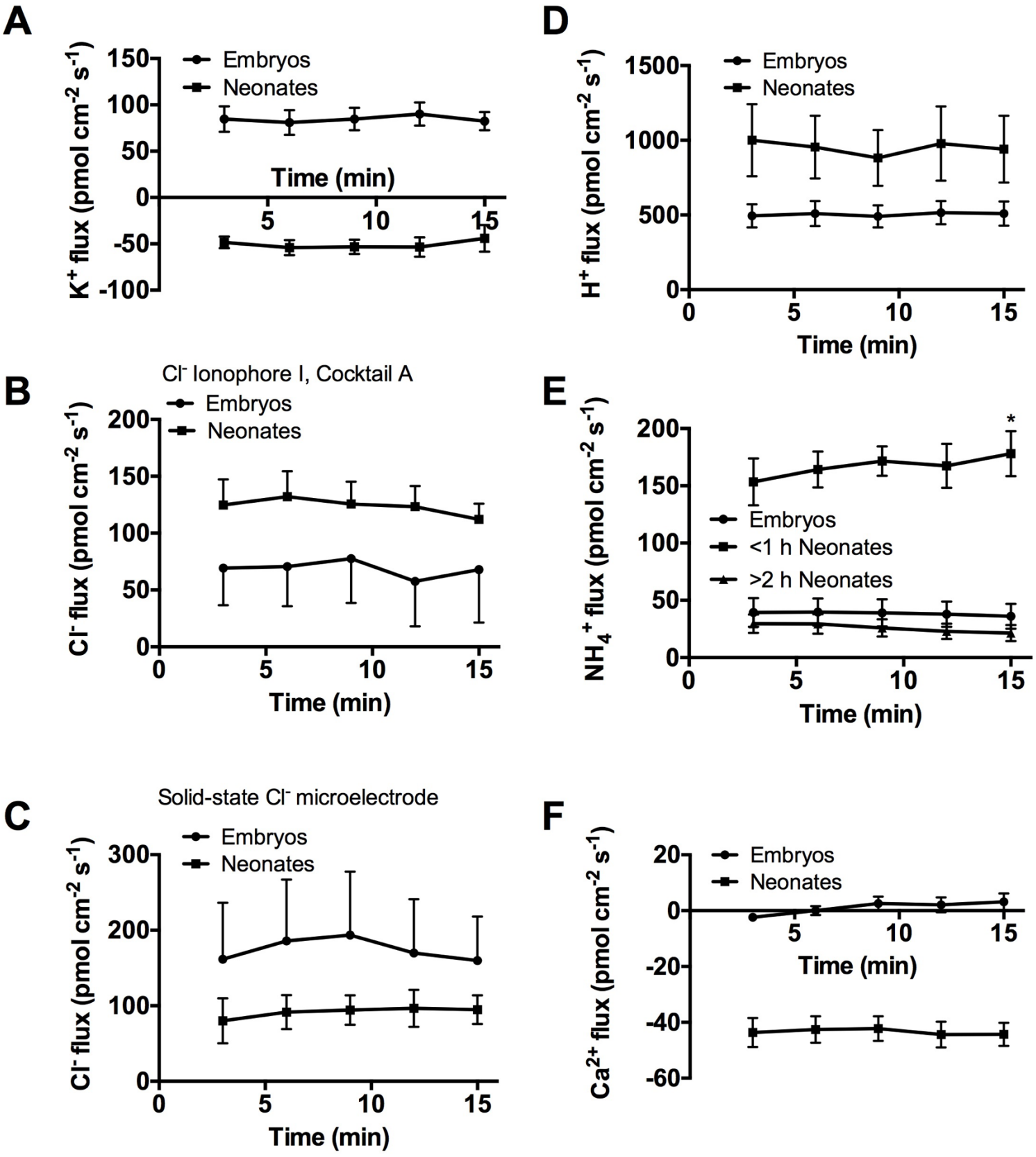

A
Embryos
Neonates
Time (min)
K+ flux (pmol cm-2 s-1)
150
100
50
0
-50
-100
5
10
15
D
Embryos
Neonates
H+ flux (pmol cm-2 s-1)
1500
1000
500
0
Time (min)
B
Cl- Ionophore I, Cocktail A
Embryos
Neonates
Cl- flux (pmol cm-2 s-1)
200
150
100
50
0
Time (min)
E
NH4+ flux (pmol cm-2 s-1)
*
Embryos
<1 h Neonates
>2 h Neonates
Time (min)
C
Solid-state Cl- microelectrode
Embryos
Neonates
Cl- flux (pmol cm-2 s-1)
300
200
100
0
Time (min)
F
Embryos
Neonates
Ca2+ flux (pmol cm-2 s-1)
20
0
-20
-40
-60
Time (min)

Figure 4-4. A) K^+ fluxes (mean ± s.e.m.) at the nuchal organ of embryos (*N*=8) and neonates (*N*=8) measured at 3 minute intervals. B) Mean Cl^- fluxes at the nuchal organ of embryos and neonates measured at 3 minute intervals with microelectrodes based on Cl^- Ionophore I, cocktail A. N=10 embryos, N=12 neonates. Error bars (s.e.m.) for some points omitted for clarity. C) Mean Cl^- fluxes at the nuchal organ of embryos and neonates with solid-state Cl^- microelectrodes. N=5 embryos, N=7 neonates. Error bars (s.e.m.) for some points omitted for clarity. D) H^+ fluxes (mean ± s.e.m.) at the nuchal organ of embryos (*N*=10) and neonates (*N*=9) measured at 3 minute intervals. E) NH_4^+ fluxes (mean ± s.e.m.) measured at 3 minute intervals at the nuchal organ of embryos (*N*=8), neonates <1 h post emergence (*N*=6) and neonates >2h post emergence (*N*=10). The asterisk denotes a significant difference between flux at the indicated time point relative to that at 3 min (2-way repeated measures ANOVA followed by Sidak's multiple comparisons test). F) Ca^{2+} fluxes (mean ± s.e.m.) at the nuchal organ of embryos (*N*=7) and neonates (*N*=8) measured at 3 minute intervals.

