UNDERSTANDING THE EFFECTS OF OSMOREGULATORY STRESS ON ION AND WATER TRANSPORT IN SELECT TERRESTRIAL AND AQUATIC ARTHROPODS

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Abstract

Arthropods including insects and crustaceans inhabit a wide range of environmental habitats and thus require fine-tuned mechanisms for ion and water balance (osmoregulation). Terrestrial insects face dehydration while freshwater insects face dilution of body fluids; both scenarios present challenges to osmoregulatory homeostasis. Adult female mosquitoes must deal with a salt and water load when blood feeding, while aquatic larval midges face a salt load from salinization of freshwater habitats. This research examined how transmembrane water channels known as aquaporins (AQPs) are involved in osmoregulation when arthropods face these challenges. The thesis focused on (1) AQP protein localization and abundance in adult mosquitoes (*Aedes aegypti*); (2) the effect of neurohormones on a specific AQP (AaAQP1) in mosquitoes; (3) cloning and characterization of a novel AQP from midge (*Chironomus riparius*) larvae; and (4) effects of road de-icers on osmoregulation in two freshwater arthropods, a crustacean (*Hyalella azteca*) and *C. riparius* larvae. In mosquitoes, AaAQP1 was localized to the apical membrane of the Malpighian tubules (MTs) while blood feeding had no effect on AaAQP1 localization or abundance. However, AaAQP1 proved to be vital for diuretic action of MTs. Starvation reduced AaAQP1 and AaAQP4 (another *Ae. aegypti* AQP) protein abundance in the male MTs, relative to sugar-fed males. In midge larvae, CrAQP2 expression was abundant in the MTs relative to other osmoregulatory organs and was salinity responsive in anal papillae and midgut. Knockdown of CrAQP2 expression resulted in dehydration of larval midges and survival decreased following 72-96hr de-icer exposure. Commercial de-icer containing beet juice caused profound changes in osmoregulatory parameters of *H. azteca*, including decreased activity of major enzymatic ion-transporters proving to be as harmful to freshwater amphipods as traditional de-icers. Overall, this thesis presents two significant advances in the field. Firstly, the characterization of AaAQP1 in the

disease vector *Ae. aegypti* revealing a previously unknown role for an AQP in the diuretic and anti-diuretic hormone control of primary urine production. Secondly, this research uncovered the deleterious impact of a beet-juice based de-icer on the osmoregulatory physiology of representatives from two important aquatic arthropods, the larval insect *C. riparius* and the amphipod *H. azteca*.

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List of Abbreviations

AB – abdomen

ADB – antibody dilution buffer

AFW – artificial freshwater

AP – anal papillae

AQP(s) – aquaporin(s)

AaAQP – *Aedes aegypti* AQP

BB – brush border

BBJD – brine beet juice de-icer

βLac - β-lactamase

BSA – bovine serum albumin

cAMP – cyclic adenosine monophosphate

cDNA – complimentary deoxyribonucleic acid

CrAQP2 – *Chironomus riparius* AQP 2

DAPI – 4', 6-diamidino-2-phenylindole

ddH_2O – double distilled water

DH_{31} – diuretic hormone 31

DIG – digoxygenin

DMEM – Dulbecco's modified eagles medium

DNA – deoxyribonucleic acid

DRIP – *Drosophila* integral protein

dsRNA – double stranded RNA

ECL – enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

Eglp – entomoglyceroporin

EP – epidermis

FB – fat body

FG – foregut

FISH – fluorescence *in situ* hybridization

FSR – fluid secretion rate

FW – freshwater

GC – gastric caeca

HEK – human embryonic kidney

HG – hindgut

HRP – horseradish peroxidase

5HT – 5-hydroxytryptamine (serotonin)

Hyb – hybridization solution

IHC – immunohistochemistry

ISME – ion-selective microelectrodes

kDa – kilodalton

LB – Luria-Bertani agar

M – mitochondria

MG – midgut

MNP – mosquito natriuretic peptide

mRNA – messenger RNA

MT(s) – Malpighian tubule(s)

MV – microvilli

NBF – non-blood fed

NKA – Na^+/K^+-ATPase

ORF – open reading frame

OV – ovaries

PBM – post-blood meal

PBS – phosphate-buffered saline

PBT – PBS with Tween-20®

PBTB – PBS with Tween-20® and blocking solution

PCR – polymerase chain reaction

PFA – paraformaldehyde

PRIP – *Pyrocoelia rufa* integral protein

PVC – polyvinyl chloride

RACE – rapid amplification of cDNA ends

RBB – reduced brush border

RIPA – radioimmunoprecipitation assay

RNA – ribonucleic acid

RP – rectal pads

RT – room temperature

SCW – salt contaminated water

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – standard error of the mean

TBST – Tris-buffered saline with Tween-20®

TEM – transmission electron miscroscopy

TS – testes

UTR – untranslated region

VA – Vacuolar-type H^+-ATPase

VD – Ventral diverticulum

WB – whole body

X-GAL – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

YPP – yolk precursor protein

Statement of Contributions

Chapter One

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Chapter One

Overview

1.1 Osmoregulation in Invertebrates

In nature, animals are typically known as osmoregulators where they activiely maintain an internal osmolarity that's different form their external environment or osmoconformers who's internal water and salt levels matches the external osmotic pressure. The process of osmoregulation is vital to osmoregulating animals, as it ensures the balance of water and ions in individual cells and bodily fluids as a whole. More importantly, the active transport of ions across cell membranes is the driving force for the movement of water and thus the coordinated movement of ions and water together are essential to the proper functioning of every eukaryotic cell (Bradley, 2009). Invertebrate animals are extremely diverse and with that comes a broad variety of ecosystems they inhabit and lifestyles they practice. Invertebrates constitute the majority of animal species and can inhabit a wide range of environments such as aquatic, semi-aquatic, and terrestrial. In particular, the phylum Arthropoda constitutes the majority of invertebrates on Earth and includes the most plentiful groups known as insects, as well as crustaceans, spiders, centipedes, and millipedes (Pisani, 2009). Arthropods are ubiquitously dispersed and occupy a variety of aquatic and terrestrial habitats. Therefore, the differential osmoregulatory challenges that each Arthropod faces is heavily dependent on the environment they reside in.

1.1.1 Terrestrial Osmoregulatory Strategies

The most abundant terrestrial Arthropods consist of insects and thus provide a prime example of the terrestrial osmoregulatory strategies. A major distinction in evolution from before and after terrestrial domination was the ability for air breathing on land, as well as the acquisition of water on land (Takei, 2015). Terrestrial animals including insects face a regular threat to desiccation and therefore the retention of water is a primary focus for life on land. A particularly

unique challenge is faced by insects due to their high surface area to volume ratio, which heavily favours water loss to the environment (Noble-Nesbiti, 1990). However, they have successfully adapted to desiccation stress and, in doing so, possess a hardened waxy cuticle that provides protection and a hydrophobic barrier to the external environment (Chown et al., 2011). In addition, they also possess a specific set of osmoregulatory organs that are designed to favour ion secretion while maintaining adequate water levels in their haemolymph (Halberg & Denholm, 2024). Furthermore, the process of increased or decreased urine production to remove excess ions or water is controlled by various hormone signalling pathways that have been extensively studied over the years (Halberg & Denholm, 2024). Although, insects are not the only Arthropods that have found success on land and developed important strategies to mitigate desiccation stress. For example, talitridean amphipods are a subset of terrestrial Crustaceans that typically live on sandy beaches and in areas of fluctuating tide (Herkul et al., 2006). These terrestrial amphipods are reliant on coastal regions because they absorb water through their skin on the underside of their bodies from moist environments (Morritt & Richardson, 2000). In this manner, they also face a lethal threat to desiccation, as a dry environment would result in a rapid loss of water through their skin and eventually lead to death. Although understudied, it has been shown that terrestrial amphipods have developed intricate ways of sequestering ions without the direct link to osmotically obliged water (Wright et al., 1997). Overall, terrestrial Arthropods have thrived on land due to their ability to conserve water in a variety of ways.

1.1.2 Aquatic Osmoregulatory Strategies

In addition to Arthropods finding success on land, there are also an abundance of insects and crustaceans that inhabit aquatic environments. Aquatic environments are osmotically

challenging because they require fine-tuned regulation of ion and water balance that is now based on a potentially unstable external osmolality. To begin, freshwater habitats are home to a diverse variety of insects and crustaceans that are subject to environmental, temperature, and salinity changes (Dijkstra et al., 2014). With that, freshwater (FW) invertebrates face an osmoregulatory challenge that is opposite to their terrestrial counterparts; with their internal osmolality being greater than that of their surrounding environment, the threat to cellular dilution is large (Halberg & Denholm, 2024). For FW insects such as the mosquito larvae of *Aedes aegypti*, the midge larvae of *Chironomus riparius*, or the mayfly larvae of *Hexagenia rigida*, they must work to actively obtain ions from their dilute external media, while also eliminating excess water (Barker & Wilhm, 1982; Bradley, 1987; Chadwick & Feminella, 2001). Similarly, FW crustaceans including the amphipods *Gammarus fasciatus* and *Hyallela azteca* must utilize strategies to ensure appropriate levels of ions such as Na^+, K^+, and Cl^- are maintained within their haemolymph (Werntz, 1963). In contrast to FW Arthropods, there are many Arthropods that reside in saltwater (SW) habitats where they face a constant threat of water loss to their hyperosmotic environment, relative to their internal osmolarity. However, there are many insects and crustaceans that can tolerate a range of osmolality, due to the presence of ion-transporting epithelia that allow for active transport of ions with osmotically obliged water movement to regulate blood and urine levels of ions and/or water.

1.2 Osmoregulatory Organs and Ion-Transporting Epithelia in Select Arthropods

1.2.1 Insects

Across both terrestrial and aquatic insects, there is a specific set of osmoregulatory organs that utilize active and passive transport of ions that can consequently facilitate movement of water across cell membranes and thus aid in maintaining internal homeostatic ion and water levels. In

insects, the main osmoregulatory organ are the Malpighian tubules (MTs), which use an apically localized V-type H^+-ATPase (VA) to drive the active transport of ions into the tubule lumen, followed by osmotically obliged water through aquaporins (AQPs) to produce an ion-rich primary urine (Clark & Bradley, 1998; Farina et al., 2022). The MTs have been thoroughly studied in many insects including aquatic FW *Ae. aegypti* mosquito larvae (Ramsay, 1951), aquatic SW *Ae. taeniorhynchus* mosquito larvae (Bradley et al., 1982), terrestrial adult *D. melanogaster* fruit flies (Dow & Davies, 2001), and adult terrestrial *Ae. aegypti* mosquitoes (Beyenbach et al., 1993), to name only a few. The number of MTs differs depending on the insect but can range anywhere from only a few tubules to over 50 in some insects. However, the overall goal of all MTs is to function in producing primary urine, to maintain haemolymph osmolarity. The MTs produce primary urine through transepithelial transport of ions and water, which in many insects including *Ae. aegypti* is made possible by the presence of two main cell types; the larger and more abundant principal cells which are connected via septate junctions to the smaller intercalated stellate cells (Berridge & Oschman, 1969; Beyenbach et al., 2010). The principal cells of the tubules are found throughout and are the main site for Na^+ and K^+ secretion into the tubule lumen (Beyenbach et al., 2010). In some insects such as adult *Ae. aegypti*, the MTs have been shown to express a number of AQP water channels that allow for the movement of water into the tubule lumen (Drake et al., 2010), while their cell-specific localization is currently unknown. However, in other insects such as adult *D. melanogaster*, various AQPs have been found and localized to the less abundant stellate cells of the MTs to aid in transepithelial secretion of water (Cabrero et al., 2020). The principal cells of the MTs are also the site for VA localization at the apical (lumen-facing) membrane, where ATP is consumed to drive the active transport of H^+ ions into the tubule lumen, which energizes the exchange of protons for cations such as Na^+ or K^+ (Beyenbach et al., 2010). At the opposite

basolateral (haemolymph-facing) membrane, there is a Na^+/K^+-ATPase (NKA) that also uses ATP to drive the exchange of Na^+ out of the cell for K^+ into the cell which powers secondary active transporters such as inward rectifying K^+ channels (*Kir*) to further secrete ions (Beyenbach et al., 2010). An important feature of the tubule principal cells is the mitochondrial-rich brush border (apical) that works to supply ample ATP to both VA and NKA. The distally-localized stellate cells are also important in transepithelial ion secretion, as they have been shown to possess Cl^- channels that allow for the secretion of Cl^- into the tubule lumen (Beyenbach et al., 2010). The next important osmoregulatory organ in insects is the posteriorly located hindgut (HG). The HG, which is divided into the ileum and rectum, is responsible for final alterations of urine before excretion which can include reabsorption or removal of ions and water (Phillips et al., 1987). As the primary urine passes from the MTs into the HG, various ion channels along with AQPs can allow for the secretion of ions or water where necessary (Bradley, 1987). Furthermore, some larval aquatic insects including *Ae. aegypti* and *C. riparius* larvae possess a set of externally located anal papillae (AP), which play a large role in actively absorbing ions from the external media. Previous work on *Ae. aegypti* AP has shown that they actively uptake ions from their FW environment, including Na^+, K^+, and Cl^- (Donini & O'Donnell, 2005). Additionally, select AQPs have been found to be colocalized with both VA and NKA at the apical and basolateral membranes, respectively (Akhter et al., 2017). The AP are of particular importance to FW aquatic insects because their need for ion uptake is vital in a hypoosmotic media. Interestingly, the AP are an important osmoregulatory organ in the response of some aquatic insects to changes in external salinity. In *Ae. aegypti* larvae exposed to increased salinity, the AP show adaptation to the environment through differential expression of ion transporters and water channels to deal with the osmotic stress (Durant et al., 2021). In addition, some aquatic insects possess specialized tracheal gills such as mayfly nymphs

of *Hexagenia rigida* that are involved in both respiration and osmoregulation (Nowghani et al., 2017). The gills of *H. rigida* have been shown to possess active ion transporters such as NKA and VA, indicative of their role in ion and water transport (Nowghani et al., 2019). Overall, terrestrial insects mitigate water loss by altering their primary urine produced in the MTs as well as active reabsorption of water in the HG; while aquatic insects face the challenge of either conserving water in SW conditions or conserving ions in FW conditions.

1.2.2 Crustaceans

In crustaceans, the balance of internal ion and water content is efficiently regulated by the use of gills. Crustacean gills are complex osmoregulatory and respiratory organs that vary greatly in their structure depending on a few factors including species and habitat (Freire et al., 2008). Typically, a crustacean gill will possess around four main cell types that aid in ion and water transport, including the thin cells, thick cells, flange cells, and pilaster cells (Freire et al., 2008). The type and number of each cell can differ across species, in addition to the overall arrangement of the gills. Crustacean gill ultrastructure is typically well suited to the animal's external environment. For example, a Japanese freshwater amphipod species *Sternomoera yezoensis* possess two distinct types of gills, the sternal gills with deep basolateral membrane infolding and the coxal gills with thick apical membrane infolding (Kikuchi et al., 1993). The distinction between the gill ultrastructure results in a differential transport of ions and water across the gill epithelium, thus improving the amphipod's ability to respirate and osmoregulate, simultaneously. A large influence on crustacean gill ultrastructure comes from the range of salinity the animal regularly tolerates in their habitat. The thin cells of crustacean gills are typically found in crabs that are considered hyperosmoregulators in their environment, as they possess apical and basolateral

infoldings to aid in ion absorption, while also being linked to serving as a respiratory epithelium based on their thin and flat organization in a squamous epithelium (Freire et al., 2008). Thick cells are also common amongst crustacean hyperosmoregulators and are identified as ionocytes due to their deep membrane infoldings that are associated with a large quantity of mitochondria, which is typical of ion-transporting epithelia (Freire et al., 2008). Interestingly, flange cells are found in both marine and FW crustaceans but may play different roles depending on the external salinity. For example, flange cells in a FW shrimp have been shown to reduce the presence of microvilli at their apical membrane upon exposure to increased salinity, while maintaining the infoldings present at the basolateral membrane to aid in ion retention (Freire et al., 2008). Additionally, the flange cells possess similar attributes to both thin and thick gill cells, therefore giving them a role in both respiration and osmoregulation (Freire et al., 2008). Finally, the pilaster (pillar) cells of crustacean gills are present in the lamellar space, consist of microtubules, and play a role in maintaining gill structure (Freire et al., 2008). However, pilaster cells have also been shown in some FW crustaceans to possess an apically located VA, which is linked to the absorption of NaCl from the dilute environment (Freire et al., 2008). Ion transport in FW and SW crustacean gills has been extensively studied and provides a clear understanding of how these aquatic animals can adapt to a wide range of salinity within their lifetimes. Typically, in FW crustacean gills, an apically located VA with a basolaterally located NKA allow for the active absorption of ions via various externally-facing Na^+ and Cl^- transporters, from the external environment into the haemolymph (Onken et al., 2003). Conversely, in marine-adapted crustaceans, their gill epithelium is primarily geared towards active NaCl secretion driven by basolaterally localized NKA, to remove excess Na^+ and Cl^- to the external environment (Freire et al., 2008). Overall, the gills are an essential

organ to maintaining internal osmolarity of FW and SW crustaceans through the expression of various ion-transporting cells in their gill epithelium.

1.3 Aquaporin Water Channels

The ability to move water into and out of living cells is essential to survival for all living things and, across all Kingdoms, aquaporins (AQPs) have been identified as relatively small transmembrane domain proteins that form a pore in which water can pass through the hydrophobic lipid bilayer (Kruse et al., 2006). AQPs are highly conserved proteins that were originally discovered as members of the major intrinsic protein (MIP) family but were later termed as AQPs because of their ability to transport water (Kruse et al., 2006). The structure of AQPs mainly consists of six transmembrane alpha helices that are connected to each other via five loops, three of which (A, C, and E) protrude at the extracellular side of the membrane and two of which (B and D) protrude at the intracellular side of the membrane (Kruse et al., 2006) (Figure 1-1). Also facing intracellularly, are the N- and C-termini of the AQP, where the C-terminus has been shown to be subjected to phosphorylation (Campbell et al., 2008). The phosphorylation of an AQP's C-terminus may lead to vesicular trafficking, which has been modeled by AQP2 (water-selective) in the renal collecting ducts of the mammalian kidney (Campbell et al., 2008). At both loops B and E, there is a highly conserved set of amino acids including asparagine, proline, and alanine (NPA) forming a motif that, upon appropriate folding of the AQP channel within the membrane, form a pore in the center to allow water passage through the membrane (Jung et al., 1994; Kruse et al., 2006). Although one monomer of an AQP protein will allow water passage, AQPs are typically organized as tetramers where they form stable 4-way pores to allow for optimal water entry (Kruse et al., 2006). AQPs are effective because they are typically highly selective for water entry, while limiting

the possibility of any non-water molecules from entering. At the channels pore, there is a conserved aromatic arginine (Arg195) residue that creates a constriction site and following Arg195, the NPA motif acts as a second constriction site, to essentially only allow water molecules to enter the channels pore (Murata et al., 2000; Kruse et al., 2006; Hub & deGroot, 2007). An important feature of the AQP pore is the positioning of each half helices (loops B and E) that direct the terminal asparagine to the center of the pore and thus the dipoles created by their positioning set up an electrostatic barrier to prevent the passage of protons through the pore (Hub & deGroot, 2007; Campbell et al., 2008).

Although AQPs are identified as water channels, there is a subset of AQPs that have the ability to transport some small molecules and these are widely known as aquaglyceroporins. It has been shown that aquaglyceroporins contain the conserved NPA motif while altering the presence of the aromatic Arg195 to non-aromatic amino acids, which in turn alters the size of the pore and can permit larger molecules such as glycerol through the channel (Kruse et al., 2006; Hub & deGroot, 2007). Aquaglyceroporins have been shown to be important in water and solute passage across many Kingdoms. In particular, thorough research has been conducted on AQPs in plants, mammals, and insects, to name a few (Ishibashi et al., 2011).

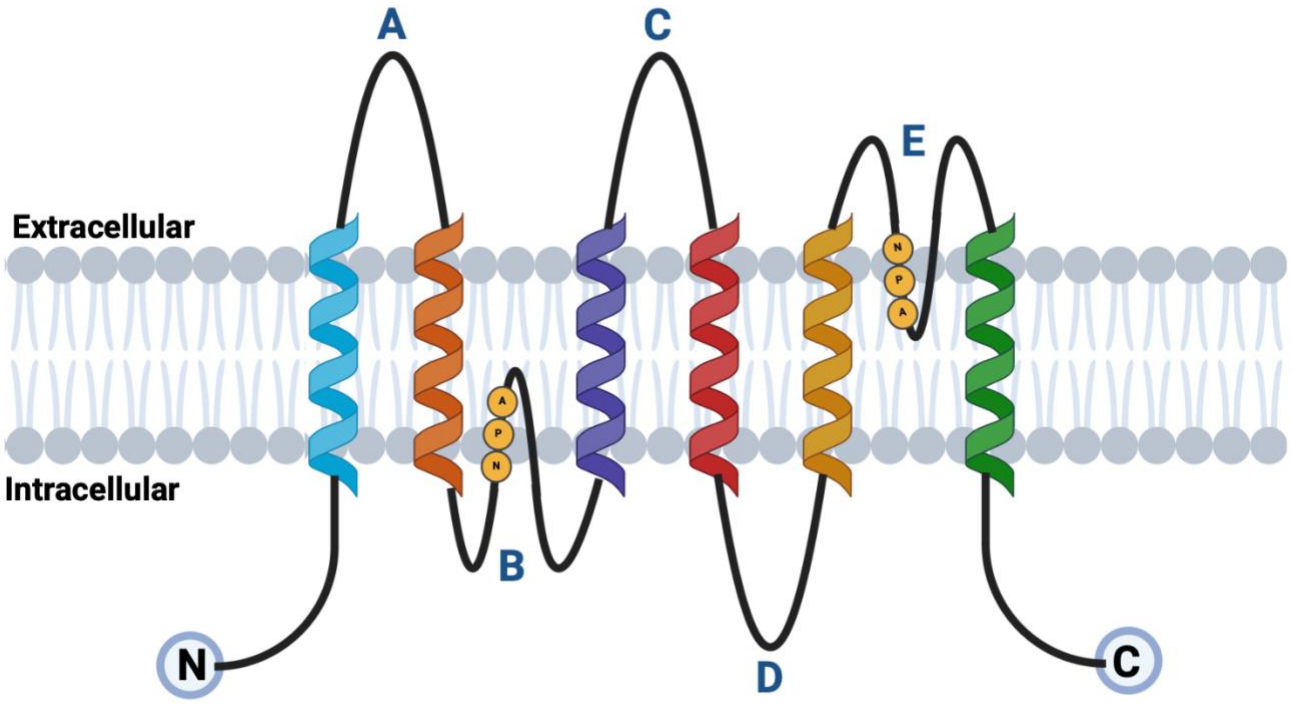

Figure 1-1. Unfolded Aquaporin Water Channel. Example diagram of an unfolded aquaporin monomer embedded in the lipid bilayer. A total of six alpha helices span the cell membrane and are connected via five loops (A-E). Loops A and C are extracellular; loop D is intracellular; loops B and E are half helices. At each half helix (B and E), there is a conserved trio of amino acids labelled as N-P-A (alanine-proline-asparagine) that meet with each other upon assembly of the aquaporin channel and form the pore through which water may pass through. Additionally, seen at the intracellular side encircled in blue are the N (amino)- and C (carboxyl)-termini of the channel. This diagram was developed using BioRender software and was adapted from Yool and Campbell, 2012.

1.3.1 Aquaporins in Insects

To date, many AQP genes have been found and characterized in a variety of insect species including the model fruit fly *Drosophila melanogaster*, the mosquitoes *Ae. aegypti*, *Anopheles gambiae*, and *Culex pipiens*, the red flour beetle *Triboleum castaneum*, the non-flying chironomid *Polypedilum vanderplanki*, and the honeybee *Apis mellifera*, to name a few (Campbell et al., 2008; Kikawada et al., 2008). Many of the discovered insect AQPs are predicted homologs to mammalian genes as well, which has allowed research to further develop an understanding into the function of many insect AQPs. One of the earliest insect AQP discoveries was on AQP*cic* in the green leafhopper, *Cicadella virdis*, where the channel was localized to a filter chamber membrane which is used for fluid and nutrient concentration from their nutrient-poor diet (Beuron et al., 1995; Spring et al., 2009). Additionally, six other AQP genes have been identified in important insects such as *D. melanogaster* and *Ae. aegypti,* particularly linked to water secretion by the MTs (Dow et al., 1995; Lee et al., 2001; Drake et al., 2010; Cabrero et al., 2020). Based on naming and characterization in *D. melanogaster*, various families and groupings of insect AQPs have been established. The DRIP (*Drosophila* integral protein) family of insect AQPs was first discovered as the DRIP AQP gene in *D. melanogaster* (Dow et al., 1995). Various DRIP homologs have been identified in other insects including but not limited to AaAQP1 in *Ae. aegypti* (Drake et al., 2010), Drip-like AQP in *Culex pipiens* (Yang & Piermarini, 2017), AQP-Bom1 in *Bombyx mori* (Kataoka et al., 2009a), and DRIP-like AQP in *Belgica antarctica* (Yashida et al., 2021). In all cases, DRIP or DRIP-like AQPs are known to be water specific (Kaufmann et al., 2005). The next family of insect AQPs is known as the PRIP family, which was named based on the *Pyrocoelia rufa* integral protein (PRIP) discovered by Lee et al., in 2001. AQPs belonging to the PRIP family such as PRIP in *D. melanogaster* (Cabrero et al., 2020), AaAQP2 in *Ae. aegypti* (Drake et al., 2010; Drake et

al., 2015), PRIP-like AQP in *B. antarctica* (Yoshida et al., 2021), and RdPrip in *Rhyzopertha dominica* (Tan & Chen, 2023) have been shown to be water-selective, similar to the DRIP family. The third group of AQPs is characteristically different than the others and is known as the Big Brain gene (BIB) family, which has been characterized in *D. melanogaster* as a non-selective cation channel, instead of a water channel (Campbell et al., 2008) and thus, is not considered a traditional AQP. More recently, sub-groups of aquaglyceroporins in insects more commonly known as entomoglyceroporins have been found in various insect epithelia. The Eglp (entomoglyceroporin) AQPs have been found to transport water as well as some solutes in various transporting epithelia in *D. melanogaster* (Crabrero et al., 2020) and *Ae. aegypti* (Drake et al., 2015).

In insects, AQP research has been primarily focused on the MTs, as they produce primary urine and are the major site for osmoregulation. A vast majority of the research surrounding AQPs in the MTs has been on *D. melanogaster* as well as some in *Ae. aegypti*. Specifically, in *D. melanogaster* MTs, the stellate cells have been identified as the primary site for AQP expression, including evidence of DRIP, PRIP, and Eglp gene expression (Cabrero et al., 2020). In *Ae. aegypti* MTs, AaAQP1 (DRIP), AaAQP2 (PRIP), AaAQP4 (Eglp2), and AaAQP5 (Eglp4) gene expression has been detected in the MTs (Drake et al., 2010). Overall, the importance of AQPs in the osmoregulatory organs of insects has been highlighted across many insect species, but a primary focus has typically been on the MTs for their critical ion and water transporting capabilities.

1.4 Osmoregulatory Challenges of Adult Terrestrial *Ae. aegypti* Mosquitoes

1.4.1 Lifestyle of *Ae. aegypti*

The disease vector mosquito *Ae. aegypti* is found in sub-tropical and tropical regions around the globe and is generally known as an urban mosquito due to their preference to reside in urban communities and feed on human hosts (Jansen & Beebe, 2010; Kraemer et al., 2015). Adult female *Ae. aegypti* are haematophagous and acquire a blood meal from vertebrate, preferentially human, hosts which results in the spread of several deadly arboviral diseases including dengue fever, Chikungunya, yellow fever, and Zika virus (Leta et al., 2018). Female mosquitoes are opportunistic animals and will acquire a blood meal from a host as they present themselves (Ponlawat & Harrington, 2005). The females must acquire a blood meal to obtain the essential nutrients and proteins needed to mature their eggs, after fertilization has occurred (MacDonald, 1956). Upon gonadal maturity, male *Ae. aegypti* will inseminate the females and, in doing so, the female can store the sperm in accessory organs known as the spermatheca, until she may find an appropriate blood meal needed for egg maturation (Hartberg, 1971). An aspect related to the success of *Ae. aegypti* is their semi-aquatic lifestyle, where the adult females will lay their eggs near bodies of freshwater which will then hatch into their aquatic larval stage (Surtees, 1967). The aquatic larval stages consist of four instar phases which ends in a non-feeding transitional pupal stage before emergence as a terrestrial adult mosquito. In urban communities, *Ae. aegypti* larvae are typically found in artificially created water basins, allowing them to have a constant close proximity to human hosts upon emergence (Cheong, 1967). In their typical freshwater environment, *Ae. aegypti* larvae are subjected to constant ion loss to their surrounding hypotonic media and thus utilize their osmoregulatory organs such as the anal papillae to actively uptake ions from the water (Sohal & Copeland, 1966). To aid in their success, *Ae. aegypti* larvae have been

found to tolerate a wide range of salinities from brackish water (3% seawater) to ~30% seawater (Clark et al., 2004), as well as high ammonia environments such as septic tanks in urban communities (Durant & Donini, 2019). It is evident that *Ae. aegypti* mosquitoes have developed a variety of tactics to ensure their success and a large component relates to their ability to respond to osmoregulatory stress throughout their lifecycle.

1.4.2 Adult Female *Ae. aegypti*

Adult female *Ae. aegypti* mosquitoes typically acquire their first blood meal within 24hr of emergence, hence why they have developed a preference for egg laying in urban communities (Jones & Pilitt, 1973). It is possible for the females to only partially feed on their vertebrate host, which could result in an additional blood meal taken from a different host shortly after the first meal (Farjana & Tuno, 2013). Typically, adult female mosquitoes reside in a desiccating environment where they are subjected to water loss (Figure 1-2A). However, a vertebrate blood meal presents a large load of water, salts, and proteins on the female's body, as she acquires about 3x her haemolymph volume in vertebrate blood and creates the opposite problem for the female (Figure 1-2B). Therefore, a coordination in ion and water transport through her osmoregulatory organs must occur rapidly post-feeding, in order to return haemolymph osmolarity to normal. It is common for females to feed anywhere from 45 to 225 seconds, however the process of diuresis where an increase in fluid and ion excretion occurs, begins while the mosquito is still blood feeding and can last up to 2hr post meal (Jones & Pilitt, 1973). The rapid post-prandial diuresis is initiated by the release of diuretic neurohormones into the haemolymph that act on sites such as the MTs to increase primary urine production to remove excess water and salts (Wheelock et al., 1988; Clark & Bradley, 1998; Beyenbach, 2012). A number of neurohormones have been shown to elicit a

diuretic response by the MTs in female *Ae. aegypti* including diuretic hormone 31 (DH_{31}), serotonin (5HT), corticotropin-releasing factor (CRF)-like diuretic hormone 44 (DH_{44}), and kinin-related peptides such as the *Culex* depolarizing peptide (CDP) (Clark & Bradley, 1998; Cady & Hagedorn, 1999; Jagge & Pietrantonio, 2008; Sajadi et al., 2018). Two of the major diuretic neurohormones are arguably DH_{31} and 5HT, which have been shown to illicit the largest magnitude of increased fluid and ion secretion post blood meal (Clark & Bradley, 1996; Clark & Bradley, 1998; Kwon et al., 2012; Sajadi et al., 2018). Additionally, an anti-diuretic neuropeptide known as *Aedae*CAPA-1 in *Ae. aegypti* has been shown to reduce DH_{31} and 5HT-stimulated secretion by the MTs in the post-prandial response (Sajadi et al., 2018).

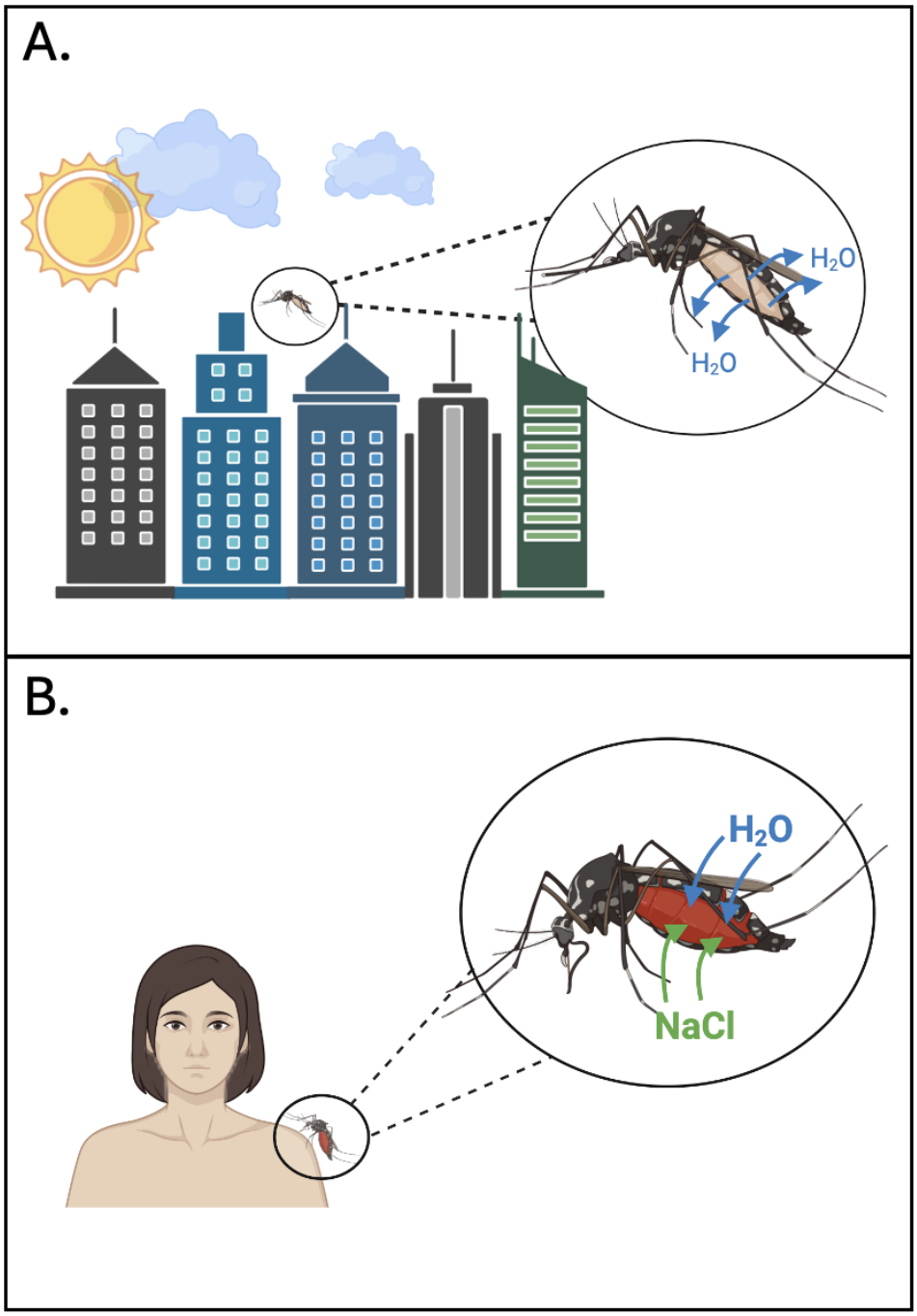

Figure 1-2. Osmoregulatory Challenges of the Adult Female Mosquito (*Aedes aegypti*). The mosquito *Ae. aegypti* is a tropical and sub-tropical terrestrial insect that is responsible for spreading several deadly diseases due to its' hematophagous lifestyle. **A.** The representation of a typical environment for adult female *Ae. aegypti*, where she is subjected to passive water loss due to the desiccating environment in which she resides. **B.** The representation of an adult female *Ae. aegypti* taking a blood meal from her human host, where she experiences a rapid increase in water and salts in her haemolymph, creating an opposing osmoregulatory challenge than her typical environment. This diagram was created using BioRender software.

Diuretic hormone 31 (DH_{31})

The calcitonin-like diuretic hormone 31 (DH_{31}) has been identified as the mosquito natriuretic peptide (MNP) that is vital to post prandial diuresis driving secretion of Na^+ and osmotically obliged water by the MTs (Coast et al., 2005). The G-protein coupled receptor (GPCR) for DH_{31} has been localized to the principal cells of *Ae. aegypti* MTs and upon activation of the receptor, the cAMP secondary messenger pathway is activated and initiates the response to increase Na^+ and water secretion (Coast et al., 2005; Kwon et al., 2012). In *D. melanogaster*, a single gene for DH_{31} has been identified that has also been found and isolated in *Ae. aegypti* (Predel et al., 2010). Recently, it has been shown that DH_{31} reaches its peak levels around 5 minutes post blood meal in the haemolymph of female *Ae. aegypti* and also appears present in the haemolymph directly after blood feeding has ended (Sajadi et al., 2025). Therefore, DH_{31} represents one of the main diuretic hormones released post blood feeding, which works to increase Na^+ transport into the tubule lumen by various transporters including the Na^+-K^+-Cl^-cotransporter while also increasing the activity of VA to ultimately move more fluid and ions into the tubule lumen (Coast et al., 2005; Karas et al., 2005; Sajadi et al., 2023).

Serotonin (5HT)

The hormone 5-hydroxytryptamine (5HT) commonly known as serotonin is a biogenic amine that is endogenous to *Ae. aegypti*. Many studies have been conducted on 5HT in *Ae. aegypti*, proving its role as a diuretic hormone that increases fluid and ion secretion by the MTs (Clark & Bradley, 1996; 1997). In larval *Ae. aegypti*, 5HT has been shown to induce a diuretic response during periods of increased salinity to drive the active secretion of ions (Clark & Bradley, 1998; Donini et al., 2006). More recently, 5HT has been shown to increase fluid secretion by adult female

Ae. aegypti MTs, potentially through the increase in K^+ secretion by tubules (Sajadi et al., 2018). The receptor for 5HT has not been characterized in *Ae. aegypti* MTs, however immunolocalization of a putative 5HT receptor in larval *Ae. aegypti* has been identified in the MTs (Petrova & Moffett, 2016).

CAPA Peptides

In order to return tubule secretion rates back to normal after diuretic activation as well as prevent dehydration, anti-diuretic neuropeptides are released to reduce stimulated-secretion rates. In *Ae. aegypti*, the peptide known as CAPA has been identified as an anti-diuretic hormone in both larvae and adults (Ionescu & Donini, 2012; Sajadi et al., 2018). The CAPA peptide was first identified in *D. melanogaster* (Kean et al., 2002) where it was later shown to elicit a dose-dependent diuretic effect on *Drosophila* MTs (Pollock et al., 2004; Davies et al., 2013). However, in the hematophagous insect *Rhodnius prolixus* as well as adult *Ae. aegypti*, CAPA peptides (CAPA-1 and CAPA-2) have been shown to elicit a dose-dependent anti-diuretic effect (Paluzzi et al., 2008; MacMillan et al., 2018; Sajadi et al., 2018). Additionally, it appears activation of the cGMP second messenger pathway is involved in the CAPA signalling cascade of *Ae. aegypti* tubules, shown in both larval and adult mosquitoes (Ionescu & Donini, 2012; Sajadi et al., 2018). In female MTs, CAPA-1 has been specifically shown to reduce DH_{31}- and 5HT-stimulated secretion (Sajadi et al., 2018) and has been shown to be responsible for the dissociation of the apically localized VA that reduces the ion gradient across the tubule membrane and thus returns secretion rates back to normal (Sajadi et al., 2023).

Ion and Water Transport Post Blood Meal in the MTs

A blood meal imbibed by female *Ae. aegypti* induces several important changes in ion and water transport throughout the body in response to the associated osmoregulatory challenge. As the MTs are the major osmoregulatory organ in the mosquito, several studies have shown the effects of blood feeding on ion transport in the tubules. Upon initiation of a blood meal, various diuretic neurohormones are released into circulation that aid in increasing Na^+, K^+, and Cl^- secretion by the MTs (Wheelock et al., 1988; Cady & Hagedorn, 1999). However, along with the ions, water secretion also increases via aquaporins. It was shown that in female *Ae. aegypti* MTs, three of the six *Ae. aegypti* AQP genes (AaAQP1, 2, and 4) were upregulated 3hr post blood meal while the entomoglyceroporin AaAQP5 was upregulated 24hr post blood meal (Drake et al., 2010). Furthermore, some AQPs in *Ae. aegypti* MTs have been shown to be phosphorylated 24hr post blood meal, which indicates regulation of the channels is necessary at different time points post blood feeding (Kandel et al., 2022).

1.4.3 Adult Male *Ae. aegypti*

Male *Ae. aegypti* exhibit quite a different lifestyle based on their diet, in comparison to their female counterparts. The main difference is that male mosquitoes do not acquire blood meals, but instead only feed on sugar-rich plant nectar to obtain their required nutrients (Barredo & DeGennaro, 2020). A noticeable difference to the male and female mosquito diet is that a nectar meal for male mosquitoes is typically less osmotically challenging than a blood meal is in female mosquitoes. With that, important osmoregulatory organs such as the MTs are much smaller and thinner in the male (Plawner et al., 1991). Also, the rate of fluid secretion by male MTs was shown to be almost 7x less in comparison to female MTs, with overall lower Na^+, K^+, and Cl^- secretion

rates as well (Plawner et al., 1991). It was also found that the second messenger of important diuretic hormones, cAMP, induced increases in Na^+, Cl^-, and fluid secretion post treatment in male *Ae. aegypti* MTs, similar to the results on female MTs (Plawner et al., 1991). Overall, a vast majority of studies on fluid and ion secretion by mosquito MTs has been completed on females due to their haematophagous lifestyle, leaving relatively little known about ion and fluid secretion in male MTs. The mechanisms behind fluid secretion by male mosquito MTs post nectar-feeding is largely unknown yet remains an important topic of study.

1.5 Osmoregulatory Challenges in the Aquatic Freshwater Arthropods

1.5.1 Salinization of Freshwater and Effects on Freshwater Fauna

Freshwater (FW) ecosystems are home to a wide variety of animals including many arthropods such as aquatic insects and crustaceans. Typically, FW contains small amounts of ions including Na^+, K^+, Cl^-, Ca^{2+}, and HCO_3^- and is generally considered a hypotonic environment for most animals residing in this aquatic environment (Iglesias, 2020). As a result, aquatic insects and crustaceans, as well as many vertebrates such as fishes, have become well equipped in their osmoregulatory physiology to deal with the dilute conditions, as they regularly experience a threat to ion loss in a hypotonic environment (Figure 1-3). In recent decades, an increased concern has been raised regarding the rapid salinization of freshwater ecosystems. Many anthropogenic activities such as, but not limited to, urbanization, land irrigation, and road salting particularly in the Northern Hemisphere have resulted in the salinization of freshwater (Kaushal et al., 2021). Studies have shown that seasonal patterns of increased salinization such as early spring in parts of eastern USA have ultimately resulted in less diversity of aquatic insects (Timpano et al., 2018). Historically, levels of ions such as Cl^- have risen to extreme concentrations (50-100x) and it has

been demonstrated that even small increases in Cl^- can cause detrimental effects on FW invertebrate populations such as reduced diversity, reduced growth rates, and more (Gillis et al., 2011; Dugan & Arnott, 2022). With time and repeated exposure to increased salinity, many invertebrate species have adjusted their osmoregulatory physiology to accommodate for higher levels of salts in their environment. Some invertebrate species including the water flea *Daphnia pulicaria* and the copepod *Eurytemora affinis* have been regularly exposed to salinization in their natural environment and, from that, have shown a higher tolerance to increased salinity (Lee, 2016; Wersebe & Weider, 2023). A large fraction of increased salinity stems from the ice and snow melt run off that occurs in nearby freshwater streams, rivers, and ponds as temperatures begin to rise following the winter months in the Northern Hemisphere (Dugan & Arnott, 2022). Typically, road salt is used in the form of NaCl pellets or NaCl brine and are heavily applied to roads, bridges, and walkways to avoid ice and snow build up. The use of NaCl-based de-icers and the resulting salinization effects on FW has been linked to osmotic stress and species decline in many FW organisms (Jones et al., 2017). Therefore, the development of natural and organic-based de-icers has become more widespread in Canada and the USA. A newly introduced brine beet juice de-icer (BBJD) has been implemented throughout North America and is mainly comprised of the extract from sugar beets mixed with a NaCl brine that typically contains lower levels of salts than traditional NaCl-based de-icers (Nutile & Sloan, 2019b). However, not enough research has been conducted to deem the BBJD as a truly eco-friendly alternative to NaCl-based de-icers, as most studies have not examined its effects on freshwater invertebrates.

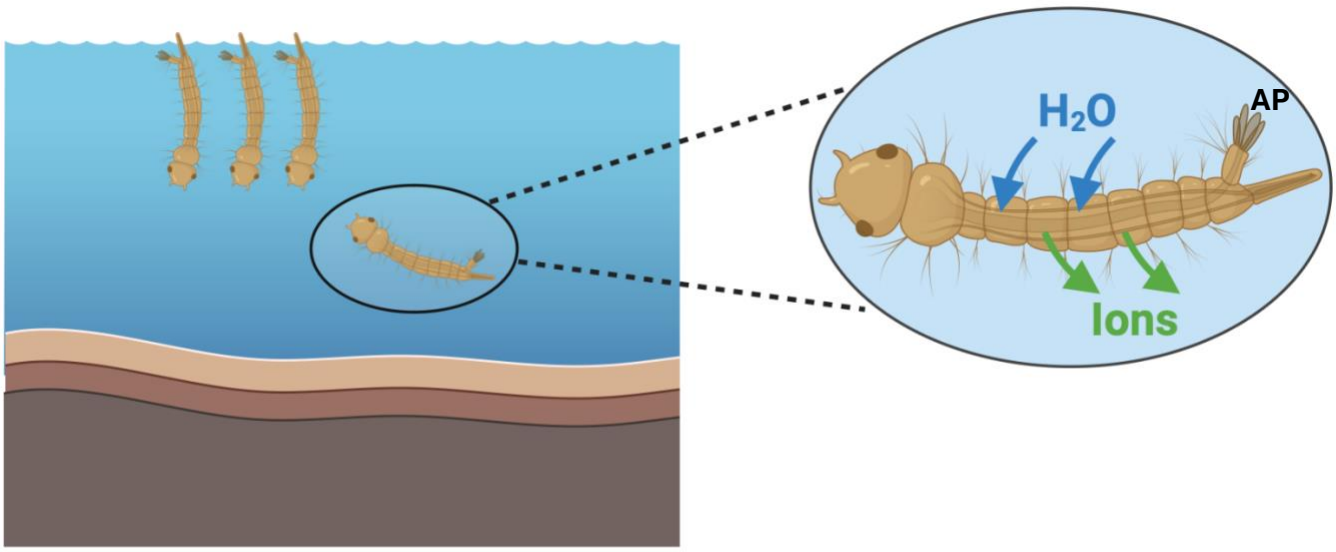

Figure 1-3. Osmoregulatory Challenge of Freshwater Larval Insects. Freshwater larval insects such as *Aedes aegypti* larvae (pictured) are hyperosmoregulators, meaning they have a higher internal osmolarity than their surrounding environment and are thus subjected to passive water entry as well as ion loss to the external environment. Their osmoregulatory physiology is equipped to mitigate the dilution of their internal haemolymph by actively uptaking ions from the water using their anal papillae (indicated by AP on inset diagram). This diagram was created using BioRender software.

1.5.2 Effects of Salinity on Freshwater Crustaceans

Crustaceans comprise the largest aquatic group of Arthropods and can be found in both marine and freshwater habitats. Crustaceans include many animals such as but not limited to shrimps, crabs, and water fleas and are widely important to the ecosystems they inhabit. For example, they serve as a food source for many larger animals, while some are involved in cleaning their environment by feeding on decaying matter, as well as serving as an important economic role in society (Szaniawska, 2017). Unfortunately, for many FW crustacean species in the Northern Hemisphere, they have had to adapt in order to survive increasing conditions of salinization in recent decades. Many studies have been conducted on the FW water flea known as *Daphnia magna*, as they are a plentiful food source for many larger invertebrates as well as fishes and are also known as a bioindicator as their survival and success in an environment has been shown to be linked to the quality of the water in which they reside (Ha & Choi, 2009). Optimal salt concentration for many FW crustaceans is anywhere between 0-2% and outside of that range is when osmotically induced stressors such as increased respiration due to higher metabolic rate, is seen (Jeppesen et al., 1994; Grzesiuk & Mikulski, 2006). Further studies on *D. magna* with prolonged exposure to NaCl-contaminated water found that increasing concentrations of NaCl (up to 8g L^{-1}) caused significant decreases in total progeny, average lifespan, and life expectancy, indicating the detrimental effects of the salinization of FW habitats (Martinez-Jeronimo & Martinez-Jeronimo, 2007). Similar findings and outcomes of salinization have been shown in a FW shrimp species *Palaemonetes antennarius*, where increased salinity made acclimation to increased ambient temperature much more challenging, resulting in excess energy expenditure (Dalla Via, 1987). Relatedly, in a FW amphipod *Gammarus fossarum*, tolerance to salinity rapidly decreased with high salt concentrations (Dorgelo, 1974). Overall, many decades of research have

concluded that the salinization of FW has had detrimental effects on the FW crustaceans that inhabit those regions, thus the importance of eco-friendly alternatives to NaCl-based road de-icers is of utmost importance.