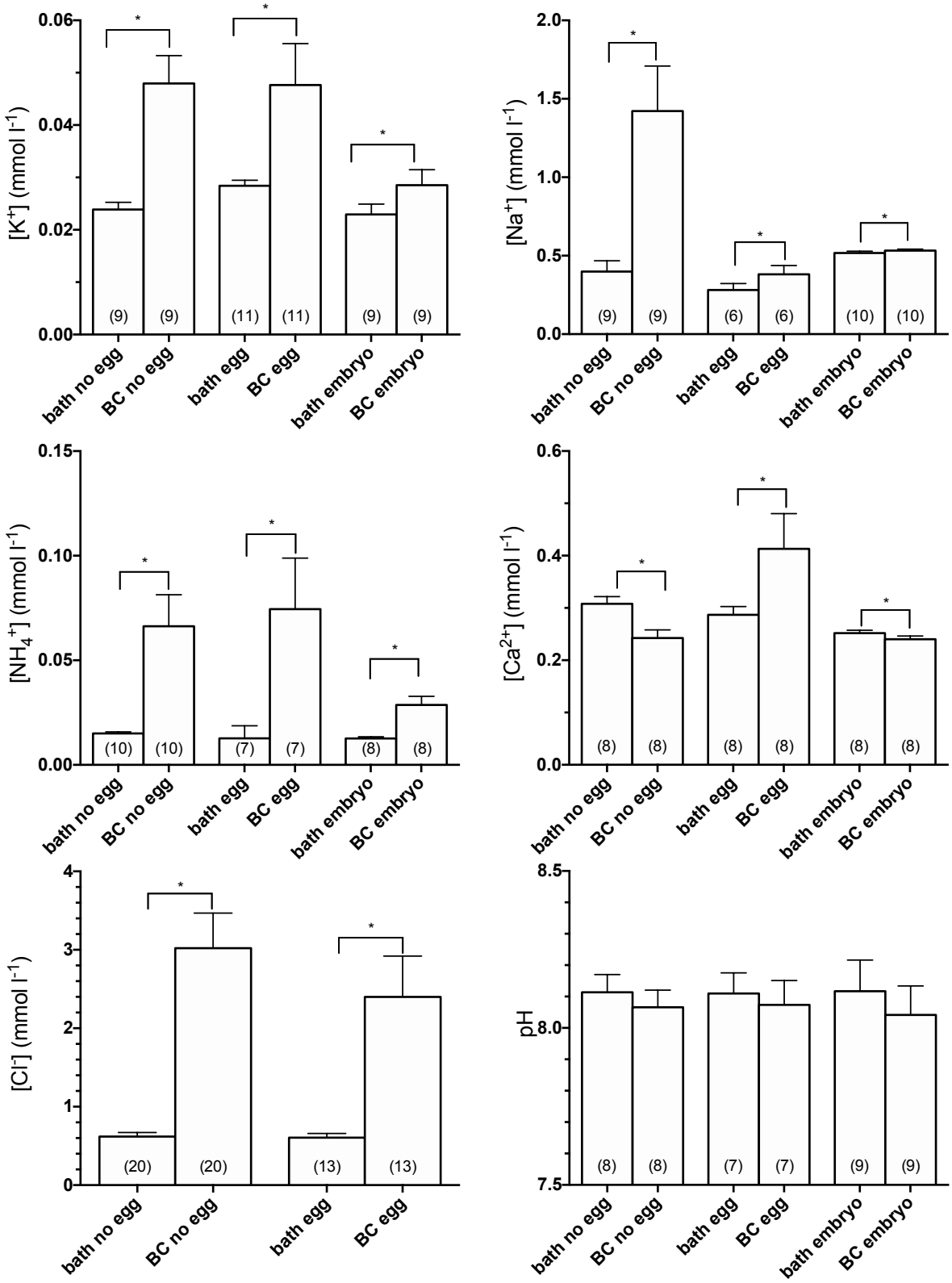
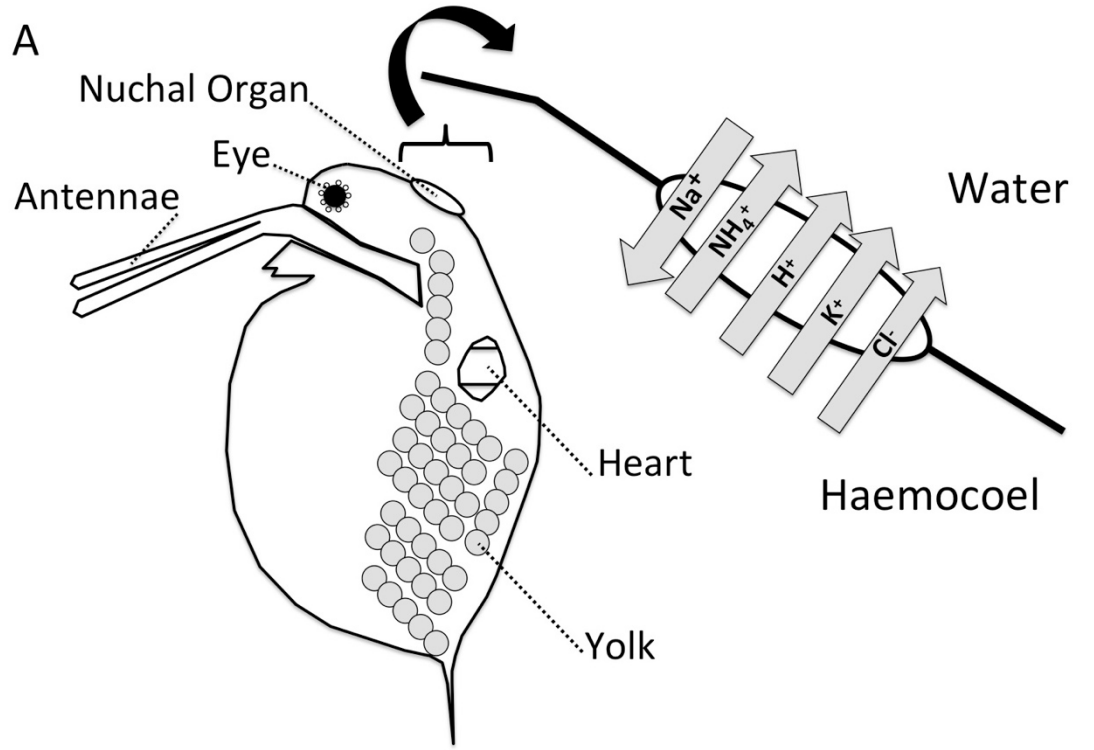

[K+] (mmol l-1)
0.06
0.04
0.02
0.00
(9) (9) (11) (11) (9) (9)
[Na+] (mmol l-1)
2.0
1.5
1.0
0.5
0.0
(9) (9) (6) (6) (10) (10)
[NH4+] (mmol l-1)
0.15
0.10
0.05
0.00
(10) (10) (7) (7) (8) (8)
[Ca2+] (mmol l-1)
0.6
0.4
0.2
0.0
(8) (8) (8) (8) (8) (8)
[Cl-] (mmol l-1)
4
3
2
1
0
(20) (20) (13) (13)
pH
8.5
8.0
7.5
(8) (8) (7) (7) (9) (9)
bath no egg
BC no egg
bath egg
BC egg
bath embryo
BC embryo

Figure 4-5. Ion concentrations and pH (mean ± s.e.m.) in the brood chamber (BC) of adult *Daphnia* with no eggs, with eggs, or with embryos. Ion concentrations and pH were measured in DHTW in the bath > 1 mm away from the *Daphnia* and compared with those measured when the ion-selective microelectrode tip was positioned within the brood chamber. Asterisks between bars linked by square brackets indicate significant differences in means as measured by a paired t-test. The numbers of animals in each condition are indicated within parentheses in each bar


A
Nuchal Organ
Eye
Antennae
Heart
Yolk
Water
Haemocoel
Na+
NH4+
H+
K+
Cl-

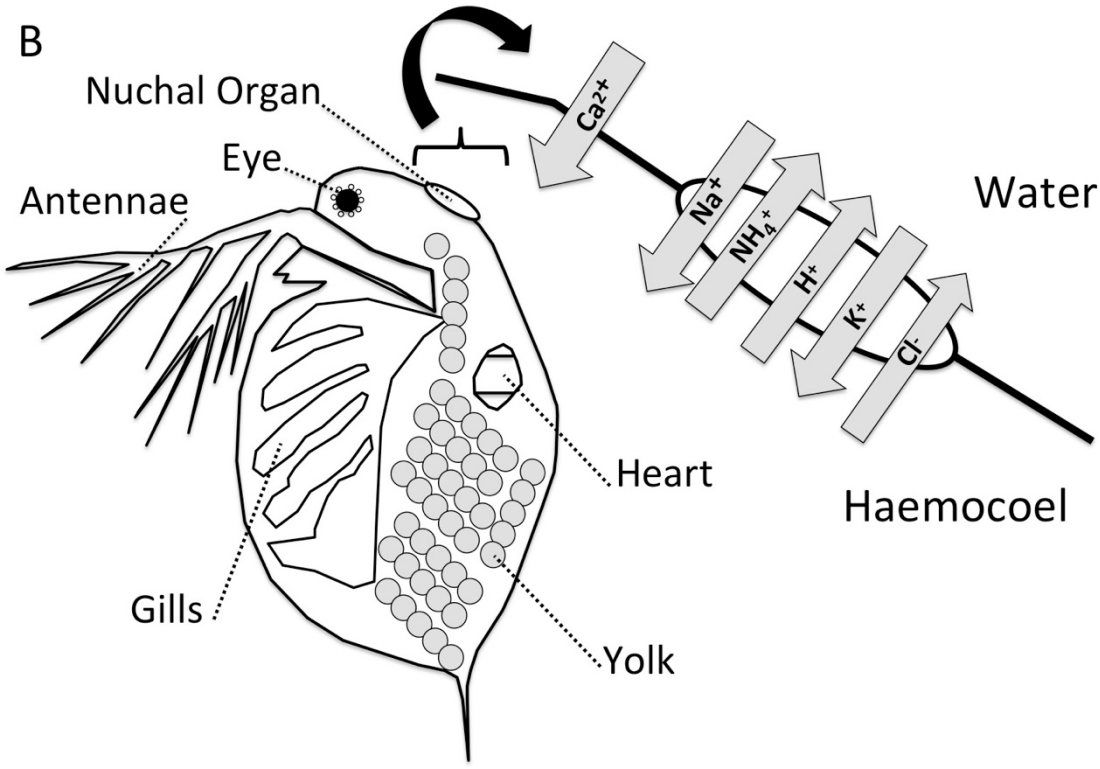

B
Nuchal Organ
Eye
Antennae
Gills
Heart
Yolk
Water
Haemocoel
Ca2+
Na+
NH4+
H+
K+
Cl-

Figure 4-6. Schematic diagram summarizing ion fluxes across the nuchal organ and body surface of *Daphnia magna* (A) embryos and (B) neonates. The nuchal organ is the site of influx of Na^+ and efflux of H^+, NH_4^+ and Cl^-. Transport of these ions across the carapace or dorsal ridge is negligible. The nuchal organ is also the site of K^+ efflux in embryos and K^+ influx in neonates. Ca^{2+} transport across the body surface and nuchal organ is negligible in embryos, but there is influx of Ca^{2+} across the body surface in neonates.

Supplementary Material

Table 1

Summary of Ion Fluxes at Sites Away From the Nuchal Organ

	Ion	Flux pmol cm^{-2} s^{-1} Mean ± s.e.m. (N)	**One-sample t-test
Embryos	Na^+	4.4 ± 4.0 (6)	N.S.*
	K^+	4.1 ± 2.3 (6)	N.S.
	Cl^-	3.2 ± 5.7 (6)	N.S.
	H^+	8.1 ± 2.0 (6)	P<0.05
	NH_4^+	1.5 ± 0.9 (6)	N.S
	Ca^{2+}	-5.2 ± 1.5 (7)	P<0.05
Neonates	Na^+	-22.4 ± 13.8 (6)	N.S.
	K^+	-0.5 ± 1.5 (6)	N.S.
	Cl^-	-2.0 ± 9.4 (6)	N.S.
	H^+	-4.7 ± 6.3 (6)	N.S.
	NH_4^+	1.0 ± 0.4 (6)	P<0.05
	Ca^{2+}	-27.7 ± 8.7	P<0.05

*N.S. not significant (P >0.05)

**One-sample t-tests were run to determine if the measured fluxes were significantly different from 0.

Supplementary Table 2

Michaelis-Menten kinetic analysis of Ca^{2+} influx at the nuchal organ or neonates.

	$[Ca^{2+}]$ mmol l^{-1}	Flux pmol cm^{-2} s^{-1}	K_m mmol l^{-1}	V_{max} pmol cm^{-2} s^{-1}	R^2
Neonate #1					
	0.042	2.57			
	0.098	-8.92			
	0.176	-14.47			
	0.314	-15.45			
	0.770	-24.34			
	1.294	-26.05	0.329	-33.6	0.91
Neonate #2	0.056	-25.62			
	0.100	-42.32			
	0.184	-59.22			
	0.428	-73.29			
	0.776	-68.83	0.098	-83.88	0.94
Neonate #3	0.036	-4.12			
	0.059	-10.38			
	0.112	-13.67			
	0.169	-13.02			
	0.312	-15.09	0.067	-19.05	0.84
Neonate #4	0.055	-34.42			
	0.103	-56.48			
	0.157	-68.21			
	0.242	-67.78			
	0.430	-79.38			
	0.630	-83.62			
	1.023	-86.51	0.076	-93.6	0.96
Neonate #5	0.044	-5.19			
	0.066	-18.46			
	0.108	-33.20			
	0.213	-46.38			
	0.366	-52.18			
	0.838	-58.95			
	1.481	-68.87	0.178	-76.21	0.95

Neonate #6	0.048	-26.42			
	0.091	-42.24			
	0.161	-57.65			
	0.271	-72.99			
	0.435	-80.13			
	0.810	-91.49			
	1.558	-94.30	0.131	-104.5	1.00
		MEAN	0.146	-68.5	0.93
		SEM	0.040	14.0	0.02

Chapter 5

GENERAL DISCUSSION

Summary of Findings

I investigated the effects of elevated ambient ion concentrations on ion regulation in adult *Daphnia magna* and the mechanisms of ion transport in juveniles. Three hypotheses were tested throughout this thesis, and the results of each test are first discussed below. I then integrate the results of these tests and associated findings to consider mechanisms of ionregulation for each of the major ions in *D. magna*.