1.5.3 Effects of Salinity on Freshwater Insects

Insects make up the majority of Arthropods and while many of the species are terrestrial, there are many aquatic insects that reside in FW environments. Aquatic insects are vital to their FW ecosystems as they serve as a food source for many vertebrates, as contributors to nutrient processing in the environment, and as important bioindicators (Suter & Cormier, 2015). Many FW aquatic insects have been shown to tolerate a relatively wide range of salinity such as the larval mosquito *A. aegypti* (Ratnasari et al., 2021) and the midge *C. riparius* (Lob & Silver, 2012); however, increases in FW salinization remain deadly to many aquatic insects. In particular, *C. riparius* is a FW benthic-dwelling midge species that has served as a bioindicator for many FW ecosystems in Canada and the USA. Studies have shown that increased salinity by NaCl-based de-icers caused significant damage on *C. riparius* populations with increasing ambient temperatures such as reduced female size and imbalanced sex ratios at 5g L^{-1}, and total lethality at 10g L^{-1} at ~12ºC (Lob & Silver, 2012). In the FW larval mosquito *Ae. aegypti*, it was found that increasing salinity caused significant decreases in larval growth rates as well as decreased body mass (Clark et al., 2004). Furthermore, research has shown that increased salinity has significant effects on the ion and water transport characteristics of important osmoregulatory organs such as the Malpighian tubules and anal papillae of some FW insects (Silver & Donini, 2021). Therefore, salinization of FW has a large impact on FW aquatic insects which in turn can have detrimental effects on macroinvertebrate populations.

1.6 Research Aims and Objectives

Arthropods such as insects and crustaceans live in a wide variety of habitats and have developed different feeding mechanisms as well as environmental tolerances. A major divide between most arthropods are the habitats they reside in, either aquatic or terrestrial, and in some cases can be both in a single species. In particular, terrestrial haematophagous insects such as adult female *Ae. aegypti* mosquitoes reside in desiccating environments but feed on vertebrate blood which introduces them to an osmoregulatory challenge that threatens the balance of their internal haemolymph osmolarity (Beyenbach, 2003a). On the other hand, adult male *Ae. aegypti* do not feed on blood but instead only on plant nectar and thus require an appropriate response to the increase in water and sugars they receive from their diet, to maintain haemolymph homeostasis (Barredo & DeGennaro, 2020). Conversely, aquatic insects such as larval *Ae. aegypti* or larval *C. riparius* as well as aquatic crustaceans such as the amphipod *Hyalella azteca* reside in dilute environments where they face a threat of major ion loss to their hypotonic environment while potentially being subjected to environmental salinization, which poses an opposite problem. Overall, these various arthropods, regardless of what class they belong to, require a specific set of osmoregulatory organs that can mitigate the osmotic challenges they face on a regular basis. Many studies have focused on ion transport in osmoregulatory organs, but it is also important to understand how these osmoregulatory challenges affect the transport of water across cell membranes. In insects, it is largely overlooked how osmotically challenging behaviours such as blood feeding in female mosquitoes alters the function and expression of aquaporins. Additionally, it is also important to understand how osmotically challenging environments that are subjected to fluctuations in salinity affect the freshwater fauna.

1.6.1 Aim 1: Determine the Role of Aquaporins in the Response to Osmotically Challenging Diets in the Adult Female and Male *Ae. aegypti* Mosquito

A majority of research on the effects of a blood meal on female mosquito physiology has focused on ion transport, with a lack of understanding on how the meal affects the AQP water channels. Thus far, it has been shown that gene transcript levels of water selective AQPs such as AaAQP1 and 2 and entomoglyceroporins such as AaAQP4 and 5 are increased in the MTs of female *Ae. aegypti* following a blood meal (Drake et al., 2010; Drake et al., 2015). However, it is not yet understood what specifically causes the increase in transcript and how that translates into the regulation of the functional proteins. Furthermore, in female *Ae. aegypti* studies have shown the effects of diuretic and anti-diuretic hormone signalling on overall ion and water secretion (Sajadi et al., 2018), but there is relatively no understanding on how these hormones affect AQPs in female *Ae. aegypti*. In addition, although there is speculation and some evidence that shows adult male *Ae. aegypti* respond similarly in their secretion of water and ions post nectar-feeding (Plawner et al., 1991), there is relatively little known about how their diet affects AQP water transport in their osmoregulatory organs. Therefore, the aim of this research was to investigate the effects of diet on AaAQP abundance and localization in female and male *Ae.* aegypti. This research aim was investigated in three main projects:

(1) Localize and measure abundance of AaAQP1,2,4,5, and 6 in whole body female *Ae. aegypti* pre- and post-blood feeding, in the short term (0.5hr) and long term (24hr) post-prandial diuresis, with specific emphasis on the MTs.

(2) Determine the role of AaAQP1 in female *Ae. aegypti* MTs in the presence of diuretic neurohormones such as DH_{31} and 5HT, as well in the presence of the anti-diuretic peptide *Aedae*CAPA-1.

(3) Localize AaAQP1,2,4,5, and 6 in whole body male *Ae. aegypti* and examine the effects of diet versus starvation on the localization and abundance of a representative water selective AaAQP (AaAQP1) and a representative entomoglyceroporin (AaAQP4) in male MTs.

1.6.2 Aim 2: Understand the Effects of Salinization by Road De-Icers on the Osmoregulatory Physiology of Freshwater Arthropods Including the Crustacean *H. azteca* and the Larval Insect *C. riparius*

The salinization of freshwater has become a popular topic of research in recent decades due to its detrimental effects on freshwater fauna. A vast majority of research has focused on the effects of brackish water conditions or salinization on the osmoregulatory physiology of FW insects such as larval *Ae. aegypti* (Ramasamy et al., 2011; Surendran et al., 2018; Misyura et al., 2020). Much less is known and studied regarding the effects of non-traditional road de-icers such as brine beet juice de-icer (BBJD) on the osmoregulatory physiology of FW invertebrates, which nonetheless also contain high salt levels. BBJD has been implemented in the Northern Hemisphere and marketed as an eco-friendly road de-icing agent; however there is not enough research to categorize it as a better option compared to the traditional NaCl-based de-icers. BBJD is naturally high in K^+ (Fay and Shi, 2012; Wruss et al., 2015) which could potentially have differential or more severe effects on FW invertebrates than traditional de-icers. For example, in a FW mussel species exposed to increasing levels of BBJD in their external environment, there was a significant elevation in internal K^+ levels, indicating that there is a definite link between BBJD and the osmoregulatory physiology of FW invertebrates (Gillis et al., 2021). Overall, the aim of this research was to determine if BBJD was more, less, or equally osmotically challenging as NaCl-

based de-icers on the osmoregulatory physiology of a FW midge species *C. riparius* and of a FW amphipod species *H. azteca*. This research objective was divided into two main projects:

(1) Identify a water specific AQP in the FW larvae of *C. riparius* and characterize its role in response to increased external salinity caused by BBJD and NaCl.

(2) Determine the effects of increased salinity by BBJD and NaCl on the osmoregulatory physiology and internal ion levels in the FW amphipod *H. azteca*.

For the first aim of this dissertation, which focused on *Determining the Role of Aquaporins in the Response to Osmotically Challenging Diets in the Adult Female and Male Ae. aegypti Mosquito*, the three main objectives have been organized into individual chapters. In this thesis, Chapter 2 focuses on Aim 1: Objective 1, which investigates the localization and abundance of AaAQPs in adult female *Ae. aegypti* pre- and post-blood feeding. Chapter 3 focuses on Aim 1: Objective 2 which involves understanding the role of the water-specific AaAQP1 in response to various neuroendocrine factors in the MTs of adult female *Ae. aegypti.* Then, Chapter 4 of this thesis covers Aim 1: Objective 3 where the localization and abundance of AaAQPs was measured in adult male *Ae. aegypti* in response to nectar feeding. The second aim of this dissertation involved *Understanding the Effects of Salinization by Road De-Icers on the Osmoregulatory Physiology of Freshwater Arthropods Including the Crustacean H. azteca and the Larval Insect C. riparius*, which was divided into two main objectives. Chapter 5 of this dissertation covers Aim 2: Objective 1, which looked into identifying an AQP (CrAQP2) in the larval midge *C. riparius* and understanding its role in response to de-icer contamination. Finally, chapter 6 of this thesis covers Aim 2: Objective 2, which focused on the effects of de-icer contamination on the osmoregulatory physiology of a freshwater crustacean, *Hyallela azteca.*

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Chapter Two

Protein localization of aquaporins in the adult female disease vector mosquito, *Aedes aegypti*

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2.1 Summary

The female *Aedes aegypti* mosquito is a vector for several arboviral diseases, due to their blood feeding behaviour and their association with urban communities. While ion transport in *Ae. aegypti* has been studied, much less is known about mechanisms of water transport. Rapid water and ion excretion occurs in the adult female mosquito post blood meal and involves a set of organs including the midgut, Malpighian tubules (MTs), and hindgut. The MTs are responsible for the formation of primary urine and are considered the most important site for active transport of ions. Within the cells of the MTs, along with various ion transporters, there are aquaporin water channels that aid in the transport of water across the tubule cell membrane. Six aquaporin genes have been molecularly identified in *Ae. aegypti* (AQP1-6) and found to be responsible for the transport of water and in some cases, small solutes such as glycerol. In this study, I used immunohistochemistry to localize AaAQP1, 2, 4, 5, and 6 in the adult female *Ae. aegypti*, in non-blood fed and post blood feeding (0.5 and 24hr) conditions. I further examined the main water transporting aquaporin, AaAQP1, using western blotting to determine protein abundance changes in isolated MTs pre- and post-blood feeding. Using fluorescence *in situ* hybridization, *aqp1* mRNA was found exclusively in the principal cells of female MTs. Finally, I used immunogold staining with transmission electron microscopy to determine subcellular localization of AaAQP1 in the Malpighian tubules under non-blood fed conditions. Interestingly, AaAQP1 was found to be predominantly in the principal cells of the MTs, dispersed throughout the brush border; however, there was also evidence of some AaAQP1 localization in the stellate cells of the MTs.

2.2 Introduction

The mosquito, *Aedes aegypti* is responsible for the spread of deadly arboviral diseases such as Zika virus (Benelli & Romano, 2017), Chikungunya (Vega-Rua et al., 2014), yellow fever (Soper, 1967), and dengue fever (Black et al., 2002). Adult *Ae. aegypti* are terrestrial and feed on plant nectar for essential nutrients. Females also obtain a blood meal from vertebrate hosts to utilize the protein for egg maturation (Ponlawat & Harrington, 2005). Upon the initiation of blood feeding, female mosquitoes intake a large quantity of water and ions, which must be dealt with quickly. Rapid excretion of water and ions, such as Na^+ and K^+, has been shown to begin before a female *Ae. aegypti* has finished taking a blood meal (Wheelock et al., 1988; Beyenbach & Piermarini, 2010). Insects, including *Ae. aegypti,* have specialized organs that are responsible for osmoregulation and rapid urine excretion, namely the Malpighian tubules (MTs), and the hindgut. The MTs are comprised of two main epithelial cell types; the more abundant principal cells and the intercalated stellate cells that together are responsible for the production of primary urine, which is driven by an apical V-type H^+-ATPase (VA) (Wieczorek et al., 2009). Specifically, the VA maintains a proton gradient that is needed for secondary active transport of ions such as Na^+ and K^+ into the tubule lumen, in exchange for protons through cation proton antiporters expressed on the apical membrane of both cell types (O'Connor & Beyenbach, 2001; Weng et al., 2003; Piermarini et al., 2015). In addition to the MTs, the posterior region of the hindgut (ie. rectum) is responsible for reabsorption of water and ions before final waste excretion (Paluzzi et al., 2014). The excretion of ions has been studied extensively, however there is much less known about the movement of water by insect excretory organs, including the MTs.

Aquaporins are transmembrane proteins that form selective channels for water and some solutes such as glycerol and trehalose (Drake et al., 2015), that make it possible for the transcellular

flow of water to occur. In *Ae. aegypti*, there have been six aquaporin genes identified [AaAQP1–6] (Drake et al., 2015; Sreedharan et al., 2016; Yang & Piermarini, 2017). In this study, the AQP nomenclature used follows that of the Hansen lab (Table 2-1) (Drake et al., 2010). Functional characterization in a heterologous system revealed AaAQP 1, 2, and 5 allow significant water permeability (Drake et al., 2015) as does AaAQP6 (Sreedharan & Sankaranarayanan, 2018), while AaAQP 4 and 5 have been identified as entomoglyceroporins, able to transport some solutes across the cell membrane (Drake et al., 2015). Sequencing (Sreedharan et al., 2016), heterologous expression data (Drake et al., 2010), and gene replacement data (Finn et al., 2015) suggest that AaAQP4 and AaAQP5 have a different amino acid composition resulting in a larger pore diameter, allowing the transport of solutes like glycerol, urea, erythritol, adonitol, mannitol, and trehalose in addition to water. AaAQP5 has a comparatively high permeability to water, similar to AaAQP1, which is significantly higher than AaAQP4 that is a poor water transporter (Drake et al., 2015).

Table 2-1. Sizes and functional characteristics of aquaporins examined in the current study found in *Aedes aegypti* along with their orthologs in the African malaria vector mosquito, *Anopheles gambiae*, and the fruit fly, *Drosophila melanogaster*. Table data, including accession numbers, was compiled based on phylogenetic data described previously (Drake et al., 2010).

Name in *Aedes aegypti*	Accession Number	Length	Putative AQP Function	*Anopheles gambiae* Homolog	*Drosophila melanogaster* Homolog
AaAQP1	XP_001656931	249	Water-selective AQP	XP_319584	DRIP-CG9023
AaAQP2	XP_001649747	264	Water-selective AQP	XP_319585	PRIP-CG7777
AaAQP4	XP_001650168	292	Entomoglyceroporin	XP_554502	Eglp2-CG5398
AaAQP5	XP_001650169	249	Entomoglyceroporin	XP_318238	Eglp4-CG4019
AaAQP6	XP_001648046	261	Water-selective AQP	XP_309823	CG12251

AaAQP1, 2, 4, 5, and 6 mRNA has been identified in the MTs of non-blood fed females (Drake et al., 2010). A blood meal in female *Ae. aegypti* increases mRNA levels of AaAQP1, 4, and 5 in the MTs, specifically between 3–48hr post-blood meal (Drake et al., 2010; Drake et al., 2015). Previous work done in larval *Ae. aegypti* established the localization of AaAQPs throughout the alimentary canal with abundance of AaAQP1, 4, and 5 shown in the MTs (Misyura et al., 2017), as well as in adult *Ae. aegypti* where AaAQP1 was localized to the tracheolar cells (Pietrantonio et al., 2000). To date, there has been no comprehensive characterization of AQPs in the adult *Ae. aegypti* mosquito; particularly at the protein level. In addition, identifying mechanisms by which AQPs in *Ae. aegypti* are regulated is important for understanding how a blood meal in female *Ae. aegypti* may affect localization, abundance and ultimately the function of AaAQPs. In *Ae. aegypti*, control and modification of MT function involves circulating hormones, including neuropeptides (Sajadi et al., 2018). The rate of fluid secretion by MTs increases with application of the neuropeptide, diuretic hormone 31 (DH_{31}) and decreases with application of the anti-diuretic hormone, *Aedae*CAPA-1 (Sajadi et al., 2018). The actions of DH_{31} and *Aedae*CAPA-1 are mediated by intracellular signaling that involves assembly and disassembly of the VA in the apical membrane of principal cells (Sajadi et al., 2023). Through the control of fluid secretion by the MTs, AaAQP regulation is also possible, however there have been limited studies on invertebrate AQP regulation. It has been proposed that AQPs can be phosphorylated during periods of stress, in addition to the possibility that they are packaged into membrane vesicles on demand, for example during diuresis (Kandel et al., 2022). The goal of this study was to provide a deeper understanding of the localization of AQPs in the adult female *Ae. aegypti* and to gain better insight on AaAQP1 expression and regulation before and after blood feeding by female mosquitoes.

2.3 Materials and Methods

Mosquito Rearing

Aedes aegypti eggs (Liverpool) were gathered from a long- standing colony reared at York University in Toronto, Ontario Canada. Filter paper was placed in small cups filled with ddH_2O, where adult females were able to lay their eggs at the surface of the water. Females were fed twice weekly with sheep's blood in Alsever's solution (Cedarlane Laboratories, Burlington, Ontario Canada) using an artificial feeding method (Rocco et al., 2017). Egg strips were then air dried and hatched as necessary, in 1L dechlorinated water baths. Larvae were fed with a 1:1 liver powder-yeast mixture dissolved in ddH_2O. As the larvae pupated, the pupae were collected in small 10mL containers and placed in mosquito cages. Each mosquito cage was provided with a 10% sucrose-soaked cotton ball to allow the mosquitoes to feed on a simulated nectar meal *ad libitum*. Male and female pupae were combined in each mosquito cage. For non-blood fed conditions, ~10–15 female *Ae. aegypti* were isolated from mosquito cages at ~10–12 days post-emergence and their Malpighian tubules (MTs) were dissected out in physiological saline (Petzel et al., 1987). Treatment conditions for female *Ae. aegypti* included 0.5hr and 24hr post blood meal (PBM), where ~10–15 females were blood fed at ~10–12 days old as described above. The females were allowed to feed for ~15min and then the time was initiated for each post-blood fed treatment with females that engorged on blood identifiable by their red abdomen. The rearing and treatments protocols were then kept consistent for each biological replicate.

Immunohistochemistry

Immunohistochemistry was completed for whole body (WB) adult female *Ae. aegypti*, to localize the different AaAQPs. Immunohistochemistry was also completed for isolated adult

female MTs from *Ae. aegypti*, localizing AaAQP1. For each treatment, NBF, 0.5hr PBM, 24hr PBM in WB and isolated MT sections, 4-5 individual mosquitoes (biological replicates) were studied. For each individual mosquito, 5–6 technical replicates were completed. Procedures were completed following previously published protocols (Akhter et al., 2017; Misyura et al., 2020; Sajadi et al., 2023). All tissues were fixed in Bouin's fixative and dehydrated in a series of ethanol and xylene. Paraffin embedded tissues were then sectioned using an Epredia HM325 manual microtome (Epredia, Kalamazoo, Michigan United States) and placed on Fisherbrand™ ColorFrost™ Plus Adhesion Microscope slides. Samples were processed such that tissue sections from non-blood fed (NBF), 0.5hr PBM, and 24hr PBM mosquitoes were placed on the same slide. The slides were processed in a multi-day procedure, with stepwise washes in 1xPBS. WB tissue sections were probed with one of the following; Anti-AaAQP1 affinity- purified primary antibody (1:1000 rabbit polyclonal antibody against CFFKVRKGDEESYDF, Genscript, NJ, USA) (Misyura et al., 2020), Anti- AaAQP2 affinity purified primary antibody (1:50 rabbit polyclonal antibody against CNGLGNTGLKENVQD, Genscript, NJ, USA) (Durant et al., 2021), Anti-AaAQP4 affinity purified primary antibody (1:500 rabbit polyclonal antibody against PAEQAPSDVGKSNQS, Genscript, NJ, USA) (Misyura et al., 2020), Anti-AaAQP5 affinity purified primary antibody (1:1000 rabbit polyclonal antibody against FRREVPEPEYNRELT, Genscript, NJ, USA) (Misyura et al., 2020), or Anti-AaAQP6 affinity purified primary antibody (1:50 rabbit polyclonal antibody against CSFRNMFLADKAKAE, Genscript, NJ, USA). WB tissue sections were also probed with a mouse monoclonal anti-a5 antibody for Na^+/K^+-ATPase (NKA) (Douglas Fambrough, Developmental Studies Hybridoma Bank, IA, USA, 1:10 dilution) as a membrane marker. Malpighian tubule tissue sections were probed with the same AaAQP1 antibody as previously listed, as well as a guinea pig anti-V1 antibody for V-type H^+-ATPase (VA)

(Ab 353-2, gifted by H. Wieczorek, Osnabruck, Germany, 1:5000 dilution) as a membrane marker, to specifically distinguish the apical membrane of the MTs. For secondary antibodies, a goat anti-rabbit AlexaFluor 594 (Jackson Immunoresearch) antibody was used (1:400 dilution) to visualize all of the AaAQPs, a goat anti-mouse secondary antibody conjugated to Cy2 (Jackson Immunoresearch) was used (1:500 dilution) to visualize NKA, and a goat anti-guinea pig AlexaFluor 488 (Jackson Immunoresearch) antibody (1:500 dilution) was used for VA. For control slides, the primary antibody was omitted to confirm an absence of staining where only secondary antibody was added. Similar controls were completed for all AaAQP primary antibodies used. Aside from omission of the primary antibody, control and experimental slides were treated identically. All samples were mounted on slides with ProLong Gold antifade reagent with DAPI (Life Technologies, Burlington, Ontario Canada). Slides were viewed and images captured using an Olympus IX81 fluorescent microscope (Olympus Canada, Richmond Hill, Ontario Canada) in combination with CellSense 1.12 Digital Imaging software (Olympus Canada). VA staining (AlexaFluor 488) and NKA staining (Cy2) were viewed using the Brightline GFP filter set and AaAQP staining (AlexaFluor 594) was viewed using the Brightline TRITC filter set (Olympus Canada). Exposure and gain settings were first determined by viewing sections from NBF mosquitoes and then the identical acquisition settings were used to view and capture images of the 0.5hr PBM and 24hr PBM mosquito sections all on the same slide. This same procedure was repeated for each slide of processed samples containing NBF and PBM mosquito sections. Identical acquisition settings were also used on control slides, to confirm that in the absence of primary antibody, no staining of AaAQPs was observed.

Probe Synthesis and Fluorescence *In-Situ* Hybridization

Investigation into the identification and localization of *aqp1* mRNA began with *de novo* sequencing of the gene in *Ae. aegypti*. The originally reported transcript of *aqp1* by Pietrantonio et al. (2000) was used as a template for this work, however it was discovered that annotations in the reference genome have reported different predicted transcript variants of the *aqp1* gene, which ultimately yield different C-termini of the AaAQP1 protein. Through standard PCR and rapid amplification of cDNA ends (RACE), I confirmed the *aqp1* gene sequence (GenBank Accession: PP003259), which matches the originally reported sequence by Pietrantonio et al. (2000) (see Supplementary Materials and Methods and Supplementary Results). I also used heterologous expression in human embryonic kidney (HEK293T) cells to verify our custom antibody against AaAQP1 specifically detects this water channel and the immunoblot results demonstrate a band size that matches that observed in protein extracted from MTs (Figure 2-S1). To synthesize a template suitable for fluorescence in situ hybridization (FISH) with DIG-labelled RNA probes, *aqp1* FISH forward and reverse primers (Table 2-S1) were used to amplify a 583bp *aqp1* fragment. Primers were designed using the Primer3 plugin in Geneious® 8.1.8 (Biomatters Ltd., Auckland, New Zealand). The *aqp1* gene was then amplified using bacterial cloning, by ligating the product into the pGEM T-Easy cloning vector (Promega, Madison, Wisconsin, USA) and the previously described standard protocol for cloning (Paluzzi et al., 2014) was followed to amplify the specific portion of the *aqp1* gene. During colony screening, *aqp1* FISH primers with added T7 promoter sequences were used to yield *aqp1* gene fragments with added T7 sequences for RNA probe synthesis. Confirmation of fragment size was done by running an agarose gel and colonies yielding PCR products with correct size were chosen for overnight inoculation and then plasmid DNA was isolated by standard column-based plasmid mini-prep (Bio Basic Inc., Markham, Ontario,

Canada), according to the protocol associated with the kit. Products were diluted 1:100 and ran on an agarose gel to confirm product size and band intensity. Then several replicate PCR reactions were completed to generate a large volume of both the anti-sense and sense DNA template products used for preparing RNA probes for experimental and control preparations, respectively. Products were pooled and purified using the Monarch PCR and DNA Cleanup Kit (5µg). The DNA template concentrations were determined using the Synergy Multi-Mode Microplate Reader (BioTek, Winooski, USA). To synthesize the digoxigenin (DIG) labeled RNA probes, the purified DNA products (either anti-sense or sense template) were added to a PCR tube with reaction buffer, a T7 polymerase, and DIG RNA Labeling Mix (Roche Applied Science, Mannheim, Germany), following the T7 RNA Polymerase Kit (New England BioLabs, Ontario, Canada). The tubes were incubated overnight at 37°C and the following day, the probe products were diluted 1:10 and were treated with a DNAse I to remove the DNA template. Products were run on a RNase-free non-denaturing agarose gel to confirm RNA probe size and band intensity. To prepare tissue samples for FISH, I followed a protocol previously described (Sajadi & Paluzzi, 2023). First, MTs were dissected from 3–4 day old adult female *Ae. aegypti* and transferred into a microcentrifuge tube containing 200µL of sterile PBS. The PBS was then replaced with freshly prepared 4% paraformaldehyde (PFA) and placed on a rotator for 1hr at room temperature to fix the tissues. The PFA was removed and tissues were washed five times with sterile PBT (sterile PBS + 0.1% Tween-20). Tissues were then quenched with 1% H_2O_2 for 20 min at room temperature to quench endogenous peroxidase activity which would otherwise result in elevated non-specific background fluorescence. Following this, tissue samples underwent permeabilization using 4% Triton X (960µl PBT + 40µL Triton X-100) with tubes set on a rocker at room temperature for 1hr. Tissues were then washed three times with PBT and followed by a second fixation with 4% PFA, with tissues

rotating at room temperature for 20 min. The MTs were then rinsed with 1:1 PBT:Hybridization solution (Hyb) (50% formamide, 5x SSC, 100ug/mL heparin, 100ug/mL sonicated salmon sperm DNA and 0.1% Tween-20), followed by a single wash with 100% Hyb. All subsequent incubations above or below room temperature were carried out on a thermocycler. Pre-Hyb solution was made during this time by aliquoting 250µL per sample of Hyb solution into PCR tubes which were then placed at 100°C for 5 min, followed by a 5 min incubation on ice. After removal of 100% Hyb from the sample tubes, pre-Hyb solution was added to the sample and tissues were incubated at 56°C for 1hr. During this time, 4ng/µL of anti-sense (experimental) or sense (control) probe was prepared in pre-Hyb solution and then applied to the tissues overnight at 50°C. Solutions for the following day were prepared at this time, including 100% Hyb, 3:1 Hyb : PBT, 1:1 Hyb : PBT, 1:3 Hyb : PBT, and 100% PBT, all incubated overnight at 50°C. The next day, tissues were washed in the following 50°C pre-warmed solutions; twice with 100% Hyb, once with 3:1 Hyb : PBT, once with 1:1 Hyb : PBT, once with 1:3 Hyb : PBT, and once with 100% PBT. Then, blocking of tissues to reduce non-specific staining was done with 1% blocking solution, PBTB (0.1g Molecular Probes block reagent; Invitrogen, Carlsbad, USA + 9.5mL PBT), rotating for 1hr at room temperature. The tissues were then incubated for 1.5hr with 1:200 mouse anti-DIG biotin-conjugated antibody (Jackson Immuno Research, West Grove, USA) diluted in PBTB, rotating at room temperature. From this step and onward, tissue samples were protected from light exposure. Tissues were then washed with PBTB four times, for 15 min each, rotating at room temperature. The tissue samples were then incubated with 1:50 HRP-streptavidin in PBTB for 1hr, rotating at room temperature to bind with the biotin-conjugated anti-DIG primary antibody. Tissues were once again washed with PBTB four times, for 15 min each, rotating at room temperature. Following manufacturer instructions, the samples were then incubated in 100µL diluted tyramide solution (Life

Technologies, Eugene, USA) for 5 min at room temperature and then immediately followed by the addition of 100µL of stop solution (Life Technologies, Eugene, USA). The solution was then removed, and ten PBS washes were completed, following by an overnight incubation with PBS, rotating at room temperature and protected from light. Tissue samples were mounted on slides the following day using in-house mounting media (1:1 PBS:glycerol containing 4µg/mL DAPI) and were imaged with the EVOS FL Auto Live-Cell Imaging System (Life Technologies, Burlington, ON). The fluorescence *in situ* hybridization experiments were completed in at least four biological replicates that each included experimental (anti-sense probe) and control (sense probe) preparations. Image acquisition settings were identical for all sample preparations including experimental samples treated with anti-sense probes and control samples treated with sense-probes.

Transmission Electron Microscopy and Immunogold Staining

Transmission electron microscopy (TEM) techniques and imaging was carried out by Dr. Ali Darbandi at the Nanoscale Biomedical Imaging Facility at The Hospital for Sick Children Research Institute – Peter Gilgan Centre for Research and Learning. The MTs from non-blood fed adult female *Ae. aegypti* were dissected in physiological saline (Petzel et al., 1987) and fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1M sodium cacodylate for 2hr at room temperature. The MTs were then rinsed in buffer and dehydrated in a graded ethanol series (50%, 70%, 90%, and 100%) for 20 min each at 4°C. Following this, two 1:1 ethanol/LR white acrylic resin changes were made for 30 min each and then tissue samples were embedded in LR white resin where blocks were left to cure overnight at 60°C. MT sections of 70nm thickness were cut on a Leica EM UC7 ultramicrotome (Ontario, Canada) and the sections were stained with uranyl

acetate and lead citrate. The grids were imaged using an electron microscope at the Nanoscale Biomedical Imaging Facility at The Hospital for Sick Children Research Institute – Peter Gilgan Centre for Research and Learning. For immunogold labeling, MT samples were prepared on TEM grids as previously described by Schwartzbach and Osafune (2010), completed at the Peter Giligan Centre for Research and Learning, Toronto CA. Then, each individual grid was placed on a 100µl droplet of 0.01M sodium citrate at 95°C for 15 min, followed by a brief 5 min cooling period and two washes with 0.15M glycine for 15 min and 1xPBS for 5 min. The grids were placed on individual 100µl droplets of antibody dilution buffer (ADB) blocking for 30 min at room temperature, followed by three washes with 1xPBS, 1% BSA, and 0.05% Tween®20 (Bio-Rad) for 5 min each and a 2hr wash in 1x PBS, 10% BSA, and 0.05% Tween®20 (Bio-Rad). The tissues were probed with 1:5 anti-AaAQP1 affinity purified rabbit polyclonal antibody in 1x PBS, 10% BSA, and 0.05% Tween®20 (Bio-Rad) overnight at 4°C. The grids were washed five times in 1x PBS with 1% BSA for 5 min each, followed by a 1hr incubation in a colloidal gold AffiniPure goat anti-rabbit secondary antibody conjugated with 18nm gold particles (Jackson Immunoresearch) in 1% BSA and 0.05% Tween®20 (1:10) (Bio-Rad). For control samples, they were treated identical to the experimental grids previous to this step. However, for control grids the primary anti-AaAQP1 antibody was omitted, to show its specificity relative to the treated samples. All tissue samples (control and experimental) were then treated with 2% glutaraldehyde in PBS for 5 min, before a series of washes with ddH_2O and the grids were stained with uranyl acetate and lead citrate. Imaging was completed at the same facility using the electron microscope.

Gel Electrophoresis and Western Blotting

Gel electrophoresis and western blotting was completed on protein samples isolated from adult female *Ae. aegypti* MTs. For each biological replicate (n=5–6), MTs were collected from 75 individual female mosquitoes under physiological saline (Petzel et al., 1987), from NBF, 0.5hr PBM, and 24hr PBM groups. Protein processing for all samples was done using the Mem-PER Plus Membrane Protein Extraction kit (Thermo Fisher Scientific, Burlington, Ontario Canada) as recently described (Picinic & Paluzzi, 2023). First, the cytosolic fraction of the MT protein was separated by adding 60µl of permeabilization buffer from the kit to each sample tube, with 1:200 protease inhibitor cocktail (Thermo Fisher Scientific, Burlington, Ontario Canada). The tubes were set to mix on a rotator at 4°C for 10min, before centrifugation at 16,000g for 15min at 4°C. The supernatant was collected as the cytosolic fraction. The remaining pellet was then re-suspended in 60µl of solubilization buffer with 1:200 protease inhibitor cocktail (as above) and set to incubate at 4°C for 30min. The samples were then centrifuged at 16,000g for 15min at 4°C and the supernatant was collected as the membrane fraction. Protein concentrations were measured using a Bradford assay, relative to bovine serum albumin (BSA) standards. The MTs samples were prepared for SDS-PAGE, where MT protein was combined with 50mM Tris buffer and 6x loading buffer [225mmol L^{-1} Tris-HCl, pH 6.8, 3.5% SDS, 35% glycerol, 12.5% ß-mercaptoethanol, and 0.01% bromophenol blue]. The samples were then heated for 5 min at 100°C, placed back on ice briefly, and centrifuged at 10,000g for 1 min. For each replicate, 5µg of protein were loaded in 12% SDS-PAGE gels. Following gel electrophoresis at 120V, a wet transfer was completed where MT protein was transferred onto a polyvinyl difluoride membrane at 90V for 2hr on ice in 1x transfer buffer (0.225g Tris, 1.05g glycine, 20% methanol in 1L of ddH_2O). Following the transfer, the membranes were placed in 5% milk blocking buffer (5g skim milk powder, 100mL 1x Tris-

buffered saline [TBS-T; 0.12g Tris, 0.9g NaCl, 0.1mL Tween®20 (Bio-Rad), 0.1mL NP-40 (Sigma-Aldrich) in 1L of ddH_2O]) at room temperature for 1hr on a rocker. The membranes were then probed with 1:1000 specific anti-AaAQP1 affinity-purified primary antibody (1.282 mg/mL) (Misyura et al., 2020) overnight at 4°C and then washed in 1x TBS-T the following day before incubation in horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody at 1:5000 in blocking buffer for 1hr at room temperature. After a second series of washes with 1x TBS-T, 2mL of prepared chemiluminescent Clarity™ Western ECL substrate kit (BioRad, Hercules, California, United States) was applied to each membrane for 5 mins. The membranes were viewed using a Chemi-Doc MP Imaging System (BioRad, Hercules, California, United States) and protein bands were normalized against Coomassie staining of total protein. The protein bands were analyzed using ImageJ 1.53a Software (USA) to quantify protein abundance, normalized to total protein.

Statistics

All data was analyzed using Prism® 9 software (GraphPad Software Inc., California, USA). Normalized protein abundance values were plotted as mean values ± standard error of the mean (SEM). For *Ae. aegypti* western blot graphs, data was normalized to non-blood fed control groups. An unpaired t-test was completed for each data set to determine if there were significant changes in AaAQP abundance values. The ROUT outlier test was used to determine if there were outlier values, which were removed accordingly.

2.4 Results

Localization of AaAQPs in Adult Female *Ae. aegypti*

AaAQP1, 2, 4, 5, and 6 were localized in whole body (WB) tissue sections of adult female *Ae. aegypti* mosquitoes with Na^+/K^+- ATPase (NKA) used as a membrane marker.

AaAQP1

AaAQP1 immunoreactivity was primarily detected in the Malpighian tubules (MTs) and the fat body (FB), under non-blood fed (NBF) conditions (Figure 2-1A). AaAQP1 immunoreactive staining in the MTs appears aggregated in some areas but, for the most part, is evenly distributed at the apical side of the cell, in the NBF conditions (Figure 2-1A). At 0.5hr post blood meal (PBM), staining of AaAQP1 appeared uniform at the apical membrane of the MTs, with stronger staining intensity compared to NBF mosquitoes (Figure 2-1B). At 24hr PBM, immunoreactive staining of AaAQP1 is similar to the 0.5hr PBM in the MTs (Figure 2-1C). AaAQP1 staining in the fat body appeared to decrease in intensity at 0.5hr PBM in comparison to the NBF group (Figure 2-1B). However, at 24hr PBM, staining intensity of AaAQP1 in the fat body appeared more intense in comparison to the 0.5hr PBM group, but less intense compared to the NBF condition (Figure 2-1C). Interestingly, prominent AaAQP1 immunoreactive staining was localized to the ovaries in both the 0.5hr and 24hr PBM groups (Figures 2-1B, C). Minimal immunoreactivity of AaAQP1 was detected in midgut sections visible in the 0.5hr PBM group (Figure 2-1B). Furthermore, AaAQP1 immunoreactivity was found in the apical membrane of the hindgut in the 0.5hr PBM group and as well as on the apical side of the cells in the rectal pads in the 24hr PBM group, showing intense staining (Figures 2-1B, B', C).

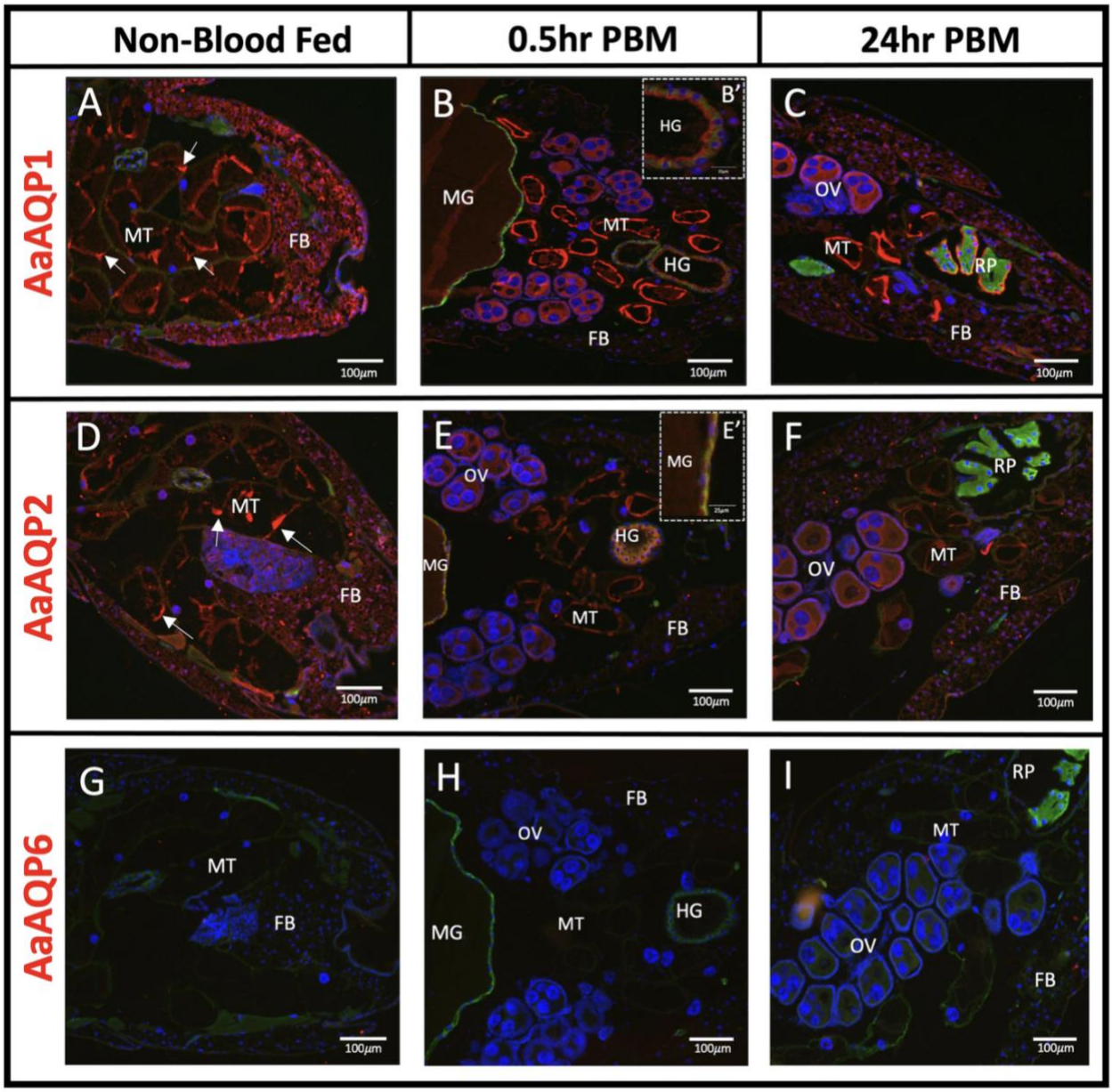

Figure 2-1. Localization of AaAQP1, 2, and 6 in whole body adult female *Aedes aegypti*. Immunohistochemical localization of water selective AaAQPs (red staining) in female *Ae. aegypti* mosquitoes (~10–12 days old). Each AaAQP (red staining) was localized under non-blood fed conditions, 0.5hr post blood meal conditions, and 24hr post blood meal conditions. Whole body sections of the abdominal segment immunolocalizing AaAQP1 in (**A–C**), AaAQP2 in (**D–F**), and AaAQP6 in (**G–I**). In (**B**), an inset image (**B'**) shows AaAQP1 membrane localization in the HG. In (**E**), an inset image (**E'**) shows AaAQP2 membrane localization in the MG. All images were taken at 10x magnification (n=3). The Na^+/K^+-ATPase (NKA) was used as a membrane marker (green staining) and nuclei were stained with DAPI (blue). MT, Malpighian tubules; FB, fat body; OV, ovaries; HG, hindgut; MG, midgut; RP, rectal pads. White arrows indicate aggregated staining of AaAQP in the MTs.

AaAQP2

Immunoreactivity for AaAQP2 was detected in the MTs, showing dispersed and apical membrane staining in the NBF group (Figure 2-1D). In both the 0.5hr and 24hr PBM groups, the overall staining intensity of AaAQP2 immunoreactivity remains the same but staining appears uniformly at the apical membrane, in comparison to the NBF group (Figure 2-1E). Immunoreactivity of AaAQP2 appeared intense in the fat body under NBF conditions, with a reduction in intensity in the 0.5hr PBM group, and a partial recovery of staining intensity in the 24hr PBM group (Figures 2-1D–F). Further, AaAQP2 immunoreactivity was found in the ovaries of the blood fed groups. Finally, AaAQP2 immunoreactive staining was also present in the midgut and hindgut tissue in the 0.5hr PBM, where it was co-localized with the NKA membrane marker appearing at the basolateral membrane (Figure 2-1E, E').

AaAQP6

Mosquito abdominal sections were void of any AaAQP6 immunoreactive staining in NBF and PBM conditions (Figures 2-1G–I).

AaAQP4

Immunoreactivity for AaAQP4 was detected in the MTs, under all three conditions (Figures 2-2A–C). Staining appeared at the apical membrane of the MTs, with similar intensity seen in the NBF and 0.5hr PBM groups, but an increase in staining intensity was observed in the MTs in the 24hr PBM group (Figure 2-2C). Immunoreactivity for AaAQP4 was also found in the fat body, appearing intense in the NBF group, reduced in the 0.5hr PBM group, and partially recovered in the 24hr PBM group (Figures 2-2A–C). AaAQP4 was also found in the hindgut of 0.5hr and 24hr

PBM tissue sections, with relatively low immunofluorescence (Figures 2-2B, C). Additionally, minimal immunoreactivity was observed in the ovaries at 0.5hr and 24hr PBM (Figures 2-2B, C).

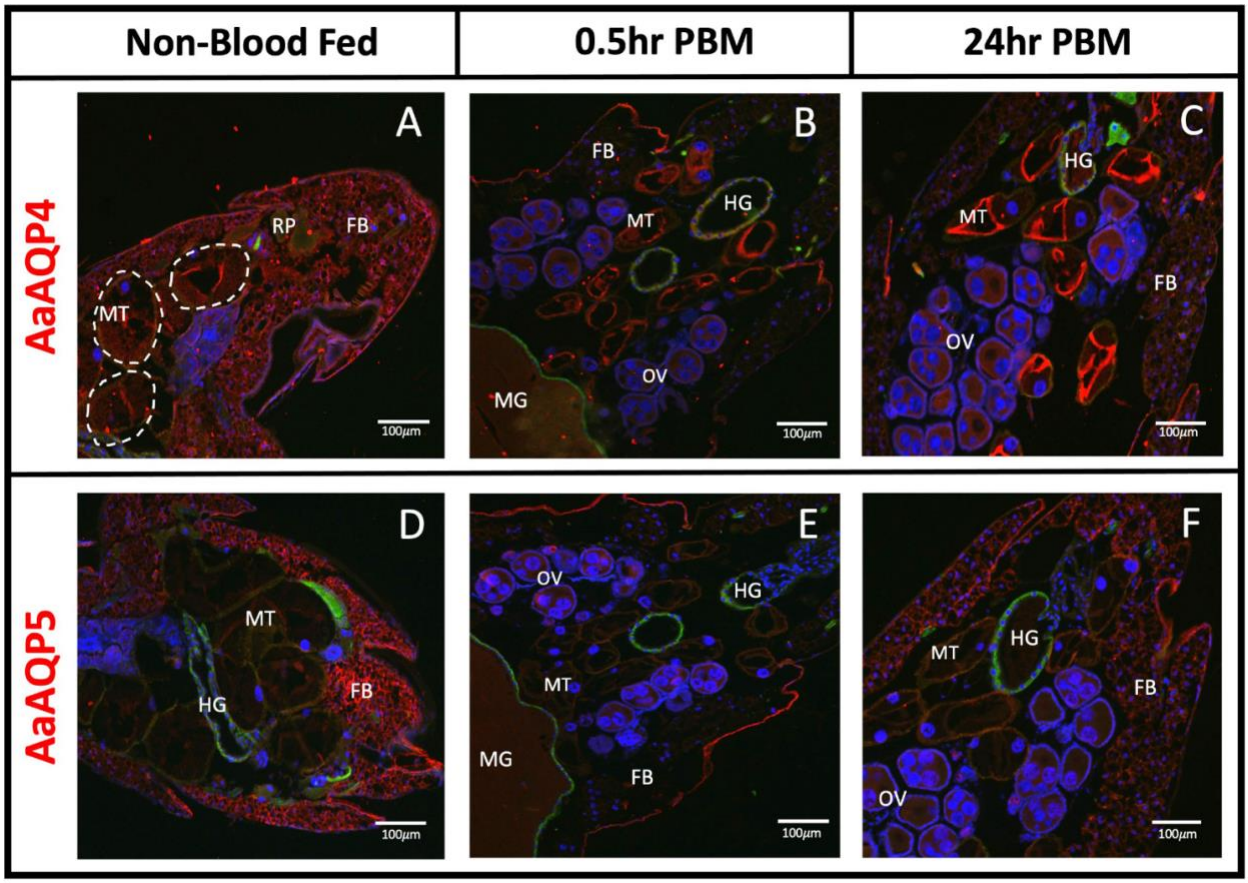

Figure 2-2. Localization of AaAQP4 and 5 in whole body adult female *Aedes aegypti*. Immunohistochemical localization of entomoglyceroporin AaAQPs (red staining) in female *Ae. aegypti* mosquitoes (~10–12 days old). AaAQP4 and 5 (red staining) were localized under non-blood fed conditions, 0.5hr post blood meal conditions, and 24hr post blood meal conditions. Whole body sections of the abdominal segment immunolocalizing AaAQP4 in (**A–C**) with basolateral membrane of MTs encircled in dashed line and AaAQP5 in (**D–F**). All images were taken at 10x magnification (n=3). The Na^+/K^+-ATPase (NKA) was used as a membrane marker (green staining) and nuclei were stained with DAPI (blue). MT, Malpighian tubules; FB, fat body; OV, ovaries; HG, hindgut; MG, midgut; RP, rectal pads. White arrows indicate aggregated staining of AaAQP in the MTs.

AaAQP5

Immunoreactivity for AaAQP5 in NBF mosquitoes appeared relatively low and uniformly distributed at both the apical and basolateral membranes of the MTs (Figure 2-2D). AaAQP5 immunoreactive staining was found mainly in the fat body in NBF conditions; however, staining was absent in the fat body in the 0.5hr PBM but was localized to the epidermis of the cuticle (Figure 2-2E). At 0.5hr PBM, there was some localized staining seen in the ovaries (Figure 2-2E). At 24hr PBM, AaAQP5 immunoreactive staining in the fat body returned (Figure 2-2F) with no additional staining observed in the midgut, ovaries and hindgut.

AaAQP1 is Expressed by Principal Cells in the MTs of Adult Female *Ae. aegypti*

Sections of isolated MTs confirmed that AaAQP1 staining is present on the apical membrane of the epithelia (Figures 2-3A–I), where co-localization with the V-type H^+-ATPase (VA) can be observed in the 0.5hr PBM mosquito tubules (Figure 2-3E). Importantly, *in situ* hybridization using an anti-sense probe demonstrated that *aqp1* mRNA in adult female *Ae. aegypti* MTs was associated with the larger and more abundant principal cells. In the proximal tubule, *aqp1* transcript was localized in the cytosol around the nuclei of principal cells (Figure 2-4A) while *aqp1* transcript staining was absent in stellate cells (Figure 2-4B). In the distal portion of the adult female tubule, very strong *aqp1* transcript levels were observed with consistent and exclusive staining to the principal cells of the MTs (Figures 2-4A, B). No staining was observed in MTs treated with the sense probe (Figure 2-4C), confirming the localization of *aqp1* transcript detected with the anti-sense probe (Figures 2-4A, B).