Increased major ion concentrations will cause disturbances to ionoregulation and alter hemolymph ion concentrations

The results from chapter 2 confirm that increases in ambient ion concentrations of Na^+, K^+ and Cl^- disrupt ionoregulation as evidenced by elevated hemolymph ion concentrations. The extent of the increases in hemolymph ion concentrations correlate well with bath water concentrations. At concentrations approaching and exceeding previously published LC50 values, increases in hemolymph ion concentrations were irreversible or only partly reversible, suggesting a link between increased concentrations of ions in the hemolymph and physiological toxicity (Mount et al., 2016). Sublethal exposure to major ions results in reversible ionoregulatory disturbances, whereby hemolymph ion concentrations are restored over time. Regulation of hemolymph $[Na^+]$ is altered upon concomitant changes in water $[Ca^{2+}]$. Likewise, regulation of hemolymph $[K^+]$ is altered when there is a simultaneous change in ambient $[Na^+]$.

The mitigating effects of Na^+ on K^+ regulation and Ca^{2+} on Na^+ regulation are discussed further below.

Transepithelial potential responses will be altered upon exposure to increases in major ion concentrations in bathing water

Depolarization of TEP occurs in elevated ambient Na^+, K^+, Ca^{2+} and Mg^{2+} salts indicating the role of diffusional gradients in TEP regulation. Ion channels have been implicated in regulation of TEP based on salt exposure and specific blocker experiments (*e.g* K^+ and Cl^-). ATP dependent pumps likely contribute to TEP based the measured effects of metabolic inhibitors (chapter 3).

Ion transport occurs at the site of the nuchal organ in embryo and neonate D. magna

The transport of K^+, Na^+, H^+, Cl^-, NH_4^+ and Ca^{2+} was measured across the nuchal organ whereby influx of Na^+ and efflux of NH_4^+ and H^+ was observed. The direction of the flux of K^+ is specific to the developmental stage. An efflux of K^+ was observed in embryos, however, later in development, K^+ is taken up through the nuchal organ in neonates. Ca^{2+} measurements indicated that there is uptake in neonates but not embryos, although influx is not localized to the nuchal organ. We have demonstrated support for the role of the nuchal organ in ionoregulation, acid-base balance and nitrogenous waste excretion in juvenile *D. magna* (chapter 4).

Ionoregulation in D. magna

Freshwater daphnids are hyper-osmoregulators living in a hypo-osmotic medium. They have a wide variety of physiological adaptations to maintain homeostasis and combat passive ion loss to the external environment while minimising the expenditure of energy. Compared to osmoconformers, osmoregulators employ active ion uptake through the nuchal organ epithelia, or epipodite epithelia, have lower permeability of the body wall to both ions and water, decreased oral or anal drinking, and increased rate of urine production (Potts and Parry 1964; Rudy 1967, Fox 1952, Robertson 1960; Lockwood 1976, 1977; Mantel and Farmer 1983). Characterizing physiological responses to increased major ions in adults and ion transport in juveniles may aid in describing the mechanisms of major ion toxicity.

Na^+

Consistent with the results from chapter 2 and 3, the presence of Na^+ channels, Na^+/K^+ ATP-ase and (2)Na^+/H^+ exchangers have been previously suggested in adult *D. magna* (Bianchini and Wood, 2008). As ambient [Na^+] increases, approaching LC50 values (Mount et al., 2016) hemolymph [Na^+] rises well above control values (chapter 2). Na^+ builds up in the hemolymph as individuals are no longer able to cope with elevated ambient [Na^+]. TEP responses of hyperpolarization upon exposure to ouabain and depolarization upon exposure to 5 increasing concentrations of NaCl, Na_2SO_2 and $NaHCO_3$ were observed. Depolarization occurred when ATP dependent processes were inhibited by CN^- and [Na^+] was increased simultaneously. These results indicate that active Na^+ transport via Na^+/K^+ ATP-ase and diffusional gradients contribute to TEP in *D. magna* (chapter 3). Na^+/H^+ exchanger activity is suggested from the

results of experiments measuring hemolymph pH and TEP in response to increased $NaHCO_3$ (chapter 2, chapter 3).

In juveniles, direct Na^+ uptake through the nuchal organ was confirmed (chapter 4). Uptake is significant and complete overturn of Na^+ within the hemocoel can be achieved in ~37hrs. In neonates a proton pump-coupled Na^+ channel plays an important role in Na^+ uptake (Bianchini and Wood, 2008). This is consistent with our results of Na^+ influx and H^+ efflux through the nuchal organ. Future studies should focus on the effects of pharmacological blockers to confirm the role of Na^+ channels, Na^+/K^+ ATP-ase and Na^+/H^+ exchangers in hemolymph ionoregulation and TEP maintenance in adults and the role of a proton pump-coupled Na^+ channel in ion regulation in juveniles.

K^+

K^+ uptake in adult daphnids is likely through K^+ channels, Na^+/K^+ ATP-ase and $Na^+/K^+/2Cl^-$ exchangers (Bianchini and Wood, 2008). Daphnids exposed to elevated $[K^+]$, showed increases in hemolymph $[K^+]$ up to 3-fold above control levels (chapter 2). Consistent with diffusive influx of K^+, TEP increased when ATP-dependent processes were inhibited and ambient $[K^+]$ was increased. The same response was observed in rising concentrations of KCl, K_2SO_2 and $KHCO_3$. $BaCl_2$, a K^+ channel inhibitor, also caused a change in TEP response (chapter 3). As previously noted, ouabain altered the TEP measurements indicating Na^+/K^+ ATP-ase activity. These results did not confirm or negate the presence of a $Na^+/K^+/2Cl^-$ cotransporter.

In embryos there is an efflux of K^+ through the nuchal organ reflecting the formation of extracellular space from intracellular space. As embryos develop into neonates, there is a coincident influx of K^+ possibly reflecting the expansion of intracellular space during development of tissues such as the gills, gut and epidermal cells. K^+ transport in juveniles is

suspected to be through K^+ channels and Na^+/K^+ ATP-ase as they have a Na^+/Cl^- exchanger rather than a $Na^+/K^+/2Cl^-$ cotransporter (Bianchini and Wood, 2008).

Cl^-

In increased ambient Cl^-, hemolymph [Cl^-] rose 1.2 times higher than control levels. Depolarization of TEP occurred in increased [Cl^-] and these effects remained when metabolism was inhibited with CN^-. In 1mM of DIDs, a $Cl^-/(2)HCO_3^-$ exchanger inhibitor and 1mM DPC, a Cl^- channel blocker that may also block $Cl^-/(2)HCO_3^-$ exchangers (Reuss et al., 1987), TEP became more negative. These results are unexpected when considering a Cl^- diffusion gradient, or a $Na^+/K^+/2Cl^-$ cotransporter but were consistent with reported TEP in perfused, isolated *Uca tangeri* gills (Drews and Graszynski, 1987). In chapter 3 an electrogenic $Cl^-/2HCO_3^-$ exchanger and likely Cl^- channels are proposed to play a role in Cl^- regulation and establishment of the TEP in adult *D. magna.*

In juveniles Cl^- moves through specific channels and Na^+/Cl^- exchangers (Bianchini and Wood, 2008). An efflux rather than an influx of Cl^- across the nuchal organ was observed and unexpected. This can be attributed to an anion surplus from high levels of HCO_3^- (Weber and Pirow, 2009), and negatively charged amino acids, peptides and proteins leading to an efflux of Cl^- across the nuchal organ.