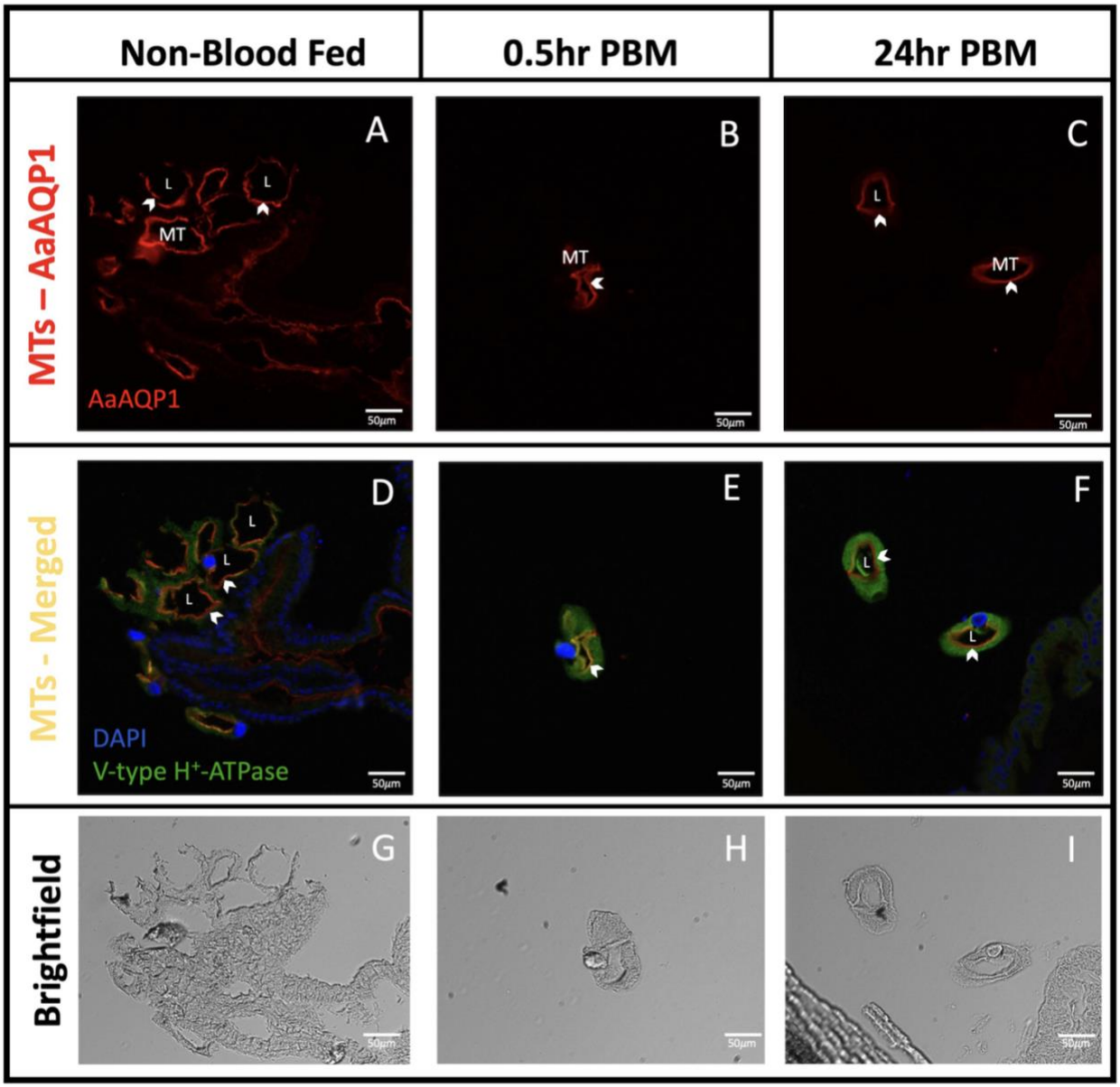

Figure 2-3. Localization of AaAQP1 in Malpighian tubules of adult female *Aedes aegypti*. Immunohistochemical localization of AaAQP1 (red staining) in isolated Malpighian tubules (MTs) of female *Ae. aegypti* (~10–12 days old) MTs were isolated from non-blood fed mosquitoes, and mosquitoes that were fed blood either 0.5hr or 24hr prior. (**A–C**) shows localization of AaAQP1 at the apical membrane of the MTs. White arrows indicate the apical membrane of the MTs. (**D–F**) shows localization of AaAQP1 at the apical membrane of the MTs, merged with staining observed for V-type H^+- ATPase (VA), which was used as an apical membrane marker (green staining). Co-localization of AaAQP1 with VA appears yellow/orange in colour. (**G–I**) shows brightfield images of MT sections, which was used to identify apical and basolateral membranes. All images were taken at 20x magnification (n=4) and nuclei were stained with DAPI (blue).

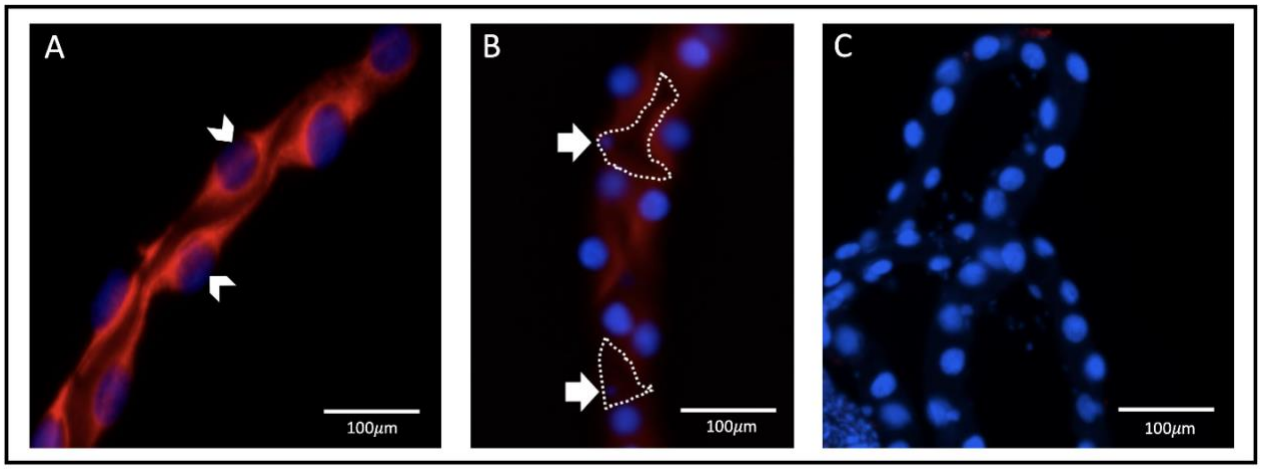

Figure 2-4. *aqp1* localization with fluorescence *in situ* hybridization in Malpighian tubules. Localization of *aqp1* mRNA (red staining) in the Malpighian tubules (MTs) of female *Ae. aegypti* mosquitoes (~3–4 days old), using fluorescence *in situ* hybridization. Tubules were dissected from non-blood fed adult female mosquitoes (n=3). (**A**) *aqp1* localization in the proximal tubule, found associated with the principal cell nuclei, indicated by the white arrow. (**B**) *aqp1* localization in the distal tubule, found associated with the principal cells. Stellate cell nuclei indicated by the white arrow, with cell structure outlined in dashed lines, showing an absence of staining in encircled region. (**C**) MTs where an absence of *aqp1* staining is seen, after incubation and treatment with the sense (control) probe.

Sub-Cellular Localization of AaAQP1 in MTs of Adult Female *Ae. aegypti*

Immunogold labeling and transmission electron microscopy were used to localize AaAQP1 at the sub-cellular level in MTs of adult female mosquitoes. In NBF MTs, immunogold particles were predominantly localized to the principal cells (PCs) and appear scattered throughout the apical region in the brush border (Figures 2-5A, B). Additionally, some immunogold particles were also detected in reduced brush border of the less abundant stellate cells (SCs) (Figure 2-5C). Control grids, where the primary antibody was omitted, showed a complete absence of gold particles, relative to the presence of gold particles in the sections treated with both primary and secondary antibodies (Figure 2-S2).

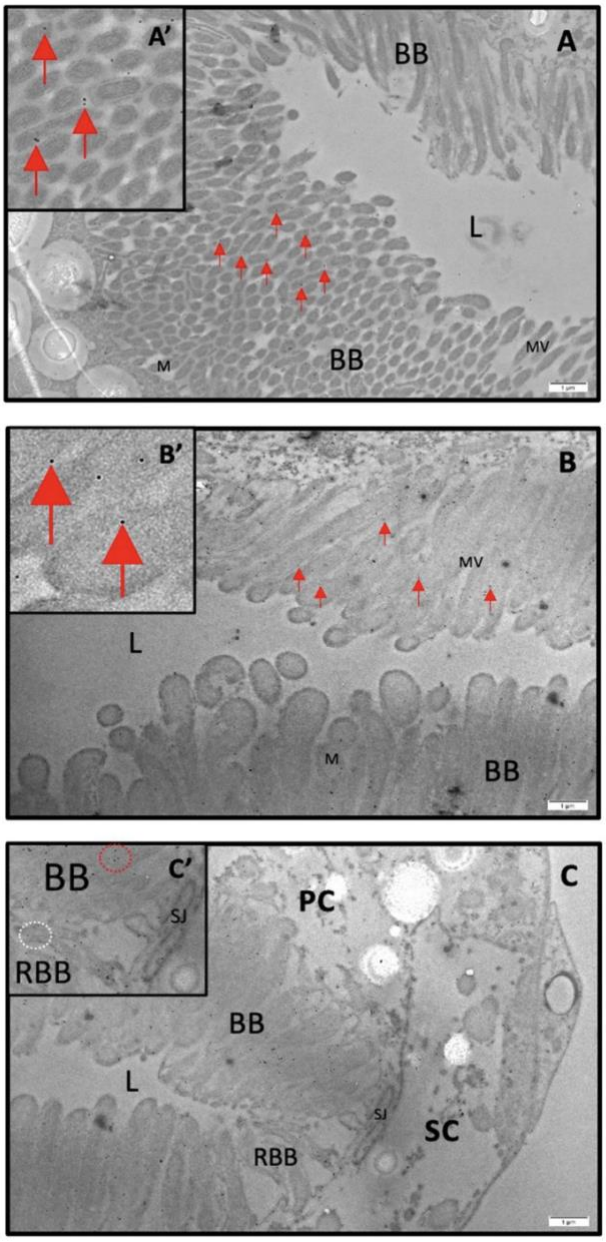

Figure 2-5. Immunogold staining of AaAQP1 in female Malpighian tubules of *Aedes aegypti*. Subcellular localization of AaAQP1 in adult female *Ae. aegypti* (~10–12 days old) Malpighian tubules, using transmission electron microscopy (TEM) with immunogold staining (n=3). AaAQP1 staining is represented by the presence of 18nm gold particles, indicated by the red arrows. Inset images magnify individual gold particles. (**A, B**) Cross sections of non-blood fed mosquito Malpighian tubules, showing the apical membrane of the principal cells. Gold particles appear dispersed through the membrane fraction specifically within the brush border, with no pattern of gold particle collection. (**C**) Cross section of non-blood fed mosquito Malpighian tubules showing predominant gold particle presence in principal cells; however, there is some presence of gold particles also within the reduced brush border of the stellate cells. BB, brush boarder; L, lumen; M, mitochondria; MV, microvilli; RBB, reduced brush boarder.

Quantitative Assessment of AaAQP1 Protein Expression in MTs of Adult Female *Ae. aegypti*

A ~25kDa band representing the putative monomer for AaAQP1 was observed in western blots of protein homogenates from MTs of adult female *Ae. aegypti* probed with custom anti-AaAQP1 antibody (Figure 2-6). Blood feeding did not result in any changes in AaAQP1 protein abundance in the MTs. There were no significant differences between AaAQP1 levels in MTs of the 0.5hr PBM and 24hr PBM groups, normalized to the NBF control. Therefore, there were no differences found in AaAQP1 protein in post blood fed female MTs relative to the NBF control. Heterologous expression of the AaAQP1 protein in human embryonic kidney cells yielded a protein of identical size, ~25kDa, confirming the specificity of the custom antibody utilized in this study (Figure 2-S1).

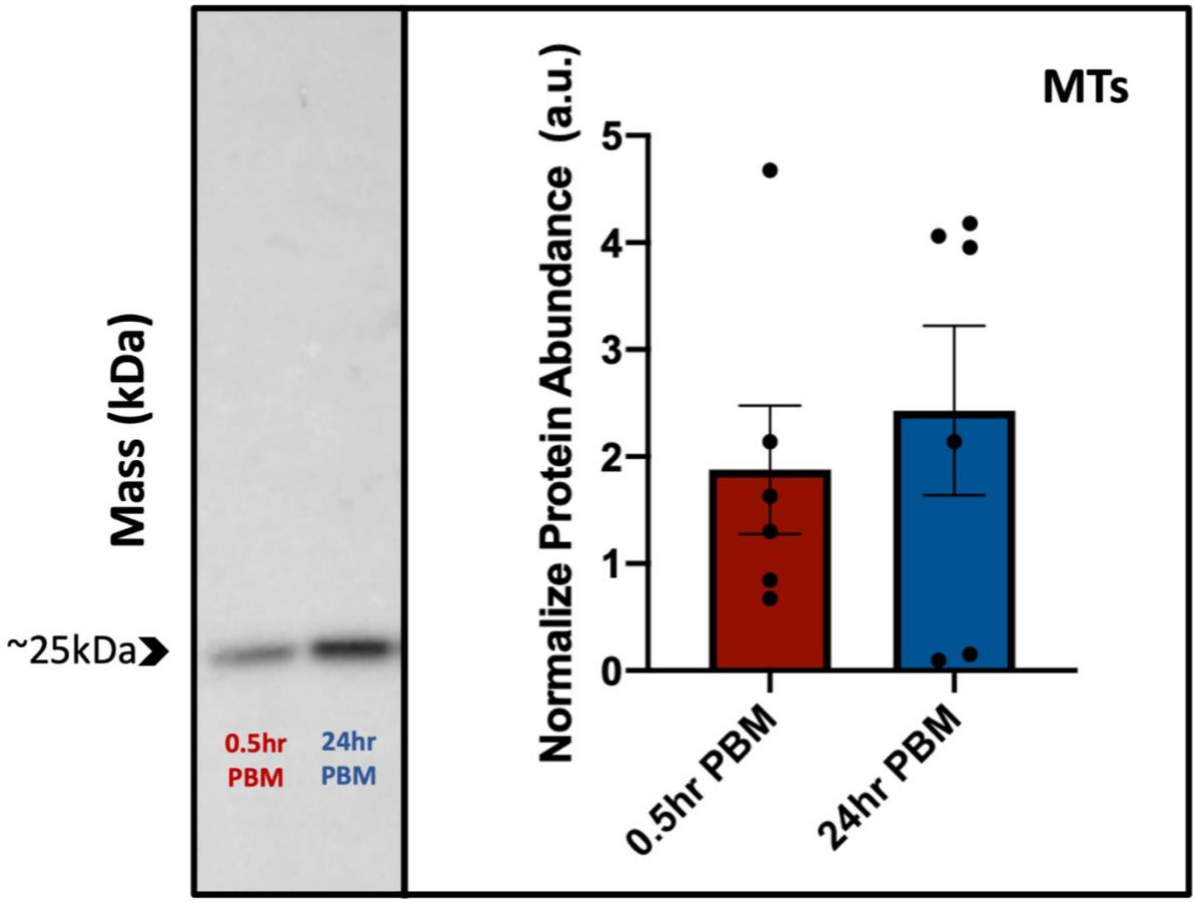

Figure 2-6. Protein abundance of AaAQP1 in female *Aedes aegypti* Malpighian tubules post blood meal. Normalized protein abundance of AaAQP1 in female *Ae. aegypti* Malpighian tubule tissue samples, in 0.5hr post-blood meal and 24hr post-blood meal groups. Values were normalized to the AaAQP1 immunoreactive band detected in Malpighian tubule protein samples from non-blood fed control mosquitoes. Malpighian tubules were isolated from ~75 individuals, for each biological replicate (n=5–6). A ~25kDa band (putative monomer) was detected for AaAQP1, and an unpaired t-test showed no significant changes in the protein abundance between the two blood fed treatment groups ($p>0.05$).

2.5 Discussion

In this study, I immunolocalized the aquaporins AaAQP1, 2, 4, 5, and 6 in organs of the adult female mosquito, *Ae. aegypti*, before and after a blood meal. Furthermore, a primary aim of this study was to better understand the function of the primary water channel, AaAQP1, in the adult female *Ae. aegypti* mosquito. For this reason, I focused more extensively on AaAQP1 expression at both the protein and transcript levels in the Malpighian tubules since earlier studies have shown its mRNA is enriched in this organ (Drake et al., 2010).

AaAQP Localization in the Fat Body

The transcript expression of AaAQPs in the fat body was previously reported where relatively low levels of *aqp1–6* mRNA were found (Drake et al., 2010; Price et al., 2011). My data demonstrated immunolocalization of AaAQP1 (Figures 2-1A–C), 2 (Figures 2-1D–F), 4 (Figures 2-2A–C) and 5 (Figure 2-2D–F) in the fat body, but did not observe AaAQP6 immunoreactive staining in this tissue (Figure 2-1G–I). The intensity of AaAQP1, 2, 4, and 5 immunoreactive staining in the fat body decreased at 0.5hr PBM compared to NBF suggesting that blood feeding leads to lowered expression of these aquaporins in the fat body. Using transcriptomic analysis, Price at al. (2011) detected three of the six mosquito aquaporins expressed in the fat body, including AaAQP1, 4, and 5 (Table 2-1). Notably, Price et al. (2011) reported an apparent decrease in the transcript abundance of AaAQP4 and AaAQP5 in the fat body 24 hours after a blood meal. My results are consistent with this data and furthermore suggest that there is an abrupt decrease in AaAQP4 and AaAQP5 protein expression after a blood meal (Figure 2-2). It has been hypothesized that a decrease in overall transporters PBM in *Ae. aegypti*, including AaAQP4 and 5, may be accounted for by the increase in transporters present in yolk proteins PBM, during embryogenesis

(Price et al., 2011). On the other hand, AaAQP1 transcript was not detected in NBF fat body although low levels were detectable in this tissue at 24hr PBM (Price et al., 2011), which is consistent with my observations of the partial recovery of AaAQP1 immunoreactive staining at 24hr PBM (Figure 2-1C). In addition, the expression of an AaAQP2 ortholog, AgAQP1 in the malaria vector, *Anopheles gambiae*, found a significant increase in the fat body, 48hr PBM (Liu et al., 2011). A similar trend in the expression of AaAQP2 is apparent in the fat body where staining intensity is largely diminished at 0.5hr PBM followed by partial recovery at 24hr PBM (Figures 2-1D–F). These findings suggest that blood feeding may lead to a temporary short- term reduction (~0.5hr PBM) in AaAQP1 and AaAQP2 expression in the fat body of mosquitoes but, the expression of these two aquaporins partially recovers within a day or two of blood feeding. It is possible that AaAQP1 and 2 proteins are more immediately relevant for the MTs or HG, during post-prandial diuresis and early-stage blood meal digestion when the animal is dealing with a large load of water associated with the 0.5hr PBM female mosquitoes. During late-stage blood meal digestion, such as 24hr PBM, increased AaAQP1 and 2 staining in the fat body might relate to roles in water transport during egg maturation.

The fat body is an important multifunctional organ for female mosquitoes participating in nutrient storage, metabolic homeostasis, and production of yolk precursor proteins for egg production (Chung et al., 2017). In the previtellogenic female mosquito that has not blood fed, the fat body stores nutrients, and the trophocytes (i.e. fat body cells) contain numerous, relatively large lipid droplets which are synthesized at the endoplasmic reticulum (Attardo et al., 2005; Price et al., 2011; Pinch et al., 2021). The fat body trophocytes are activated by a blood meal, shifting their function into yolk precursor protein (YPP) factories, which includes vitellogenin and lipophorin that are secreted into the haemolymph where they are then taken up by developing oocytes in the

ovaries (Attardo et al., 2005; Price et al., 2011; Pinch et al., 2021). During this time of YPP synthesis, the size of lipid droplets fluctuates, but by 24hrs PBM, they are reduced in size and by 48hrs PBM, they have recovered to pre-blood meal sizes (Pinch et al., 2021). Furthermore, by 36hrs PBM, the fat body trophocytes revert back to a nutrient storage organ (Attardo et al., 2005). Abundant immunoreactive staining of AaAQP4 and 5 correlates to the nutrient storage stage of the fat body trophocytes where lipid synthesis and storage are likely occurring at higher rates. When trophocytes are activated to synthesize YPPs for secretion into the haemolymph, AaAQP4 and 5 immunoreactive staining is low. Since AaAQP4 and 5 are entomoglyceroporins which have been shown to transport glycerol in a heterologous system, their function in the trophocytes may be to facilitate glycerol transport for the synthesis of lipids when the main function of the fat body is nutrient storage (Drake et al., 2015). AaAQP1 and 2 are the orthologs of *Drosophila* DRIP and PRIP, respectively and both have been shown to exhibit a preference for water transport (Drake et al., 2010; Drake et al., 2015). The intensity of AaAQP1 and AaAQP2 immunoreactive staining suggests that similar to the entomoglyceroporins, their most abundant expression in the fat body coincides with nutrient storage and metabolic function of the trophocytes. Since lipid metabolism requires water, these aquaporins may play a critical role in regulating water content of the trophocytes as they synthesize fats. Comparatively, diminished AaAQP1 and 2 immunoreactive staining coincides with when trophocytes are synthesizing YPPs and secreting these proteins and stored fats into the haemolymph, a time when water regulation may not be as vital. AaAQP1 and 2 immunoreactive staining recovers as the fat body reverts back to nutrient storage and lipid synthesis.

AaAQP Localization in the Ovaries

The ovaries are the site for oocyte production and maturation. In *Ae. aegypti*, follicles in the ovaries containing oocytes are kept in a previtellogenic state until a blood meal is imbibed (Zhang et al., 2023). Upon blood feeding the ovaries take up proteins and fats from the haemolymph, originating mainly from the fat body, to produce mature oocytes (Troy et al., 1975; Pinch et al., 2021). In general, oocyte maturation is also accompanied by water uptake in follicles and the AaAQP2 ortholog of *Bombyx mori* was implicated in this process (Maruyama et al., 2015). In particular, Maruyama et al. (2015) showed that the highest expression of the AaAQP2 ortholog in *Bombyx mori* was during vitellogenesis, aiding in water transport and hydration of oocytes. Furthermore, the localization of an AaAQP1 ortholog in *B. mori* oocytes suggests that this AQP may function to prevent dehydration, as its highest expression is found during late-stage oogenesis known as choriogenesis, when the vitelline envelope is developed to protect the egg from water loss, which occurs ~2 days pre-eclosion (Maruyama et al., 2015). In the current study, AaAQP1 (DRIP ortholog) and AaAQP2 (PRIP ortholog) immunoreactive staining was identified in the ovaries of PBM mosquitoes (Figure 2-1), which is consistent with earlier reported transcript expression of these AQPs in the ovaries (Drake et al., 2015). Additionally, it was found that AgAQP1A, the ovarian-specific AaAQP2 ortholog in *Anopheles gambiae*, was found to be abundant in non-blood fed female ovaries, with significant increases in abundance 24–48hr post blood meal (Tsujimoto et al., 2013). Earlier, Liu et al. (2011) examined AgAQP1 abundance in vivo and found that expression was significantly increased 48hr post blood feeding. Together, this suggests that both AaAQP1 and AaAQP2 may participate in water uptake during oocyte maturation or prevention of dehydration of oocytes in *Ae. aegypti*. Further studies to pinpoint the exact localization of these AQPs in the ovaries of *Ae. aegypti* is needed. Additionally, the current

study found an entomoglyceroporin ortholog to the *D. melanogaster* Eglp1, known as AaAQP4, was immunolocalized in the ovaries after blood feeding (Figure 2-2). Relatively low levels of AaAQP4 transcript has been shown to be present in adult female *Ae. aegypti* ovaries, pre- and post-blood meal (Drake et al., 2010). A role for AaAQP4 in the ovaries is still unclear and requires further investigation.

AaAQP Localization in the Midgut

The posterior midgut of the female mosquito stores and digests the blood meal and is the gut region where fluid and nutrients are absorbed into the haemolymph (Cui & Franz, 2020; Hixson et al., 2022). Immunoreactivity for AaAQP2 was found on the haemolymph-facing basolateral surface of the posterior midgut in 0.5hr PBM mosquitoes (Figure 2-1E, E'). Since the large volume load imbibed with a blood meal is absorbed into the haemolymph and subsequently secreted by the Malpighian tubules, basolaterally localized AaAQP2 may participate in transporting water across the midgut epithelium into the haemolymph during this time. Similarly, it was found that the PRIP ortholog, BcAQP2 in the insect *Bactericera cockerelli* was found to be highly expressed in the gut of the animal, likely responsible for transport of water post-food digestion (Ibanez et al., 2014). It has also been recently found that in *Ae. aegypti*, *aqp2* mRNA is significantly downregulated 3hr PBM (Holmes et al., 2023). In addition, some immunoreactivity of AaAQP1 was found on the lumen-facing apical surface of the posterior midgut in 0.5hr PBM mosquitoes. It is possible that AaAQP1, a water-specific aquaporin, is important in the midgut epithelial tissue post blood feeding, to aid in removal of water from the midgut. Furthermore, various DRIP orthologs have been identified in the midgut of insects such as in larval midge *Belgica antarctica* (Yi et al., 2011), the rice striped stem borer, *Chilo suppressalis*, with confirmation of expression

in whole adult female animals (Lu et al., 2018), 5th-instar *Rhodnius prolixus* posterior midgut samples (Staniscuaski et al., 2013), and in the columnar midgut cells of the silkworm *Bombyx mori* (Maruyama & Azuma, 2015).

AaAQP Localization in the Hindgut and Rectal Pads

The hindgut of adult female *Ae. aegypti* contains an elongated anterior segment, known as the ileum, and a more posterior region called the rectum, which contains six luminal rectal pads. Both segments have been implicated in ion and water absorption through the localization of major ion-transporting pumps such as Na^+/K^+- ATPase and V-type H^+-ATPase (Patrick et al., 2006). After a blood meal, alterations to the basolateral membrane of rectal pads suggest an increase in absorptive activity (Hopkins, 1967). Not surprisingly, transcript expression of AaAQP1 and AaAQP2 was detected in the ileum and rectum of female mosquitoes (Drake et al., 2015) and I detected immunoreactivity of both in the hindgut (Figure 2-1). Specifically, AaAQP1 was detected on the apical membrane of the ileum and in the apical membrane of the rectal pads (Figure 2-1) where it could play a role in absorbing water from the gut contents prior to excretion. In particular, the strong immunoreactive staining on the apical membrane of rectal pads 24hr PBM fits with the implied increased absorptive activities during this time (Piermarini et al., 2017; Kandel et al., 2022). AaAQP2 was detected in the ileum at 0.5hr PBM but, I could not verify if AaAQP2 was in the rectal pads because I did not have sections of rectal pads for NBF or 0.5hr PBM. Notably, AaAQP2 immunoreactivity was not observed in the rectal pads at 24hr PBM (Figure 2-1F), which is consistent with the very lower transcript abundance in the rectum reported earlier (Drake et al., 2015). Taken together, these findings indicate that AaAQP2 may also aid in water absorption shortly after the mosquito takes a blood meal.

AaAQP Localization in the Malpighian Tubules

The Malpighian tubules (MTs) in insects are responsible for the production of ion-rich primary urine through the secretion of ions and water from the haemolymph. Using immunohistochemistry, I localized AaAQP1 (Figures 2-1A–C), 2 (Figures 2-1D–F), 4 (Figures 2-2A–C), and 5 (Figures 2-2D–F) in the MTs of adult female *Ae. aegypti* while AaAQP6 immunoreactivity was not detected in this organ (Figures 2-1G–I). I detected AaAQP2 immunoreactivity at the apical membrane of the MTs which manifested as discrete aggregated staining in the NBF group and more evenly distributed staining around the luminal circumference of the MT in post-blood fed mosquitoes (Figures 2-1E, F). There is a possibility that the aggregated staining is indicative of AaAQP2 expression in the small stellate cells of the MTs in NBF mosquitoes, although the evenly distributed staining observed after blood feeding is more akin to expression in the principal cells. AgAQP1, the AaAQP2 ortholog in *Anopheles gambiae*, was localized to the proximal principal cells as well as the distal stellate cells, in the MTs of NBF mosquitoes (Liu et al., 2011; Tsujimoto et al., 2013). In *Drosophila melanogaster* MTs the expression of the ortholog of AaAQP2 (PRIP) is enriched in the stellate cells but is also expressed by principal cells (Cabrero et al., 2020; Xu et al., 2022). The transition from aggregated staining to an even distribution of AaAQP2 at the apical membrane of the MTs after blood feeding suggests that expression of AaAQP2 in the MTs may become associated with the stellate cells as well as the principal cells, to deal with an increased demand for water transport through the tubule epithelium. This may be necessary because principal cells are the more abundant cell type in the MTs of *Ae. aegypti* making up the majority of the tubule, whereas the relatively small volume that stellate cells make up may not be able to handle the large volume of fluid secretion that occurs after blood feeding (Satmary & Bradley, 1984).

Entomoglyceroporin immunoreactivity was detected in the principal cells of the Malpighian tubules. AaAQP4 immunoreactivity was localized to the apical side while AaAQP5 immunoreactivity appeared at the basolateral membrane (Figure 2-2). Two entomoglyceroporin orthologs are also expressed in the principal cells of *Drosophila* MTs (Cabrero et al., 2020). In *Aedes albopictus* AQP4 transcript abundance is decreased with blood feeding in the Malpighian tubules while there is a short-lived increase in AQP5 transcript abundance (Esquival et al., 2014). Conflicting observations have been reported in *Ae. aegypti* with both a short-lived increase in AaAQP4 transcript or a more sustained increase (Drake et al., 2010; Drake et al., 2015). On the other hand, a sustained increase in AaAQP5 transcript was reported and later confirmed (Drake et al., 2010; Drake et al., 2015). It appears that the increase in AaAQP4 mRNA may result in a greater protein abundance since I detected an increased intensity of AaAQP4 immunoreactive staining at 24hr PBM. I did not detect any changes in the intensity of AaAQP5 immunoreactive staining after blood feeding, which might suggest that the brief increase in *aqp5* mRNA seen by Esquivel et al. in 2014, is required to maintain the baseline AaAQP5 protein levels. AaAQP5 was shown to be an efficient water transporter in a heterologous system and its knockdown increases adult mosquito survival under desiccation stress and reduces fluid secretion by MTs in larvae (Drake et al., 2015; Misyura et al., 2017). Previous studies using an antibody generated against *Cicadella viridis* AQPcic reported that AQP1 expression is confined to the tracheolar cells of *Ae. aegypti* female MTs (Pietrantonio et al., 2000; Duchesne et al., 2003); however, the antigen region which this antibody was generated against shares relatively low identity (4/15 residues, ~27%) with the *Ae. aegypti* AaAQP1. However, in the present study, using a custom AaAQP1 affinity-purified antibody, immunoreactivity was detected at the apical membrane of the MTs, showing aggregated staining under NBF conditions followed by distinct continuous staining along the circumference

of the apical membrane of the MTs in PBM female mosquitoes (Figures 2-1A–C). AaAQP1 antibody specificity was confirmed by de novo confirmation of the full *aqp1* coding sequence with rapid amplification of cDNA ends (RACE), followed by the heterologous expression of AaAQP1 in HEK293T cells (see Supplementary Results). Through western blotting using our custom AaAQP1 antibody, expression of AaAQP1 in HEK293T cells revealed a single ~25kDa band, representing the putative AaAQP1 monomer (Figure 2-S1). Using FISH, the *aqp1* transcript was localized to the principal cells in NBF female MTs. Furthermore, subcellular localization of AaAQP1 in NBF female MTs using immunogold labeling found AaAQP1 dispersed within the principal cells, specifically to the extensive brush boarder which is thinner in stellate cells (Figure 2-5). In both *Ae. aegypti* and *Anopheles gambiae*, transcript abundance of AQP1 is significantly higher in blood fed versus sugar fed mosquito MTs suggesting that AaAQP1 is important in voiding the excess water load imbibed with a blood meal (Drake et al., 2015; Overend et al., 2015), which is supported by reduced diuresis in AaAQP1 knockdown mosquitoes (Drake et al., 2010). My findings show that staining of AaAQP1 in the MTs appears uniformly at the apical membrane after a blood meal but no changes in immunoreactive staining intensity were observed. TEM with immunogold labeling confirmed that AaAQP1 is predominantly localized to the principal cells of the MTs, although some presence was detected in the smaller and less abundant stellate cells. Through western blotting, I confirmed that the protein abundance of AaAQP1 in MTs is unchanged after blood feeding in comparison to non-blood fed mosquitoes (Figure 2-6). This raises the possibility that activity of AaAQP1 is regulated by translocation to the membrane and/or through post translational modifications rather than expression. A recent study by Kandel and colleagues (2022) examined AQP regulation in *Ae. aegypti* by phosphorylation and concluded that it is more

likely that AQPs are regulated by membrane translocation during the post-blood feeding period in adult female mosquitoes.

In conclusion, this study established a comprehensive localization of AaAQP1, 2, 4, 5, and 6 in adult female *Ae. aegypti*, during NBF, 0.5hr PBM, and 24hr PBM conditions. In particular, AaAQP1 (DRIP ortholog) was localized to the MTs, fat body, ovaries, and hindgut pre- and post-blood feeding. It was also found that AaAQP1 is specifically localized to the principal cells of the MTs. Furthermore, AaAQP2 (PRIP ortholog) was localized to the MTs, fat body, ovaries, midgut, and hindgut. The entomoglyceroporin AaAQP4 (Eglp1 ortholog) was found primarily in the MTs, with immunoreactivity also observed in the fat body, ovaries, and hindgut. The entomoglyceroporin AaAQP5 (Eglp2 ortholog) was found primarily in the fat body, with minimal immunoreactivity also found in the MTs and ovaries. AaAQP6 immunoreactivity was found to be absent throughout the abdominal tissue sections, which aligns with earlier studies that demonstrated enrichment of this aquaporin within the foregut of the alimentary canal (Drake et al., 2010) that was not investigated herein. Further studies on the role of each AaAQP in the various organs of female *Ae. aegypti* will provide a better understanding of the mechanisms by which water and other solutes are transported within mosquitoes.

2.6 References

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2.7 Supplementary Materials and Methods

AaAQP1 Sequence Confirmation and Heterologous Expression in HEK293T Cells

The current *aqp1* (AAEL024675) gene model in the improved reference genome of *Aedes aegypti* (Matthews et al., 2018) contains two annotated transcripts (AAEL024675-RA and AAEL024675-RB) that do not match the cloned sequence previously reported (Pietrantonio et al., 2000), leading to dissimilar deduced proteins. Importantly, these predicted transcripts yield a deduced protein that excludes ~90 amino acids on the N-terminal half of the protein and possessing a different C-terminus, which has implications for the custom antibody we designed that used a specific antigen on the C-terminus of the originally reported sequence (Pietrantonio et al., 2000). In order to independently determine and confirm the 3' end of the *aqp1* gene in *Ae. aegypti*, specific primers were designed (Table 2-S1). Malpighian tubule RNA was isolated from 4-day old adult females and cDNA was synthesized. Utilizing various reverse primer combinations (Table 2-S1), a partial *aqp1* product containing the full open reading frame (ORF) and 273bp of the 3' untranslated region, UTR) was successfully amplified by PCR using Q5® High-Fidelity DNA Polymerase and base accuracy was confirmed by Sanger sequencing revealing a partial *aqp1* matching that previously reported (Pietrantonio et al., 2000). To improve the gene model and determine the transcripts derived from the *aqp1* gene, the FirstChoice® RLM-RACE kit (Thermo Fisher Scientific, Mississauga, ON) was utilized to amplify the complete 5'-UTR. Malpighian tubules were dissected from 4-day old adult female *Ae. aegypti* (n = 100) and total RNA was purified using the Monarch Total RNA Miniprep Kit (New England Biolabs, Whitby, ON). The RACE kit protocol was optimized for a small-scale reaction requiring 1µg of total RNA. For each nested PCR, both positive and negative control reactions were included involving two gene-specific primers with and without the template, respectively. Following successful amplification

of *aqp1* with the 5' inner RACE primer (provided in the kit) and two nested gene-specific reverse primers (Table 2-S1), PCR products were purified using the Monarch DNA Gel Extraction kit (New England Biolabs, Whitby, ON) and ligated to pGEM T-Easy cloning vector. An aliquot of this ligation reaction was used to transform DH5α-strain *Escherichia coli* and grown on ampicillin-selective LB plates (with X-GAL). Recombinant colonies were screened by colony PCR using small volume reactions (10µl) containing standard Taq polymerase and two gene-specific primers, specifically *aqp1* 5' RACE nested forward and *aqp1* 5' RACE reverse 2 (or *aqp1* 5' RACE reverse 3) (Table 2-S1). The individual toothpicks used to screen isolated colonies on the plates were placed into microcentrifuge tubes containing 250µl of ampicillin selective LB media, and bacteria were grown overnight at 37°C, with constant shaking. The following day, an aliquot of each overnight culture was diluted 10-fold with sterile water and samples were re-screened with the 5' inner RACE primer (provided by kit) and 5' RACE reverse 2 (or *aqp1* 5' RACE reverse 3) primers. Several colonies that yielded the largest PCR product sizes (representing the most complete 5'UTR) were selected and larger scale bacterial cultures were grown overnight, shaking at 37°C. Plasmid DNA samples were then purified from the bacterial overnight cultures using the Monarch Plasmid Mini Prep kit (New England Biolabs, Whitby, ON, Canada) and their base characteristics were confirmed by Sanger sequencing (The Centre for Applied Genomics, Sick Kids, Toronto, ON, Canada). Upon confirmation of the full *aqp1* sequence using RACE, the ORF was amplified with Q5® High-Fidelity DNA Polymerase. Reamplification was completed with a forward primer containing a start codon preceded by a Kozak consensus translation initiation sequence and *Hind*III restriction site and reverse primer with a stop codon and *Xba*I restriction site for directional cloning (Table S1). The PCR products were then purified with a Monarch PCR Clean Up Kit (New England Biolabs, Whitby, ON, Canada) following the kit protocols, and 1µg of each sample was digested

(37°C for 40min) with the *Hind*III and *Xba*I restriction enzymes, using the AnzaTM Red Buffer (Thermo Fisher Scientific, Burlington, ON, Canada). The digested products were then run on an agarose gel and purified by gel extraction using the Monarch Gel Extraction kit (New England Biolabs, Whitby, ON, Canada), following the kit protocols. The *aqp1* ORF product was then ligated into a mammalian expression vector (pcDNA3.1+), using a 3:1 molar ratio of the *aqp1* insert to the plasmid, with the AnzaTM T4 Ligase Master Mix (Thermo Fisher Scientific, Burlington, ON, Canada). The ligated products were transformed into NEB® 5-alpha Competent *E. coli* (New England Biolabs, Whitby, ON, Canada) and grown at 37°C overnight on ampicillin-selective LB plates. The following day, colonies containing the correct amplicon size were grown overnight in liquid culture before plasmid DNA samples were purified using the Monarch Plasmid Mini Prep kit (New England Biolabs, Whitby, ON, Canada). Prior to using these constructs in heterologous expression, base accuracy was confirmed with Sanger sequencing (The Centre for Applied Genomics, Sick Kids, Toronto, ON, Canada). Human embryonic kidney (HEK293T) cells were grown in complete growth media (Dulbecco's modified eagles' medium: nutrient F12 (DMEM) media, 10% heat inactivated fetal bovine serum, and 1x antimycotic-antibiotic) within sterile T25 flasks, which were kept in an incubator at 37°C, 5% CO_2. Cells were monitored for growth and once they reached 100% confluency, the cells were re-seeded into a sterile 6-well plate (Thermo Fisher Scientific, Burlington, Ontario Canada) and left to grow overnight. The following morning, the cells were re-seeded at 90% confluency in a sterile 6-well plate and left to grow for 6hrs at 37°C. After the growth period, the cells were transfected with the mammalian expression *aqp1* construct previously made, in a 6-well plate. For transfection, Lipofectamine 3000 transfection reagent (Life Technologies, Carlsbad, CA, Unites States) was used in a 3:1 (µl:µg) ratio with DNA construct.

Table 2-S1. Oligonucleotides used for *Aedes aegypti aqp1* gene amplification including rapid amplification of cDNA ends (RACE), fluorescent *in situ* hybridization probe synthesis, and plasmid preparation for heterologous expression in HEK293T cells.

Forward primer name & sequence:	**Reverse primer name & sequence:**	**Function:**
aqp1 forward 1: GCTGTTTTACAAGGTCCCAT *aqp1* forward 2: CAGCAGGAGAGACGAAAAGC	*aqp1* 3'end reverse: CCCGATGTATGATAGGCTCAAG	3' End Sequence Confirmation
aqp1 5' RACE nested forward: CGAGAACCGCAACATATGGC	*aqp1* 5' RACE reverse 1: CGTCGGTTAGTCACTTGTCC *aqp1* 5' RACE reverse 2: AAGGGGAAAAATCAAGCACA *aqp1* 5' RACE reverse 3: TTTTGGTTAGTGGGGGATGC	5' FirstChoice® RLM-RACE
aqp1 FISH forward: CGAGAACCGCCAACATATGGC	*aqp1* FISH reverse: TTACCCATAACGACGGCTGG	Fluorescent *In Situ* Hybridization probe synthesis
aqp1 start+**Kozak**: **GCCACC**ATGACTGAAAGCGCAGGC *aqp1* start+**Kozak** +HindIII: AAGCTT**GCCACC**ATGACTGAAAGCGCAG	*aqp1* Stop+XbaI: TCTAGATTAAAAATCGTAAGATTCC	Plasmid construct with *aqp1* for heterologous expression in HEK293T cells

Preparation of Protein from Cell Samples

At 48hr after transfection, the complete media from each well of the 6-well plate was removed and placed in individual 15mL centrifuge tubes (Thermo Fisher Scientific, Burlington, ON, Canada) to collect any non-adherent cells floating in the culture media. Cells were detached from the plate surface using 2mL per well of sterile PBS/EDTA mixture (PBS + 5mM EDTA). After approximately 10min, the mixture containing the dissociated cells was then transferred to the centrifuge tubes, and samples were centrifuged at 400xg for 7 min at room temperature. The supernatant was discarded, and the cell pellets were re-suspended in 2mL per well of sterile PBS. Cell samples were centrifuged again at 400xg for 7 min at room temperature. The supernatant was discarded and then cells were re-suspended in 200µL of RIPA buffer with 1:100 protease inhibitor cocktail. Immediately after resuspension, cells in RIPA buffer were transferred to RNAse and DNAse-free microcentrifuge tubes and placed on ice. Cell samples were then sonicated at high frequency for ~15 sec, on ice. The samples were then loaded into 12% SDS-PAGE gels and the previously described western blot protocol (see Materials and Methods section in article) for AaAQP1 abundance was followed.

2.8 Supplementary Results

aqp1 Sequence Confirmation in *Ae. aegypti* and Heterologous Expression of *aqp1* in Mammalian Cells

Utilizing a variety of molecular techniques, I confirmed that the sequence of *aqp1* in *Ae. aegypti* mosquitoes (Genbank accession#: PP003259) matches the previously described sequence (Pietrantonio et al., 2000) and conclude that the current gene model and two predicted transcripts are inaccurate (https://vectorbase.org/vectorbase/app/record/gene/AAEL024675) in light of the empirically determined *Ae. aegypti aqp1* sequence in two separate studies. In addition, I utilized heterologous expression to confirm that our custom specific AaAQP1 antibody detects the ectopically expressed AaAQP1 protein while no such band is observed in HEK293T that do not express AaAQP1 (Figure 2-S1). Using western blotting, we confirmed that our AaAQP1 antibody successfully bound to HEK293T-expressed protein with a band at the correct molecular weight of ~25kDa (Figure 2-S1).

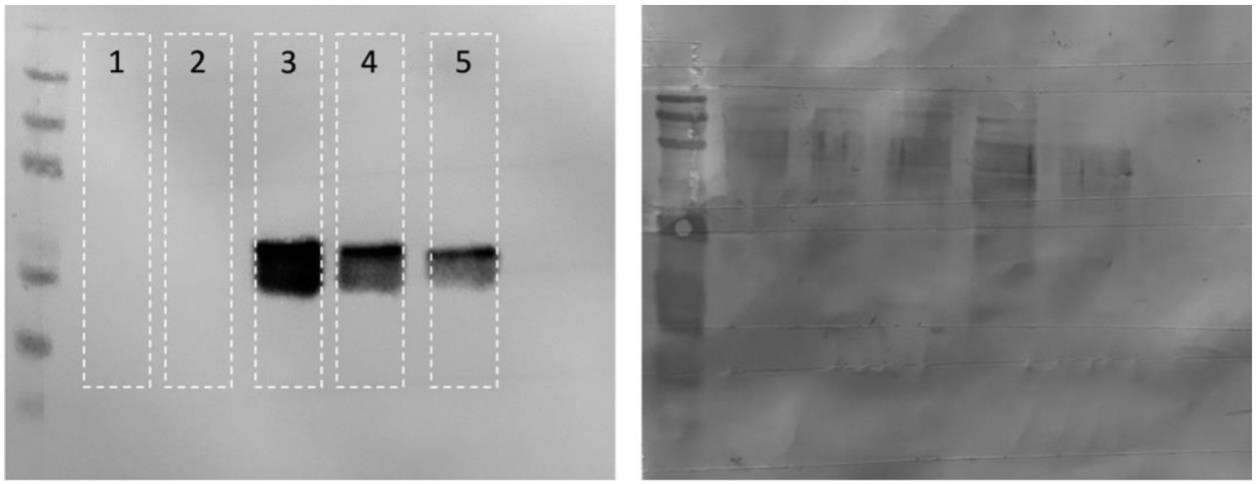

Figure 2-S1. Verification of AaAQP1 antibody specificity with HEK293T cells expressing AaAQP1. Western blot analysis of AaAQP1 protein expressed and isolated from HEK293T cells (left), with associated Coomassie stained membrane for total protein (right). Lane 1 represents protein isolated from untransfected cells, with no band detected. Lane 2 represents protein isolated from HEK293T cells transfected with pcDNA3.1+ plasmid containing mCherry, with no band produced. Lanes 3-5 are separate independent transfections of HEK293T cells with the pcDNA3.1+ AaAQP1 construct, which was confirmed a single band at ~25kDa detected by the custom antibody for *Ae. aegypti* AQP1 (n=3).

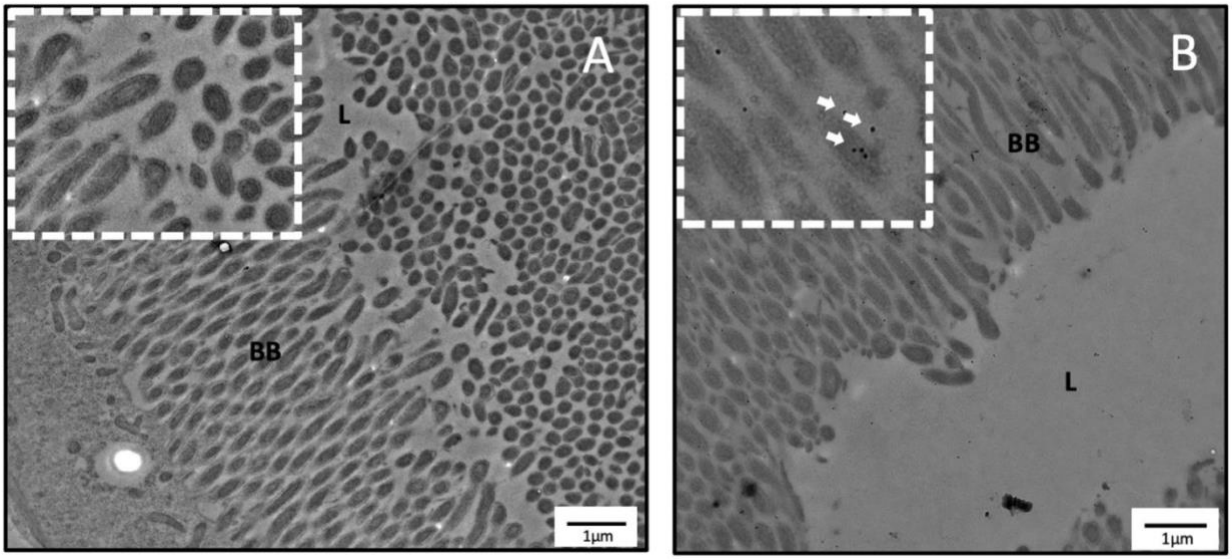

Figure 2-S2. Control for TEM with immunogold localization of AaAQP1 in female Malpighian tubule principal cells. **(A)** showing control grid of MT principal cell tissue section, where a colloidal gold AffiniPure goat anti-rabbit secondary antibody conjugated with 18nm gold particles (Jackson Immunoresearch) was applied to samples and our specific AaAQP1 antibody was omitted. An absence of gold particles is seen, which can be better visualized in the higher magnification inset image enclosed in the white dashed lines. **(B)** MT principal cell tissue section processed with our specific AaAQP1 antibody as well as the secondary antibody conjugated with 18nm gold particles, showing AaAQP1 localization scattered throughout the brush border. Goal particles can be visualized in the higher magnification inset image enclosed in the dashed white lines and indicated with individual white arrows.

2.9 Supplementary References

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Chapter Three

Understanding the role of AaAQP1 in the diuretic and anti-diuretic response of the adult disease vector mosquito, *Aedes aegypti*

This chapter has not yet been submitted for publication.

3.1 Summary

Adult female *Aedes aegypti* mosquitoes are an important model of study due to their ability to spread many deadly arboviral diseases. Their blood feeding behaviour has been extensively studied due to the osmotic stress that it places on the female mosquito's body. Along with a number of other osmoregulatory organs, the Malpighian tubules (MTs) are responsible for producing an ion-rich primary urine via the secretion of Na^+, K^+, and osmotically obliged water. The movement of water into the tubule lumen is made possible by water channel proteins known as aquaporins. In *Ae. aegypti*, there are six aquaporin genes identified (AaAQP1-6) and in particular, AaAQP1 has been identified as a water-specific aquaporin that is present in the MTs of female *Ae. aegypti*. To date, the neuroendocrine control of urine production by the tubules has been extensively studied and with that, much is known about the movement and secretion of ions post blood meal in female mosquitoes. However, less is known and understood about the neuroendocrine control of water secretion via aquaporins in the MTs. In this study, I examined the role of AaAQP1 in water secretion by female *Ae. aegypti* MTs under the influence of two known diuretic hormones, the calcitonin-like diuretic hormone 31 DH_{31} (*Drome*DH_{31}) and the biogenic amine 5-hydroxytryptamine (5HT or serotonin). In addition, I also studied the role of AaAQP1 in the anti-diuretic response of the MTs by treating diuretic-stimulated MTs with the anti-diuretic hormone, *Aedae*CAPA-1. To conduct this research, I first measured AaAQP1 protein abundance and observed protein localization in adult female MTs in the presence of two diuretics of focus in this study (DH_{31} and 5HT) as well as with the addition of CAPA onto diuretic-stimulated MTs. Then, to determine the role of AaAQP1 in fluid secretion following diuretic and anti-diuretic stimulation, AaAQP1-specific dsRNA was microinjected into 1-day old adult female *Ae. aegypti*. With knockdown of AaAQP1 achieved, fluid secretion rates and cation (Na^+ and K^+) concentrations

were measured using a modified Ramsay assay and ion selective microelectrodes (ISME), respectively. I found that AaAQP1 was localized to the apical membrane of the MTs post diuretic and anti-diuretic stimulation, with no overall changes in AaAQP1 protein abundance in the presence of each neuroendocrine factor. Interestingly, that data revealed that AaAQP1 has an important role in water secretion following 5HT stimulation since stimulated secretion was significantly reduced after the application of this diuretic following AaAQP1 knockdown. In comparison, increased fluid secretion caused by DH_{31} does not appear to rely on the function of AaAQP1 alone, as knockdown did not have significant effects on water or ion secretion in MTs treated with this peptidergic diuretic hormone. Furthermore, this data revealed that in unstimulated MTs as well as tubules treated by DH_{31} + CAPA, AaAQP1 appears to be important in basal secretion of the MTs, as knockdown caused significant decreases in fluid secretion.