Ca^{2+}

Dissolved Ca^{2+} is taken up from the environment and is very important in juvenile and adult *D. magna* as their carapace is largely reinforced by calcium salts (Tan and Wang, 2009). Particular transport mechanisms have yet to be described (Giardini et al., 2015) although electrogenic, active influx of Ca^{2+} in crayfish gills has been suggested (Kirschner, 1994).

Previous studies have shown that Ca^{2+} decreases diffusive permeability and differentially decreases Na^+ permeability relative to Cl^- permeability in freshwater fish as well as *D. magna* (Pane et al., 2003). Depolarization of TEP in response to increased $CaCl_2$ and $CaSO_4$ concentrations suggest that diffusional gradients of Ca^{2+} also contribute to TEP. Measurements of Ca^{2+} transport in embryos at the nuchal organ and body surface was negligible while Ca^{2+} influx in neonates was noted but was not localized to the nuchal organ. Daphnids are efficient at Ca^{2+} uptake because of the need to calcify the cuticle during development and post-moult.

NH_4^+

Increases in ambient [NH_4^+] did not alter TEP (chapter 3). Given that ammonia excretion in adults is increased at low ambient pH relative to the rate of excretion at neutral pH future studies should measure ammonia excretion in response to increased major ion concentrations (Al-Reasi et al., 2013). The nuchal organ, like many other ion transporting epithelia such as the fish gill and the anal papilla in mosquitos, plays a role in nitrogenous waste excretion (Donini and O'Donnell, 2005; Evans et al., 2005; Wright and Wood, 2009). The breakdown of protein from yolk granules into amino acids for energy production may explain the large efflux of NH_4^+ across the nuchal organ that was observed in both embryos and neonates (chapter 4).

Binary salts and ionoregulation in adult D. magna.

Previous studies have shown that some cations can protect against toxicity caused by other cations, for example an increase in [Ca^{2+}] in the water has been shown to mitigate Na^+ toxicity and increased [Na^+] in the water mitigates K^+ toxicity (Mount et al., 2016).

The effect of Ca^{2+} on Na^{+} regulation

The relationship between Ca^{2+} and Na^{+} is complex and an increase in water [Ca^{2+}] increases the LC50 value of NaCl (Mount et al., 2016). Previous findings show that Na^{+} uptake is decreased in low [Ca^{2+}] which is consistent with the findings in chapter 2. Low [Ca^{2+}] is not associated with competitive interactions between Na^{+} and H^{+}, whereas high [Ca^{2+}] is associated with a competitive reaction between Na^{+} and H^{+} (Glover and Wood, 2005; Havas et al., 1984). The mortality observed in low [Ca^{2+}] may be due to decreased Na^{+} influx, insufficient to compensate for the high rate of Na^{+} depletion through passive ion loss to the external environment. Consistent with mitigation of Na^{+} toxicity by Ca^{2+}, a coincident increase in Ca^{2+} and Na^{+} mitigates the increase in hemolymph [Na^{+}] (chapter 2) (Mount et al., 2016).

The results from Ca^{2+}/ Na^{+} experiments measuring TEP (appendix, figure 1A-C) are complex and potentially conflicting. TEP was measured in response to an increasing series of [Na^{+}] with 0.04mM Ca^{2+} in each test solution, ~25 times less than in DHTW. There was no correlation between TEP and increased [Na^{+}] in water containing 0.04mM Ca^{2+} or 0.4mM Ca^{2+}. In low [Ca^{2+}], the effect of [Na^{+}] on TEP seen in DHTW (1mM Ca^{2+}) was lost. [Ca^{2+}] appears to influence the permeability of Na^{+}, possibly affecting the paracellular pathway (Kirschner, 1994; Wheatly, 1999). It has been previously described that Na^{+} uptake is dependent on ambient [Ca^{2+}] and pH in *D. magna* (Glover and Wood, 2005). The results are consistent with Na^{+} uptake being affected by altered [Ca^{2+}] as changes in ambient [Ca^{2+}] changed how TEP responded to increased [Na^{+}]. In water containing 4mM Ca^{2+}, a positive correlation was seen between TEP and [Na^{+}]. The slope of 3.0mV/decade change in [Na^{+}] compared to 11.01mV/decade change in [Na^{+}] in DHTW (1mM Ca^{2+}), indicates that there is a lower relative permeability to Na^{+} in water containing 4mM Ca^{2+}. These results are thus consistent with increased [Ca^{2+}] being protective

over Na^+ toxicity (Mount et al., 2016), in that in higher ambient [Ca^{2+}], increased [Na^+] has less effect on TEP (appendix, figure 1A-C).

The effect of Na^+ on K^+ regulation

An increase in [K^+], up to 10mM, with a simultaneous increase in water [Na^+] mitigates the increase in hemolymph [K^+], a finding which is consistent with mitigation of K^+ toxicity by Na^+ (Mount et al., 2016). It appears that when ambient [Na^+] is increased, KCl exposure is less of a challenge to hemolymph K^+ homeostasis. In 20mM KCl and 10mM NaCl there is an increase in both hemolymph [Na^+] and hemolymph [K^+] suggesting a link between regulation of hemolymph [Na^+] and hemolymph [K^+]. Membrane transporters linking the transport of Na^+ and K^+ have been suggested to contribute to ion regulation in adult *D. magna* including Na^+/K^+ ATP-ase and $Na^+/K^+/2Cl^-$ (Bianchini and Wood, 2008).

The slope of the relationship between TEP and [K^+] is significantly lower in 5 increasing [K^+] with an additional 10mM Na^+ (1.36 mV/decade) than in the same 5 increasing [K^+] with no added Na^+ (3 mV/decade) (appendix figure 1D). The lower slope is thus evidence of reduced K^+ permeability in the presence of 10mM Na^+ and is consistent with the protective effect of Na^+ on the toxicity of K^+ (Mount et al., 2016).

The relationships between Na^+/Ca^{2+} and K^+/Na^+ are complex and further experiments are required to fully comprehend how binary salts interact and influence physiological responses to increased major ion concentrations.

The Effect of the Conjugate Anion in Major Ion Toxicity

Although there have been conflicting descriptions of the role of conjugate anions in major ion toxicity, different LC50 values have been reported for salts sharing the same cation paired with different anions (Mount et al., 1997; Mount et al., 2016). The results from chapter 2 and 3 suggest that while physiological disturbances caused by major ions may be cation dominated, conjugate anions do play a significant role based on both the changes in hemolymph ion concentrations and TEP responses. Animals exposed to 30mM NaCl for 24hrs showed a 1.2-fold increase in Cl^- and a 1.5-fold increase in Na^+ (chapter 2), demonstrating that although the rise hemolymph [cation] is greater than the rise in hemolymph [anion], anion hemolymph regulation is likewise disturbed. The anion species altered both the time course and extent of changes in hemolymph [Na^+] when *D. magna* were exposed to Na^+ salts (Cl^-, HCO_3^- and SO_4^-) (chapter 2). Testing the protective effect of Ca^{2+} on the elevation of hemolymph [Na^+] revealed that the anion associated with Na^+ changed the extent of the elevation of hemolymph [Na^+]. The TEP response for a 10-fold concentration change was significantly different between the three Na^+ salts (Cl^-, HCO_3^- and SO_4^-) tested (chapter 3). The TEP response for K^+ and Mg^{2+} salts were also found to be significantly different between their respective anion groups (*i.e.* between Cl^-, HCO_3^- and SO_4^-). The effect of increased [Cl^-] on TEP response was unexpected. If Cl^- diffusional channels were the primary contributor to TEP the expected result from an increase in external [Cl^-] would be hyperpolarization rather than depolarization. As $Na^+/K^+/2Cl^-$ and K^+/Cl^- exchangers are neutral, Cl^- is likely contributing to TEP through an electrogenic $Cl^-/2HCO_3^-$

exchanger, which is consistent with the anion exchangers (*e.g.* $Cl^-/(2)HCO_3^-$) that have been extensively described in freshwater crustaceans (Genovese et al., 2005; Lucu, 1990; Onken et al., 1991; Onken et al., 2000). The discrepancies between the change in hemolymph ion concentration and TEP response for salts with the same cation but different conjugate anions could reflect the contribution of that anion.

Acid-Base Regulation in D. magna

Acid-base regulation in *D. magna* appears to be complex and the results from chapter 2, 3 and 4 do not demonstrate a clear association between pH and the actions of one particular ion transporter. There are likely other mechanisms and combination of ion transporters working to establish and maintain acid-base balance. Cation exchangers (e.g. K^+/H^+ exchanger and Na^+/H^+ exchangers) and anion exchangers (e.g. Cl^-/HCO_3^-) are the primary transporters regulating acid-base balance in aquatic crustaceans (Genovese et al., 2005; Lucu, 1990; Onken et al., 1991). There are likely three primary exchangers working to maintain acid-base balance in adult *D. magna*; $Cl^-/2HCO_3^-$, K^+/H^+ and Na^+/H^+ exchangers (chapter 2, chapter 3).

A $Cl^-/2HCO_3^-$ exchanger is likely contributing to the maintenance of acid-base balance in *D. magna* but not contributing to the rise in pH in animals exposed to 10mM KCl as there is no change in hemolymph $[Cl^-]$. In 30mM NaCl the decrease in pH at 24hrs could be attributed to greater uptake of Cl^-, as noted by an increase in hemolymph $[Cl^-]$ and in turn an efflux of HCO_3^- potentially through an electrogenic $Cl^-/2HCO_3^-$ exchanger (chapter 2). The response of a $Cl^-/2HCO_3^-$ exchanger to an 20mM increase in $[Cl^-]$ lends explanation to the decrease in hemolymph pH at 24hrs in 30mM NaCl that was not seen at 24hrs in 10mM KCl. Changes in TEP were larger when Cl^- was the associated anion. TEP responses in increased $[HCO_3^-]$ are likely influenced by the change in pH, therefore, the transporters contributing to TEP in these

experiments maybe those involved in acid-base balance. An increase in water [HCO_3^-] from either $KHCO_3$ or $NaHCO_3$ would lead to decreased efflux of HCO_3^- through an electrogenic $Cl^-/2HCO_3^-$ exchanger, causing TEP to shift negative. However, TEP responses are complicated by the activity of other exchangers.

The activity of a K^+/H^+ exchanger is supported by hemolymph pH measurements in daphnids exposed to 10mM KCl (chapter 2) and TEP measurements in increasing [$KHCO_3$] (chapter 3). A K^+/H^+ exchanger is indicated as individuals exposed to 10mM KCl had higher hemolymph [K^+] and lower hemolymph [H^+], potentially contributing to the observed increase in internal pH. The pattern of pH change does not exactly parallel the time course changes in hemolymph concentrations of K^+ or Cl^-, suggesting that there may be other mechanisms working to regulate internal pH and that hemolymph pH regulation is likely prioritized over K^+ hemolymph regulation. The TEP response of hyperpolarization in increasing [$KHCO_3$] is likely due to electrogenic $Cl^-/2HCO_3^-$ and electroneutral K^+/H^+ exchangers resulting in net inside negative potentials (appendix figure 2A). Increases in ambient [Na^+] have complex effects on *D. magna.* As in many other invertebrates, it has been suggested that Na^+uptake can be through an electroneutral Na^+/H^+ exchange as well as an electrogenic $2Na^+/1H^+$ exchange (Glover and Wood, 2005). In 30mM NaCl, a sustained increase in hemolymph [Na^+] and [Cl^-] was observed and hemolymph pH stayed within control levels with a significant decrease at 24hr. Na^+ uptake is saturable, and uptake is slowed when saturation is approaching. Hemolymph [Na^+] elevations therefore reduce the activity of Na^+/H^+ exchangers, which transport Na^+ into the cells and H^+ out, such that H^+ may accumulate inside the animal decreasing hemolymph pH (Glover and Wood, 2005). TEP in $NaHCO_3$ becomes more positive, rather than negative as seen in $KHCO_3$, possibly reflecting the contribution of electrogenic ($Cl^-/2HCO_3^-$, $2Na^+/H^+$) versus electroneutral (K^+/H^+,

Na^+/H^+) exchangers (appendix figure 2B). The difference in TEP responses in $KHCO_3$ and $NaHCO_3$ may also be due to the effects of the changes in hemolymph pH on ion channels or paracellular conductance, and these effects may be different for Na^+ versus K^+.

Freshwater animals are sensitive to changes in pH, specifically aquatic acidification which has caused the disappearance of fish, molluscs and crustaceans (Leivestad and Muniz, 1976). The breakdown of Na^+ or K^+ regulation through K^+/H^+, Na^+/H^+ exchangers may be the mechanism of these mortalities (Vangenechten et al., 1989, Wood, 1989). Overall, my results do not show a clear association between pH regulation and hemolymph ion concentration or TEP response and are not consistent with a single mechanism of pH regulation but rather a combination of exchangers. One explanation is that *D. magna* prioritize the regulation of hemolymph pH even if this entails a rise of hemolymph ion concentrations.

In juveniles, the nuchal organ is involved in acid-base balance (chapter 4). The large efflux of H^+ across the nuchal organ could indicate the activity of V-ATP-ase coupled Na^+ uptake, a Na^+/H^+ exchanger or carbonic anhydrase activity which would hydrate metabolic CO_2 passing out through the nuchal organ.