3.2 Introduction

The adult mosquito *Aedes aegypti* is a tropical and sub-tropical Dipteran that is infamous for its ability to spread several arboviral diseases including yellow fever, dengue fever, and Zika virus (Black et al., 2002; Murray et al., 2013). The adult female mosquitoes utilize the proteins and iron from a vertebrate blood meal to produce mature eggs (Ponlawat & Harrington, 2005). The eggs are then laid near the surface of water and, upon hatching into freshwater, give rise to aquatic larvae that grow through four instar stages. Larval *Ae. aegypti* reside in a hypotonic environment, relative to their internal haemolymph and therefore face the osmoregulatory challenge of ion loss and water accumulation in their body (Bradley, 1987). By contrast, based on their environment, adult terrestrial *Ae. aegypti* regularly experience the threat of desiccation and must work to conserve water in their haemolymph (Patrick et al., 2006). However, upon blood meal ingestion by the female mosquito, there is a load of water and ions that must be dealt with, in order to return haemolymph osmolarity back to normal homeostatic levels (Coast, 2009).

Overall, the unique osmoregulatory challenges that both the larval and adult *Ae. aegypti* mosquitoes face are dealt with by a specific set of osmoregulatory organs, namely the midgut, Malpighian tubules (MTs), the hindgut, and in larvae only, the anal papillae. In adult *Ae. aegypti*, the midgut is mainly responsible for digestion of a blood or nectar meal, but also plays a role ion and water balance (Clark et al., 1999; Jonusaite et al., 2013; Linser & Dinglasan, 2015). The hindgut is responsible for alterations of urine before excretion such as further uptake of water into the haemolymph or removal of ions (Bradley & Phillips, 1977). The MTs, located at the junction between the midgut and hindgut, are the main osmoregulatory organ and are responsible for producing an ion-rich primary urine in adult *Ae. aegypti* (Beyenbach et al., 1987; Beyenbach et al., 1993; Wieczorek et al., 2009; Beyenbach et al., 2010). The MTs function in producing primary

urine through transepithelial active transport. There are two main cell types that make up the MTs; the larger more abundant principal cells that drive the active transport of Na^+ and K^+ and water by passive diffusion into the tubule lumen through a gradient established by the apically located V-type H^+-ATPase (Wieczorek et al., 1989; O'Connor & Beyenbach, 2001; Weng et al., 2003; Wieczorek et al., 2009) and the smaller stellate cells that facilitate transport of Cl^- and diffusion of water across the tubule membrane (O'Connor & Beyenbach, 2001). Water moves through the tubule membrane via aquaporins (AQPs) which are water channel proteins that are made up of six transmembrane domain subunits, separated by five loops (Spring et al., 2009). In female *Ae. aegypti*, six AQP genes have been identified and are denoted AaAQP1-6 (Drake et al., 2010; Drake et al., 2015; Picinic et al., 2024). AaAQP1-5 have been localized to the MTs, aiding in primary urine production (Duchesne et al., 2003; Drake et al., 2015; Picinic et al., 2024). In particular, AaAQP1 has been shown to be a water-specific AQP (Drake et al., 2015). AaAQP1 in female *Ae. aegypti*, is localized to the apical membrane of the principal cells of MTs, both pre- and post-blood meal (Picinic et al., 2024). It has been shown that AQP1 gene transcript is upregulated post blood feeding in *Ae. aegypti* as well as in another mosquito species, *Anopheles gambiae* (Drake et al., 2015; Overend et al., 2015), which highlights its prominent role in water removal from the haemolymph during post-prandial diuresis.

The regulation of secretion by the MTs and overall increased urine production post blood meal in female *Ae. aegypti* is attributed to the circulation of diuretic hormones in the haemolymph (Williams et al., 1983; Wheelock et al., 1988; Cady & Hagedorn, 1999; Beyenbach, 2003; Sajadi et al., 2018). Almost immediately upon the initiation of a blood meal, a calcitonin-like diuretic hormone 31 (DH_{31}), also known as mosquito natriuretic peptide (MNP), is released into circulation and acts as the main diuretic and natriuretic hormone in female *Ae. aegypti* (Petzel et al., 1986;

Wheelock eet al., 1988; Coast et al., 2005; Sajadi et al., 2025). DH_{31} induces diuresis/natriuresis through the cAMP second-messenger pathway to drive Na^+ secretion into the lumen of the MTs and thus increase the production of Na^+-rich primary urine (Petzel et al., 1986; Petzel et al., 1987). Additionally, the biogenic amine 5-hydroxytryptamine (5HT) or serotonin has been shown to stimulate increased MT secretion in larval and adult *Ae. aegypti* (Veenstra, 1988; Clark & Bradley, 1997; Sajadi et al., 2018), with its secretion mechanism shown to work through the cAMP second messenger pathway with possibility to work through alternative pathways to ultimately increase kaliuretic activity in later-stage diuresis (Clark & Bradley, 1998; Sajadi et al., 2018; Sajadi & Paluzzi, 2021). In order to avoid excessive water and ion loss, anti-diuretic factors are released into haemolymph circulation to inhibit diuretic action and return tubule secretion rates back to normal. Previous studies have linked the CAPA peptides to inhibiting diuretic-stimulated secretion in insects such as *Rhodnius prolixus* and *Aedes aegypti* (Paluzzi et al., 2008; Ionescu & Donini, 2012; Sajadi et al., 2018). In femtomolar doses, *Aedae*CAPA-1 has been shown to inhibit both DH_{31}- and 5HT-stimulated secretion in adult female *Ae. aegypti* MTs (Sajadi et al., 2018). The aim of this study was to investigate whether the distribution and abundance of AaAQP1 changes in the response to diuretic factors, including *Drosophila melanogaster* DH_{31} (*Drome*DH_{31}) and 5HT, as well as the anti-diuretic, *Aedae*CAPA-1. Overall, this study provides a novel understanding of how a major water transporting protein, AaAQP1 in *Ae. aegypti* is involved in the rapid removal of water post-exposure to neuroendocrine factors.

3.3 Materials and Methods

Mosquito Rearing

Ae. aegypti mosquitoes were reared and maintained in the lab at York University in Toronto, Canada. Eggs were hatched in dechlorinated tap water and larvae were fed 2-3 times per week with larval food (1:1 liver powder to yeast). The pupae were then isolated from the larval baths in 10mL plastic cups and placed in small mosquito cages with a dish of sugar water which was provided *ad libitum*. For all studies, both female and male *Ae. aegypti* were combined within the individual containers, but female adults were selected for experiments.

Diuretic and Anti-Diuretic Treatments

For all experiments involving neuropeptide and/or biogenic amine (serotonin) treatments, hormone and peptide doses were selected based on previously published work that has characterized these factors and their use in *Ae. aegypti* (see Sajadi et al., 2018). The diuretics used in this study were serotonin (5-hydroxytryptamine or 5HT) (Sigma Aldrich, ON, Canada) and *Drome*DH$_{31}$, the latter of which was gifted by Michael J. O'Donnell (McMaster University, ON, Canada) and bears high similarity with *Aedae*DH$_{31}$. The anti-diuretic peptide used in this study was *Aedae*CAPA-1 (GPTVGLFAFPRV-NH$_2$) synthesized by a contract research organization (Genscript, NJ, USA). All diuretic and/or anti-diuretic incubation times for isolated MTs were ~1hr at room temperature (RT) based on methods from Sajadi et al., 2018. Serotonin was used at 100nmol l^{-1} which was previously established to elicit secretory responses in *Ae. aegypti* MTs (Clark & Bradley, 1998; Sajadi et al., 2018). Additionally, *Drome*DH$_{31}$ was used at 25nmol l^{-1} based on previous work that showed stimulatory activity at this dose (Sajadi et al., 2018), in addition to mosquito natriuretic peptide (MNP) dose-response work completed in the mosquito

Anopheles gambiae (Coast et al., 2005). Finally, the anti-diuretic *Aedae*CAPA-1 was used at 1 fmol l^{-1} which was chosen based on previous work that showed anti-diuretic effects at this concentration in both larval and adult *Ae. aegypti* (Ionescu & Donini, 2012; Sajadi et al., 2018; Sajadi & Paluzzi, 2021).

Immunohistochemistry

Immunohistochemistry (IHC) was completed on isolated MTs from ~5-day old adult female *Ae. aegypti* mosquitoes. MTs were dissected from ~15 adult female mosquitoes under physiological saline [150mM NaCl, 25mM Hepes, 3.4mM KCl, 7.5mM NaOH, 1.8mM $NaHCO_3$, 1mM $MgSO_4$, 1.7mM $CaCl_2$, and 5mM glucose, pH 7.1] (Petzel et al., 1987) and incubated in either 100nmol l^{-1} 5HT, 100nmol l^{-1} 5HT with 1 fmol l^{-} *Aedae*CAPA-1, 25nmol l^{-1} *Drome*DH_{31}, or 25nmol l^{-1} *Drome*DH_{31} with 1 fmol l^{-1} *Aedae*CAPA-1 for 1hr at RT. As a control for each group, MTs were also isolated and incubated in physiological saline alone for 1hr at RT. Following treatment, MTs were then placed in Bouin's fixative for 1hr at RT and then washed twice with 70% ethanol and stored in the fridge (5-7 days) until a stepwise procedure of dehydration with EtOH and xylene was completed. IHC was carried out according to previously established protocols (Akhter et al., 2017; Misyura et al., 2020; Sajadi et al., 2023; Picinic et al., 2024). Embedded MTs were cut into ~4μm thin sections using an Epredia HM325 manual microtome (Epredia, MI, USA) and placed on Fisherbrand™ ColorFrost™ Plus Adhesion Microscope slides. In order to compare control (saline-incubated) MTs to the diuretic-treated or diuretic with anti-diuretic-treated MTs, sections of each treatment were placed on the same slide to comprise one biological replicate. For each set of sections completed for 5HT and *Drome*DH_{31} samples, 3-4 biological replicates were completed along with 4-5 technical replicates. Slide processing was completed following

previously established methods (Sajadi et al., 2023; Picinic et al., 2024). All MT slides were treated identically and were probed with the custom *Ae. aegypti* Anti-AaAQP1 affinity-purified primary antibody (1:1000 rabbit polyclonal antibody against CFFKVRKGDEESYDF, Genscript, NJ, USA) (Misyura et al., 2020; Picinic et al., 2024) and a guinea pig anti-V_1 antibody for V-type H^+-ATPase (VA) (Ab 353-2, gifted by H. Wieczorek, Germany, 1:5000 dilution) as the apical membrane marker (Sajadi et al., 2023; Picinic et al., 2024). For slides with abdominal tissue sections with AaAQP1 knockdown, a mouse monoclonal anti-a5 antibody for Na^+/K^+-ATPase (NKA) (Douglas Fambrough, Developmental Studies Hybridoma Bank, IA, USA, 1:10 dilution) was used as a membrane marker. AaAQP1 staining was visualized with a goat anti-rabbit AlexaFluor 594 (Jackson Immunoresearch) secondary antibody (1:400), VA staining was visualized using a goat anti-guinea pig AlexaFluor 488 (Jackson Immunoresearch) secondary antibody (1:500), and a goat anti-mouse antibody conjugated to Cy5 (Jackson Immunoresearch) secondary antibody was used to visualize NKA (1:500). Slides were mounted using ProLong® Gold antifade reagent with DAPI (Life Technologies, ON, Canada) and imaged using an Olympus IX81 fluorescent microscope (Olympus Canada, ON, Canada) with CellSense® 1.12 Digital Imaging software (Olympus Canada). All acquisition settings were kept the same for each slide and individual channel images were merged using ImageJ 1.53k software.

Protein Processing, Gel Electrophoresis, and Western Blotting

Sets of MTs were isolated from ~4-5-day old female *Ae. aegypti* under physiological saline (Petzel et al., 1987) pooling MTs from 75 individual mosquitoes for each biological replicate (n=4). Protein was isolated from the membrane fraction of the MTs as previously described using the Mem-PER Plus Membrane Protein Extraction kit (Thermo Fisher Scientific, ON, Canada)

(Picinic & Paluzzi, 2023; Picinic et al., 2024). The extracted membrane protein was then quantified relative to bovine serum albumin (BSA) standards, using a Bradford assay.

The gel electrophoresis and western blotting was also completed as previously described (Picinic & Paluzzi, 2023; Picinic et al., 2024). The membrane fraction protein samples from MTs were prepared for SDS-PAGE by adding 50mM Tris buffer and 6x loading buffer (Picinic et al., 2024) and denaturing the protein at 100°C for 5 min. After the protein was run on a gel, a 1.5hr wet transfer was conducted on ice, where the protein was transferred onto a polyvinyl difluoride membrane. The membrane was then placed in 5% skim milk blocking buffer prepared in 1xTris-buffered saline for 1hr at RT. The membrane was then probed with the *Ae. aegypti* specific anti-AaAQP1 affinity-purified primary antibody (1:1000 rabbit polyclonal antibody against CFFKVRKGDEESYDF, Genscript, NJ, USA) prepared in blocking buffer and left to incubate and rotate at 4°C for 12hr. After a series of 10min washes with 1xTris-buffered saline, the membrane was then probed with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:5000; BioRad, ON, Canada) rotating for 1hr at RT. To visualize the protein bands on the membrane, the Clarity™ Western ECL chemiluminescent substrate (BioRad, CA, USA) was used and the membrane was imaged using a Chemi-Doc MP Imaging System (BioRad). Individual AaAQP1 protein bands were normalized to total protein using Coomassie staining (BioRad) and the bands were quantified based on relative density using ImageJ 1.53a Software (USA).

dsRNA Synthesis and Delivery by Microinjection

In order to knockdown AaAQP1 in the adult female *Ae. aegypti*, gene-specific primers were designed to amplify a large portion of the gene (GenBank Accession #: XP_001656931) (See Table 3-1 for primers). The target region of the AaAQP1 gene was then amplified, purified, and

cloned into a pGEM T-Easy Vector which was used to transform standard lab-strain bacteria (Dh5alpha). Plasmid DNA was isolated by Mini-Prep (BioBasic, ON, Canada) according to the manufacturer's instruction. Sequence of the amplified product was verified by Sanger sequencing (The Centre for Applied Genomics, Sick Kids, ON, Canada) and confirmation of base accuracy was completed using Geneious Software (Dotmatics, MA, USA). The same sequence of steps was completed to amplify a portion of the *β-lactamase* (βLac) gene (Table 3-1) that was used as a knockdown control.

Synthesis of dsRNA was completed as previously described by Chasiotis et al., 2016 and Misyura et al., 2017. The concentrated mini-prep products for both AaAQP1 and βLac were diluted 1:100 and then re-amplified with gene specific primers that have an additional T7 promoter sequence added on the 5' end of the oligo (Table 3-1). The addition of T7 primers onto the sense and anti-sense primers was completed in two separate reactions. Several replicate PCR reactions were prepared using the amplified AaAQP1 product and the Forward AaAQP1 primer with the Reverse AaAQP1 T7 primer. Similarly, separate replicate PCR reactions were completed using the amplified AaAQP1 product and the Forward AaAQP1 T7 primer with the Reverse AaAQP1 primer. Products were purified and then run on a gel to confirm appropriate band size. Following this, the Promega T7 RiboMAX Express RNAi System Kit (Promega, Madison, WI, USA) was utilized to synthesize the double stranded RNA, as per the manufacturer's instructions. dsRNA for AaAQP1 and βLac were purified with the Monarch RNA Purification kit (NEB, ON, Canada), spun in an RC10.10 Jouan© vacuum centrifuge (Winchester VA, USA) for 2hr at RT to concentrate the product, and then dsRNA concentration and purity were measured with a NanoDrop2000 spectrophotometer (Thermo Scientific, DE, USA).

To induce knockdown, 1μg of AaAQP1 (or βLac as control) dsRNA resuspended in sterile water (5μg/μl) and 200nL was microinjected using a Nanoject III Programmable Nanoliter Injector (Drummon Science, PA, USA) into 1-day old adult female *Ae. aegypti* mosquitoes. The injected females were then placed in small mosquito cages with sugar water as a simulated nectar meal provided *ad libitum* and left at RT until knockdown was to be assessed.

Table 3-1. Oligonucleotides used for *Aedes aegypti* AaAQP1 gene amplification and dsRNA synthesis. The oligo name is listed with the corresponding forward and reverse primer sequences used to generate the specific AaAQP1 amplicon. Each set of primers is listed with the appropriate experiments they were used in.

Oligo Name	Forward Primer Sequence	Reverse Primer Sequence	Expected Band Size	Experiment Used For
AaAQP1	CGAGAACCGCAACATATGGC	TTACCCATAACGACTGGCTGG	583bp	PCR
dsAQP1 Reverse T7	CGAGAACCGCAACATATGGC	TAATACGACTCACTATAGGGAGATTACCCATAACGACTGGCTGG	603bp	dsRNA Synthesis
dsAQP1 Forward T7	TAATACGACTCACTATAGGGAGACGAGAACCGCAACATATGGC	TTACCCATAACGACTGGCTGG	603bp	dsRNA Synthesis
βLac dsRNA	ATTTCCGTGTCGCCCTTATTC	CGTTCATCCATAGTTGCCTGAC	800bp	dsRNA Synthesis
AaAQP1 qPCR	ATCGGATTCAGCAGGAGAGA	CCGATCGACACCAGGAAAAA	153bp	qPCR
RP49 Reference Gene	ACAAGCTTGCCCCCAAC	GCGATTTCGGCACAGTAGA	215bp	qPCR
rpS18 Reference Gene	TAAAAATGTCGCTCGTGATCC	AATCGGGGATCTTGTACTGG	253bp	qPCR

RT-qPCR

To test for AaAQP1 gene knockdown in adult female *Ae. aegypti*, RT-qPCR was used and primers used are listed in Table 3-1. Pupae were taken from lab-reared colonies and placed in small mosquito cages with sugar water provided *ad libitum*. Upon emergence at ~1-day old, female mosquitoes were injected with 5μg/μl of either dsAQP1 or dsβLac and placed in a new small mosquito cage with fresh sugar water provided *ad libitum*. The mosquitoes were left in their cages for 72hr and then the MTs were isolated from each individual under physiological saline and placed into cooled lysis buffer prepared from the PureLink RNA minikit (Invitrogen, MA, USA). For each replicate, MTs were collected from ~25 individuals (n=3) and sonicated on ice for ~10 seconds using the XL 2000 Ultrasonic Liquid Processor (QSONICA – LLC, CT, USA). RNA extraction was then completed as per the PureLink minikit (Invitrogen) protocol, followed by cDNA synthesis (~1μg RNA) using the iScript qPCR cDNA synthesis kit as per manufacturer's instructions (BioRad, ON, Canada). The cDNA was then diluted 10x and each sample was prepared in a reaction with SsoFast EvaGreen Supermix (BioRad, ON, Canada) as per the manufacturer's recommendations. The qPCR was run using the CFX Duet Real-Time PCR System (BioRad, ON, Canada) and each biological replicate was loaded in triplicates. Relative expression levels of AaAQP1 in MTs from the dsAQP1-injected versus the dsβLac-injected mosquitoes was determined using the ΔΔCt (Livak & Schmittgen, 2001) and were normalized to Ribosomal protein 49 (rp49) and 40S Ribosomal Protein L8 (rpS18) reference genes which were previously determined to be effective endogenous controls (Paluzzi et al., 2014).

Ramsay Fluid Secretion Asay

To determine the effects of AaAQP1 knockdown on secretion rate by MTs under both diuretic and anti-diuretic treatments, individual MTs were isolated from adult female mosquitoes and a modified Ramsay assay was completed (Ramsay, 1954; Lajevardi et al., 2021). The 1-day old female mosquitoes were injected with dsRNA for AaAQP1 or βLac and left in small mosquito cages for 72hr to allow for knockdown to occur. An additional group of non-injected female mosquitoes were also prepared, in the same way as the dsRNA-injected females. Then, the tubules were isolated under physiological saline (Petzel et al., 1987; Sajadi et al., 2018). Additionally, a Ramsay plate was prepared which consisted of a Sylgard-lined dish with rows of carved wells and two Minuten pins situated close by each well to mount individual MTs onto. Each well was filled with 20µl of bathing saline, submerged under hydrated mineral oil. The proximal end of individual MTs was wrapped 1-2 times around the pin, with the distal end placed in the bathing saline, to allow for urine secretions to collect at the tip of the pin.

To test the effects of AaAQP1 knockdown on MT secretions, each Ramsay plate was prepared with saline droplets consisting of physiological saline alone, the diuretic (100nmol l^{-1} 5HT or 25nmol l^{-1} *Drome*DH_{31}) prepared in physiological saline, or the diuretic with the anti-diuretic 1 fmol l^{-1} *Aedae*CAPA-1 prepared in physiological saline. Then, several individual MTs were taken from non-injected, dsβLac-injected, and dsAQP1-injected female *Ae. aegypti* and placed in each of the previously listed saline droplets. The MTs were then left to secrete in the dish for 1.5hr at RT covered with a lid and following this period, secreted droplet sizes were measured using the eyepiece micrometer of a dissecting microscope. The fluid secretion rate (FSR) was then calculated as previously described (Donini et al., 2008) comparing FSR by MTs from the non-

injected, dsβLac-injected, and dsAQP1-injected groups under saline alone, diuretic alone, and diuretic with anti-diuretic treatment.

Ion-Selective Microelectrode Technique

The ion-selective microelectrode technique (ISME) was used to measure [Na^+] and [K^+] in the secreted primary urine of female *Ae. aegypti* MTs, both pre-and post-AaAQP1 knockdown under stimulated and unstimulated conditions. In order to do so, ion-selective microelectrodes were constructed. Glass capillaries (TW-150-4, Word Precision Instruments, FL, USA) were pulled into microelectrodes using a Sutter P-97 Flaming Brown pipette puller (Sutter Instruments, CA, USA) and silanized using *N,N*-dimethyltrimethylsilylamine. To measure [Na^+], the Na^+ electrode was backfilled with 100mmol l^{-1} NaCl and then front filled with Na^+ ionophore (Sodium ionophore II cocktail A; Fluka, Switzerland). To measure [K^+], the K^+ electrode was backfilled with 100mmol l^{-1} KCl and then front filled with K^+ ionophore (Potassium ionophore I cocktail B, Fluka, Switzerland). All ion-selective microelectrodes were thinly coated with 3.5% (w/v) polyvinyl chloride (PVC) prepared in tetrahydrofuran to maintain ionophore placement in the electrode (Rheault and O'Donnell, 2004). To complete the circuit, reference electrodes were constructed using glass capillaries (1B100F-4, World Precision Instruments) pulled with a very fine tip and then backfilled with 500mmol l^{-1} KCl, for all ion measurements.

To obtain voltage recordings, the ion-selective microelectrodes and the reference electrodes were mounted onto micromanipulators, along a silver chloride wire, which conveyed voltage recordings to a PowerLab data acquisition system (ADInstruments Inc.). Na^+ microelectrodes were calibrated with 5µl droplets of 20mmol l^{-1} NaCl and 200mmol l^{-1} NaCl standards and K^+ microelectrodes were calibrated with 5µl droplets of 15mmol l^{-1} KCl and 150mmol l^{-1} KCl

standards. Then, respective ion concentrations were measured in each of the secreted urine droplets from previously prepared Ramsay assay plates by placing the ion-selective microelectrode along with the reference electrode into the urine droplet and voltage readings relative to the lower calibration value were recorded. [Na^+] and [K^+] in the secreted urine was calculated using a previously described method (Donini et al., 2008; Paluzzi et al., 2012), as well as the transport rate of each ion was calculated as previously described (Rheault and O'Donnell, 2004).

3.4 Results

AaAQP1 Localization and Abundance Post Diuretic and Anti-Diuretic Stimulation

AaAQP1 localization in the MTs of adult female *Ae. aegypti* was observed using immunohistochemistry with thin tissue sections. Figure 3-1 shows the localization of AaAQP1 in MTs that have been treated with physiological saline as a control, 25nmol l^{-1} *Drome*DH_{31}, or 25nmol l^{-1} *Drome*DH_{31} with 1 fmol l^{-1} *Aedae*CAPA-1. Immunoreactivity was detected at the apical membrane of the MTs in all three conditions (Figure 3-1A-C). Interestingly, AaAQP1 staining appeared brightest in the *Drome*DH_{31}+*Aedae*CAPA-1 group, relative to the saline alone and *Drome*DH_{31} groups (Figure 3-1C). Additionally, AaAQP1 appears localized to the principal cells of the MTs in all three conditions, as staining is present in large sections of the apical membrane with gaps in staining present where stellate cells may be localized (Figure 3-1). The apically localized cytosolic subunit for the V-type H^+-ATPase (VA) was used as a membrane marker as shown in the merged images (Figure 3-1D-F). Figure 3-2 represents the localization of AaAQP1 in MTs that have been treated with physiological saline as a control, 100nmol l^{-1} 5HT, or 100nmol l^{-1} 5HT with 1 fmol l^{-1} *Aedae*CAPA-1. Immunoreactivity was also found to be localized to the apical membrane of the principal cells of the MTs in all three conditions (Figure 3-2A-C). Across all three treatments, no changes in staining intensity of AaAQP1 were observed. Similar to *Drome*DH_{31} treatments, apically localized cytosolic subunit for the V-type H^+-ATPase (VA) was used a membrane marker as shown in the merged images (Figure 3-2D-F).