Life history and provisioning of ions

The primary ionoregulatory organs of adult daphnids (maxillary gland, gut and epiopodites) are different from those of juveniles who use the nuchal organ. Juvenile daphnids used in this thesis to investigate ionoregulaory mechanisms were produced through parthenogenesis, whereby eggs are released into the brood chamber, develop as embryos and emerge as free swimming neonates (Mittmann et al., 2014). The nuchal organ, like the epipodites

have mitochondrion-rich ion transporting cells involved in osmoregulation. Unlike, *Monia brachiate, Polyphemus pediculus,* and *Bythotrephes longimanus* which have a closed brood chamber, daphnids have open brood chambers. They are generally unable to nourish embryos by secretion of nutrients into the marsupial liquid making the development of the nuchal organ significant (Aladin and Potts, 1995). However, ion measurements in empty, egg occupied, and embryo occupied brood chambers suggest maternal provisioning of some ions may be occurring (chapter 4). Ions released from the female lends explanation to elevated K^+, Na^+ and Cl^- in the empty brood chamber with respect to the bathwater. Lower Ca^{2+} in the empty brood chamber may be due to Ca^{2+} influx. The open brood chamber is in contact with the external water and ion concentrations could reflect the combined effects of diffusive exchange with the environment, ions released from the female, and uptake or release by the egg or embryo. Although it is suspected that the egg is impermeable to ions, increases in the concentrations of Na^+, K^+, Cl^- and Ca^{2+} in the egg occupied brood chamber could reduce passive ion loss from the egg itself. Consistent with our findings of complex patterns of Ca^{2+} within the brood chamber, a previous study has suggested maternal provisioning of Ca^{2+} based on the ability to trace maternal ^{45}Ca to embryos (Giardini et al., 2015). It is possible that while the egg itself may not be permeable, Ca^{2+} uptake is sustained in the ionic and hormonal milieu within the brood chamber.

Future Directions

I set out to determine the effects of increased major ion concentrations in ambient water on hemolymph ion concentrations and TEP in adult *Daphnia magna*. Additionally, I investigated ion flux through the nuchal organ in juvenile *D. magna.* While the results from chapter 2 and 3 provide insight into the physiological responses that may correlate to toxicity and physiological

measurements to support the future development of EPRI's MIT model, it has highlighted uncertainties that should be the focus of future studies. The results from chapter 4 confirm ion transport through the nuchal organ in juvenile *D. magna* with the first measurements of near real time ion-flux.

Future hemolymph experiments could examine the effects of ion transport inhibitors and increased major ion concentrations. This will aid in determining the transporters involved in responding to the osmoregulatory challenge of increased major ions and how this affects hemolymph ion concentrations. Additionally, the effect of pH on Na^+ and K^+ hemolymph concentrations would give further information on the complex mechanism of acid-base balance in *D. magna.*

The results of chapter 3 reveal the differences between TEP regulation in *D. magna* and freshwater fish. Previous studies have identified differences in ion transporters that are functionally similar (*e.g*. $H^+/2Na^+$ (or Ca^{2+})-exchanger in crustaceans differs from Na^+/H^+-exchanger in teleost fish) (Griffith et al., 2012). Our findings suggest that freshwater fish and daphnids regulate TEP quite differently. The mechanisms of TEP regulation in daphnids more closely relates to those in saltwater fish. TEP in freshwater fish is entirely a diffusion potential whereas both ATP dependent pumps and diffusion potential contribute to TEP in saltwater fish and freshwater daphnids. This difference between freshwater fish and daphnids may challenge the principles that regulatory models such as the EPRI MIT model is built on. As suggested with future hemolymph studies, an important next step would be TEP experiments that focus on identification of ion transporters that contribute to TEP and are affected by increased major ions. Additional ion transporter blockers could be used to confirm transporters contributing to TEP, for example phenamil and ethyl isopropyl amiloride (EIPA) may indicate the diffusive transport

of Na^+ through Na^+ channels and Na^+/ H^+ exchangers, respectively. Likewise, further investigation of acid-base balance using TEP measurements in solutions of NaCl, KCl, $NaHCO_3$ and $KHCO_3$ in varying pH would be beneficial. Changing the pH of the bathing solution would alter the TEP response, reflecting the change in activity of the transporters. For example, TEP measured in the previously tested 5 [Na^+] in low pH rather than a neutral pH would likely be less positive. A broader scope of binary salt experiments may clarify the relationship between ambient Na^+/Ca^{2+} and K^+/Na^+ and physiological disturbances. Our hemolymph and TEP measurements provide the first physiological measurements of these end points in *D. magna.*

Future studies of the nuchal organ should also focus on determining, through pharmacological blockers, the ion transporters involved in ion flux through the nuchal organ. Further assessment of the role of Ca^{2+} and the influence of water chemistry (pH, HCO_3^-, hardness) and temperature may be relevant in considering possible impacts of freshwater acidification resulting from anthropogenic increases in atmospheric CO_2. As juveniles are more sensitive than adult *D. magna* it would be valuable to measure ion flux through the nuchal organ while exposing juveniles to increased major ion concentrations and changes in ambient P_{CO2} to determine how ion transport is affected during osmoregulatory stress.

Evaluating the physiological responses to increased ambient ion concentrations and mechanisms of major ion toxicity, including the ion transporters involved, furthers the understanding of ionoregulatory mechanisms and the integration of such knowledge into regulating pollution by major ions. Predictive models such as the EPRI MIT model will aid in establishing environmental regulations for major ions in aquatic ecosystems, but development of these models requires extensive physiological data.

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APPENDIX

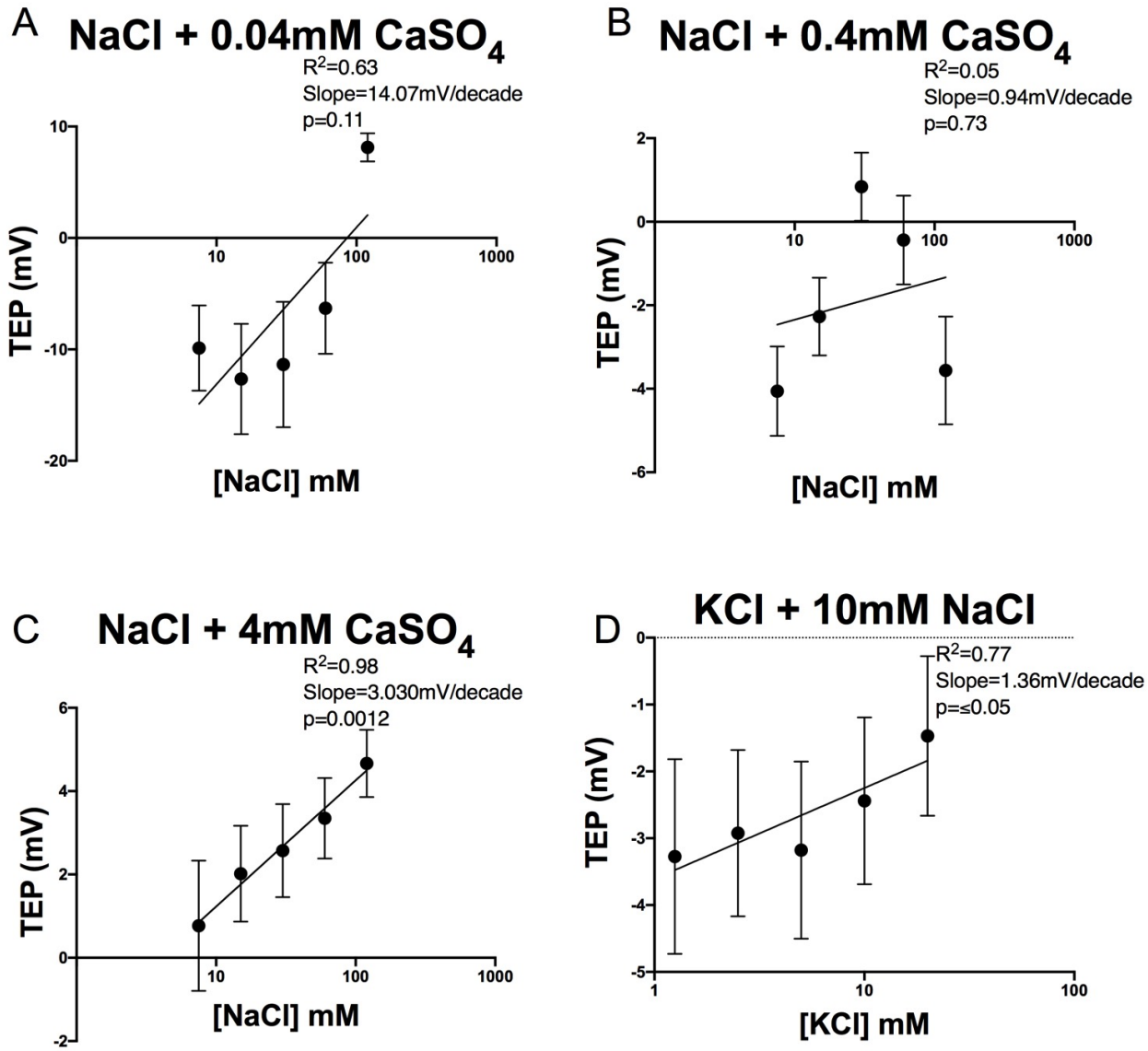
A
NaCl + 0.04mM CaSO4
R2=0.63
Slope=14.07mV/decade
p=0.11
TEP (mV)
[NaCl] mM
B
NaCl + 0.4mM CaSO4
R2=0.05
Slope=0.94mV/decade
p=0.73
TEP (mV)
[NaCl] mM
C
NaCl + 4mM CaSO4
R2=0.98
Slope=3.030mV/decade
p=0.0012
TEP (mV)
[NaCl] mM
D
KCl + 10mM NaCl
R2=0.77
Slope=1.36mV/decade
p=≤0.05
TEP (mV)
[KCl] mM

Figure 5-1. The effects of binary salt solutions on TEP in *D. magna.* Logarithmic scale with linear regression, significant correlation indicated by $p \leq 0.05$ (A) 7.5mM NaCl + 0.04mM Ca_2SO_4, 15mM NaCl + 0.04mM Ca_2SO_4, 30mM NaCl + 0.04mM Ca_2SO_4, 60mM NaCl + 0.04mM Ca_2SO_4, 120mM NaCl + 0.04mM Ca_2SO_4. N=7. (B) 7.5mM NaCl + 0.4mM Ca_2SO_4, 15mM NaCl + 0.4mM Ca_2SO_4, 30mM NaCl + 0.4mM Ca_2SO_4, 60mM NaCl + 0.4mM Ca_2SO_4, 120mM NaCl + 0.4mM Ca_2SO_4. N=6. (C) 7.5mM NaCl + 4mM Ca_2SO_4, 15mM NaCl + 4mM Ca_2SO_4, 30mM NaCl + 4mM Ca_2SO_4, 60mM NaCl + 4mM Ca_2SO_4, 120mM NaCl + 4mM Ca_2SO_4. N=6. (D) 1.25mM KCl + 10mM NaCl, 2.5mM KCl + 10mM NaCl, 5mM KCl + 10mM NaCl, 10mM KCl + 10mM NaCl, 20mM KCl + 10mM NaCl. N=6.

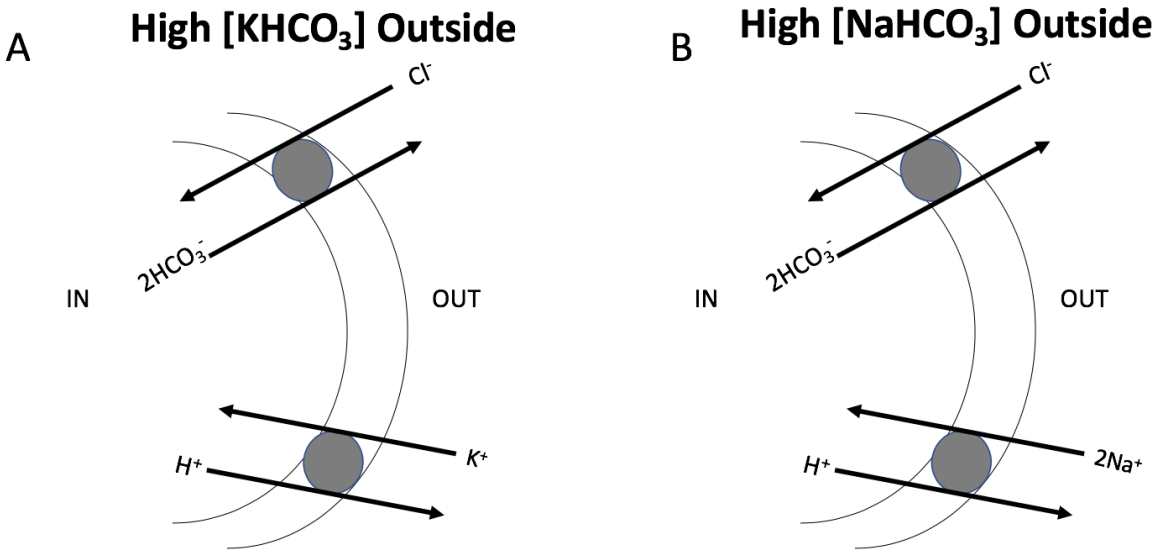
A
High [KHCO3] Outside
Cl-
2HCO3-
IN
OUT
H+
K+
B
High [NaHCO3] Outside
Cl-
2HCO3-
IN
OUT
H+
2Na+

Figure 5-2. Schema of potential transporters contributing to TEP response in high [$KHCO_3$] and high [$NaHCO_3$]. (A) In high [$KHCO_3$] the reduction in $Cl/2HCO_3^-$ removes depolarization from outward movement of negative charge, so TEP shifts negative. (B) In high [$NaHCO_3$] the reduction in $Cl/2HCO_3^-$ removes depolarization from outward movement of negative charge, but 2Na+/H exchange is still depolarizing, so TEP shifts positive.