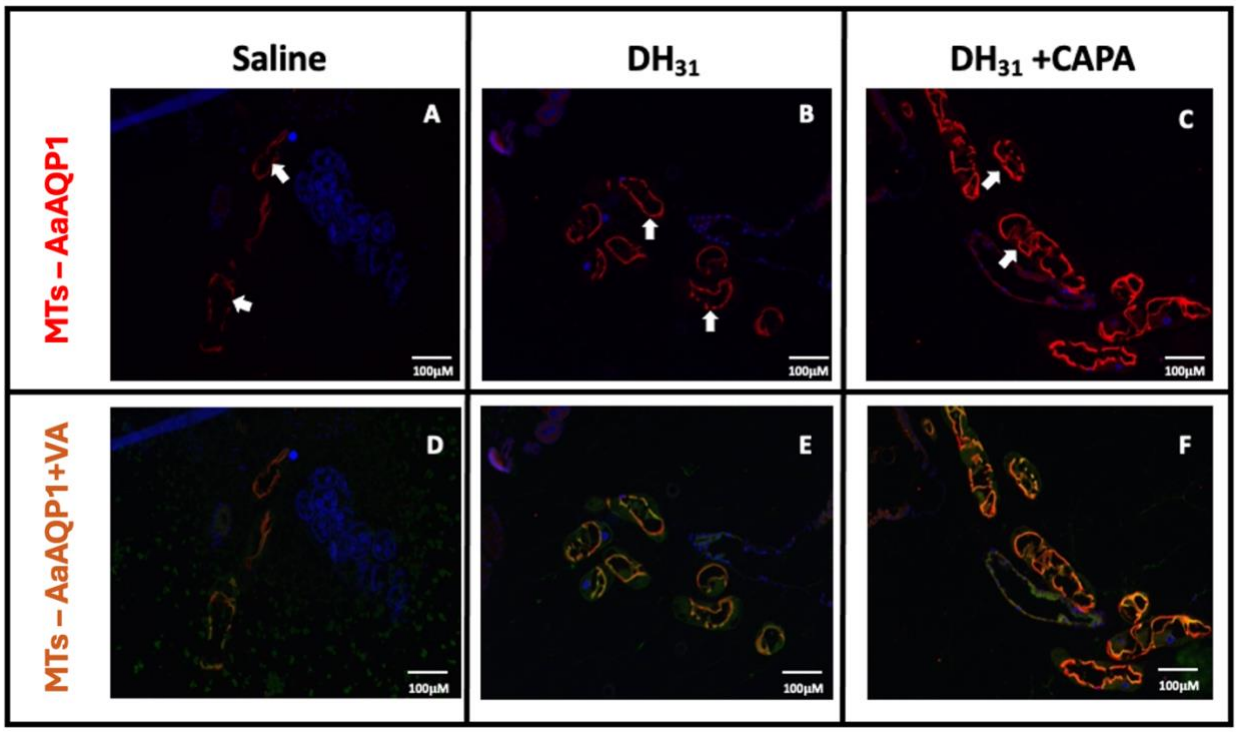

Figure 3-1. Immunolocalization of AaAQP1 in adult female *Aedes aegypti* MTs, under *Drome*DH$_{31}$ and *Drome*DH$_{31}$ with *Aedae*CAPA-1 stimulation. Localization of AaAQP1 (red) at the apical membrane (indicated by white arrow) in unstimulated MTs (**A**), *Drome*DH$_{31}$-treated MTs (**B**) and in *Drome*DH$_{31}$ with *Aedae*CAPA-1-treated MTs (**C**). Panels **D-F** show the merged images of AaAQP1 localization in the MTs with all three conditions merged with the VA membrane marker (orange) with co-localization apparent as yellow-orange staining. For each treatment, 3-4 biological replicates and 4 technical replicates were completed.

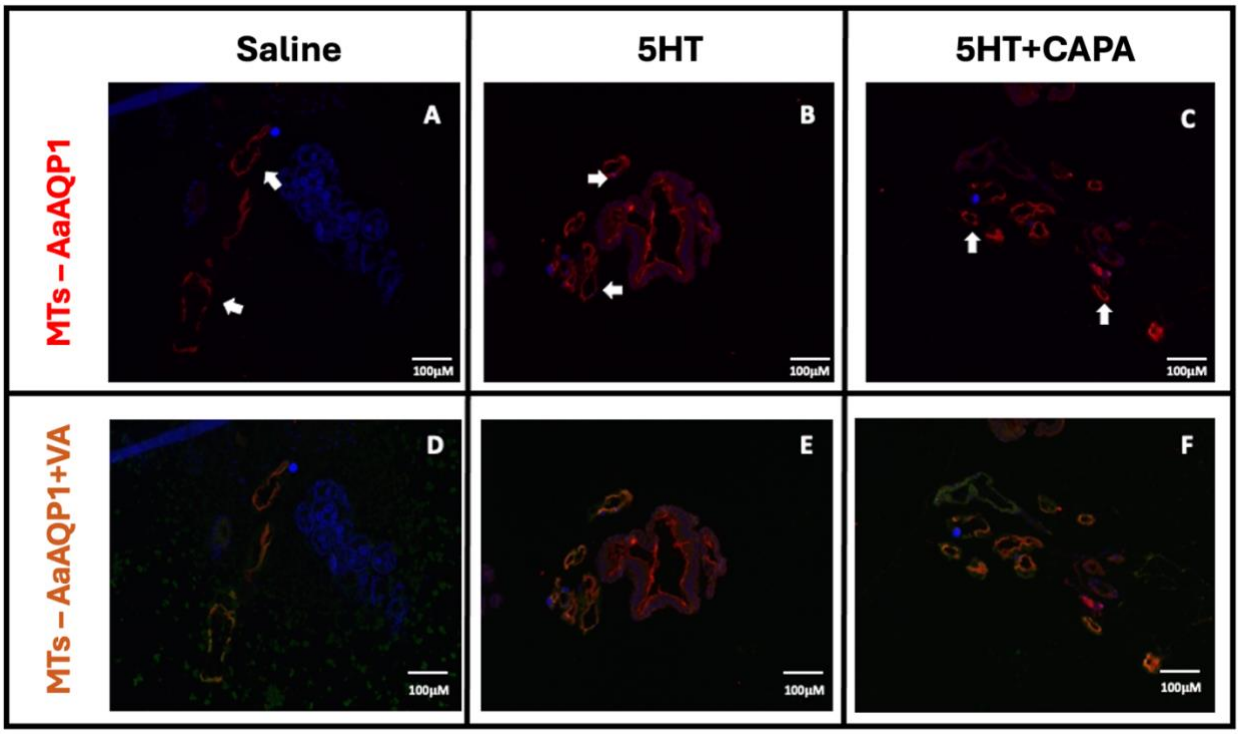

Figure 3-2. Immunolocalization of AaAQP1 in adult female *Aedes aegypti* MTs, under 5HT and 5HT with *Aedae*CAPA-1 stimulation. Localization of AaAQP1 (red) at the apical membrane (indicated by white arrow) in unstimulated MTs (**A**), 5HT-treated MTs (**B**) and in 5HT with *Aedae*CAPA-1-treated MTs (**C**). Panels **D-F** show the merged images of AaAQP1 localization in the MTs with all three conditions merged with the VA membrane marker (orange) with co-localization apparent as yellow-orange staining. For each treatment, 3-4 biological replicates and 4 technical replicates were completed.

AaAQP1 protein abundance was also analyzed in the membrane fraction of adult female *Ae. aegypti* MTs using western blotting, under the same conditions described above. Figure 3-3A shows that AaAQP1 protein abundance was significantly decreased in MTs that were treated with *Drome*DH$_{31}$ alone or *Drome*DH$_{31}$+*Aedae*CAPA-1, in comparison to the saline control group and all normalized to total protein with Coomassie staining. In contrast, the same membrane fraction samples from MTs were run in a western blot but a β-actin antibody was used to probe the membrane, with results shown in Figure 3-3B. Although not statistically significant, it appeared that β-actin was upregulated in both the *Drome*DH$_{31}$ alone and *Drome*DH$_{31}$+*Aedae*CAPA-1 groups, relative to the saline control (Figure 3-3B). Additionally, AaAQP1 protein abundance was also observed in 5HT and 5HT+*Aedae*CAPA-1 treated female MTs (Figure 3-4). Relative to the saline control group, both the 5HT and 5HT+*Aedae*CAPA-1 treated MTs showed no significant changes in AaAQP1 normalized protein abundance (Figure 3-4).

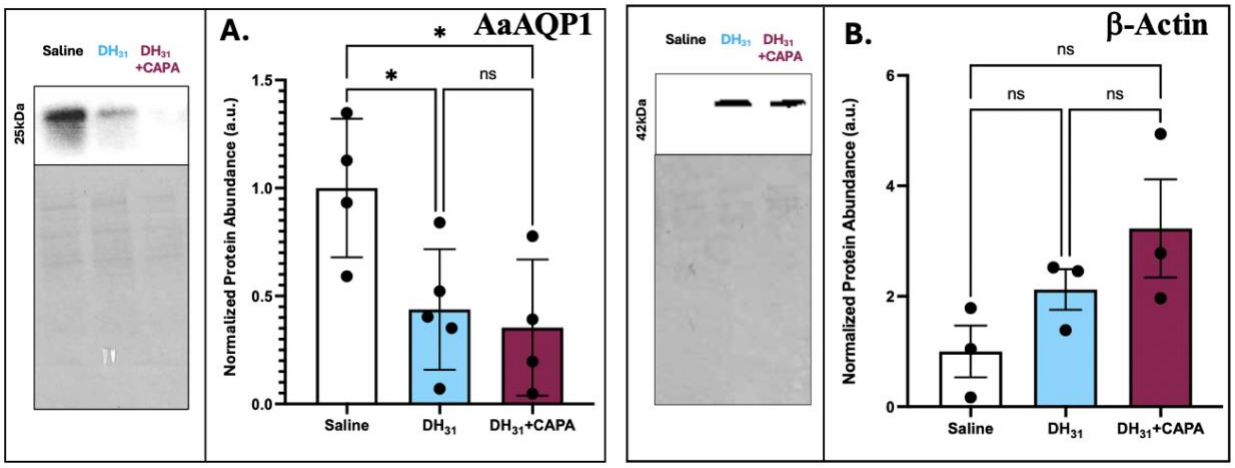

Figure 3-3. Protein abundance of AaAQP1 and β-Actin in female *Aedes aegypti* Malpighian tubules under *Drome*DH31 and *Drome*DH31 with *Aedae*CAPA-1 stimulation. The normalized protein abundance of AaAQP1 and β-Actin in the membrane fraction of female MTs post stimulation with *Drome*DH31 and *Drome*DH31 with *Aedae*CAPA-1 (n=3-4). **A.** Normalized protein abundance of AaAQP1 in the MTs, showing a significant decrease in protein abundance with *Drome*DH31 and *Drome*DH31 with *Aedae*CAPA-1 treatment, relative to the saline-treated (unstimulated) control group. **B.** Normalized protein abundance of β-Actin in the MTs, showing an apparent increase in protein abundance with *Drome*DH31 and *Drome*DH31 with *Aedae*CAPA-1 treatment, relative to the saline-treated (unstimulated) control group. For each set of data, a Kruskal-Wallis non-parametric ANOVA test with Tukey's multiple comparisons was run and an * indicates a significant difference between groups ($p<0.05$).

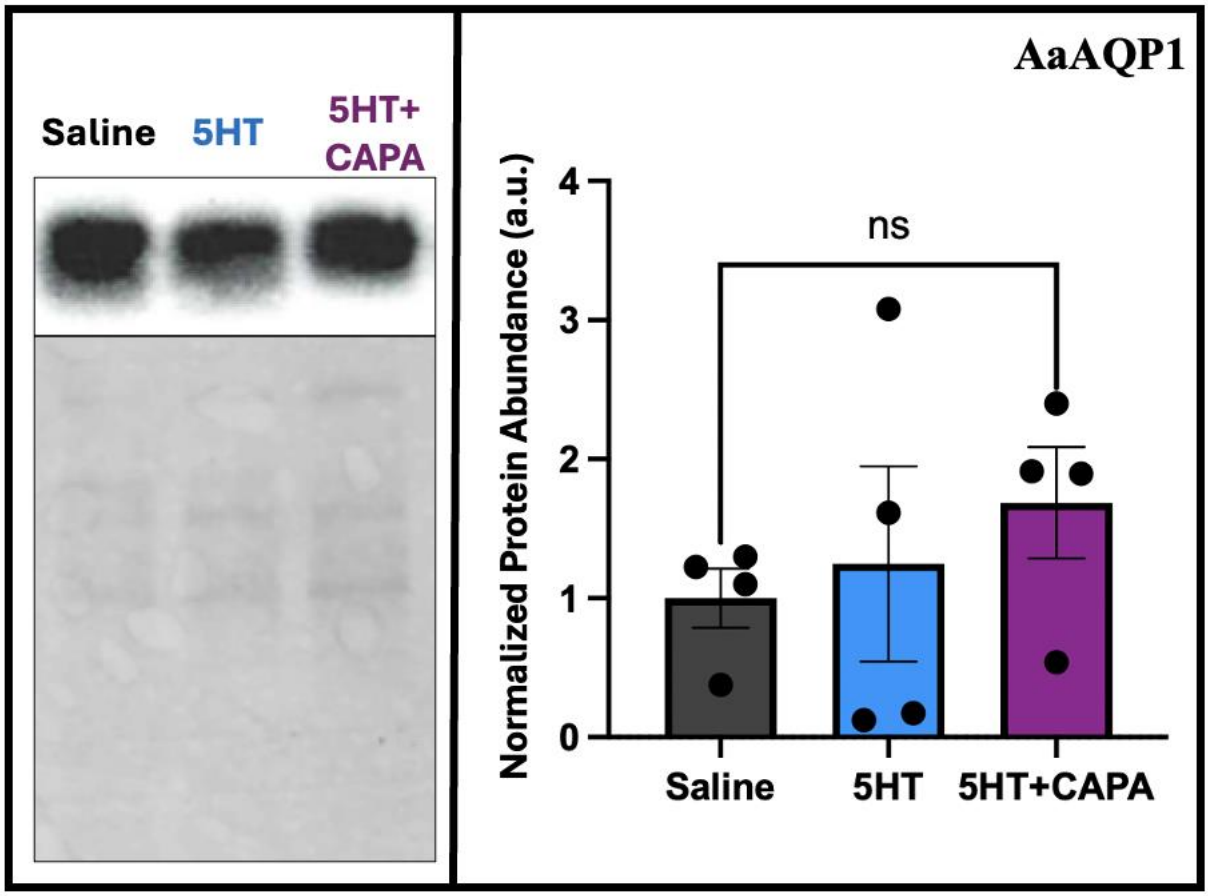

Figure 3-4. Protein abundance of AaAQP1 in the membrane fraction of female *Aedes aegypti* Malpighian tubules under 5HT and 5HT with *Aedae*CAPA-1 stimulation. The normalized protein abundance of AaAQP1 in the membrane fraction of female MTs post stimulation with 5HT and 5HT with *Aedae*CAPA-1 (n=3-4). Normalized protein abundance of AaAQP1 in the MTs, showing no changes in protein abundance with both 5HT and 5HT with *Aedae*CAPA-1 treatment, relative to the saline-treated (unstimulated) control group. A Kruskal-Wallis non-parametric ANOVA test with Tukey's multiple comparisons was run and no significant differences were found ($p>0.05$).

Effect of AaAQP1 Knockdown on Secretion Rate by MTs and Ion Concentration in the Primary Urine

AaAQP1 knockdown was verified in female *Ae. aegypti* MTs 72hr post dsRNA-injection with ~5-fold decrease in transcript expression relative to the dsβLac-injected MTs, confirmed with qPCR for transcript abundance and using immunohistochemistry for protein knockdown (Figure 3-5). Clear AaAQP1 immunoreactivity is evident in the sections of MTs from non-injected (Figure 3-5B) and dsβLac-injected mosquitoes (Figure 3-5C) while no immunoreactive staining was observed in MTs from dsAQP1-injected mosquitoes (Figure 3-5D).

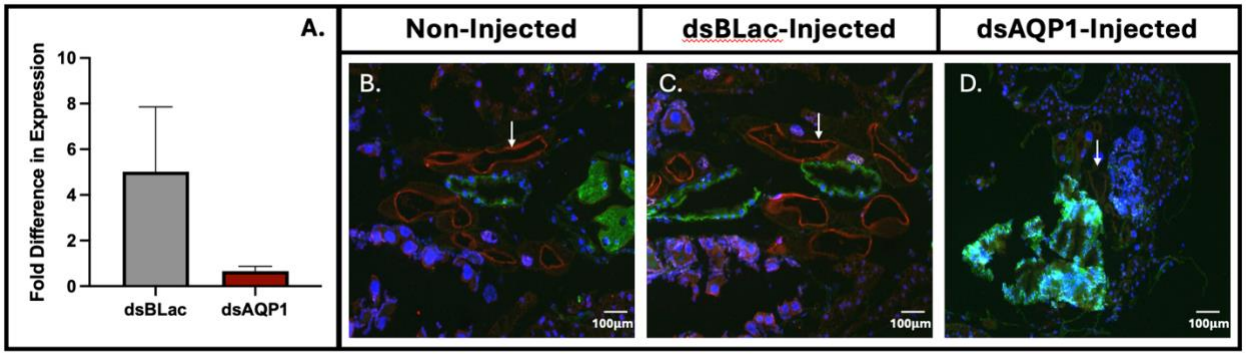

Figure 3-5. Evidence of AaAQP1 knockdown in female mosquito Malpighian tubules following dsRNA microinjections. **A.** The fold difference in expression of AaAQP1 mRNA 72hr post dsAQP1 injection in female *Ae. aegypti* MTs measured with qPCR, relative to the dsβLac-injected samples (n=4). An unpaired t-test was used to assess changes in expression with a significant difference denoted with an * ($p<0.05$). **B-C.** Immunoreactivity (red) of AaAQP1 at the apical membrane of the MTs (indicated by white arrow) in both non-injected and dsβLac-injected mosquitoes (n=3), with DAPI (blue) staining the nuclei and the Na^+/K^+-ATPase (green) used as a membrane marker. **D.** Absence of AaAQP1 immunoreactivity in the sections of MTs from dsAQP1-injected mosquitoes (apical membrane indicated with white arrow) (n=3).

Following confirmation of knockdown, the functional consequences of AaAQP1 knockdown were assessed in female MTs using modified Ramsay assays and ISME. In addition, the effects of AaAQP1 knockdown were assessed in the presence of diuretic and anti-diuretic factors. The effects of AaAQP1 knockdown on secretion rate and cation (Na^+ and K^+) concentration in secreted fluid of MTs post knockdown and with the addition of the diuretic alone (*Drome*DH_{31}) or in combination with *Aedae*CAPA-1. MTs treated with physiological saline as the control (unstimulated) condition, experienced a significant decrease in secretion rate post dsAQP1-injection, in comparison to dsβLac-injected MTs (Figure 3-6A); however, no changes were seen in [Na^+] or [K^+] post AaAQP1 knockdown under saline conditions (Figure 3-6B, C). When MTs were stimulated with the diuretic *Drome*DH_{31}, AaAQP1 knockdown had no effect on secretion rate and [Na^+] or [K^+] in the secreted fluid by MTs (Figure 3-6D-F). Additionally, when MTs were treated with *Drome*DH_{31} and the anti-diuretic *Aedae*CAPA-1, a significant decrease in secretion rate and [Na^+] was observed post AaAQP1 knockdown, in comparison to dsβLac-injected MTs (Figure 3-6G, H), while there was no change observed in secreted fluid [K^+] post AaAQP1 knockdown (Figure 3-6I).

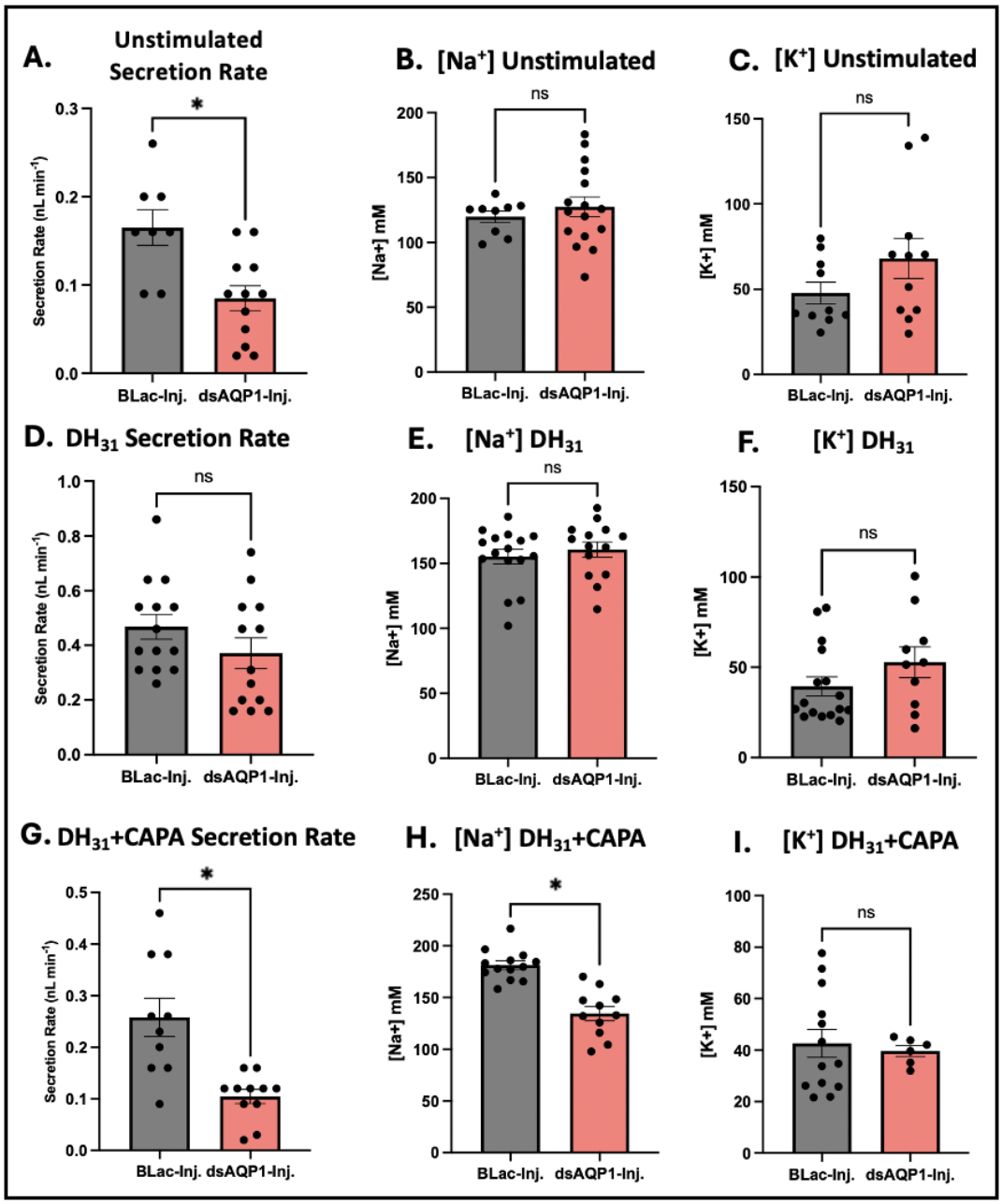

Figure 3-6. The effects of AaAQP1 knockdown on female mosquito Malpighian tubule secretion rate and cation concentration with the addition of *Drome*DH$_{31}$ and *Drome*DH$_{31}$ with *Aedae*CAPA-1. MTs from females injected with dsβLac or dsAQP1 were treated with physiological saline alone (unstimulated), or saline containing either 25nmol l^{-1} *Drome*DH$_{31}$, or 25nmol l^{-1} *Drome*DH$_{31}$ with 0.1 fmol l^{-1} *Aedae*CAPA-1, each for 1hr at room temperature before measurements were taken. **A, D, G.** Secretion rates measured over 1hr (nL min^{-1}) in each treatment. **B, E, H.** [Na^+] measured in the secreted droplets of female MTs. **C, F, I.** [K^+] measured in the secreted droplets of female MTs. All values presented are mean±SEM (n=8-16). For each panel, an unpaired t-test was used to assess changes between treatments and significant differences are denoted with an * ($p<0.05$).

The effects of AaAQP1 knockdown on secretion rate and cation (Na^+ and K^+) concentration in secreted fluid of MTs post knockdown was also assessed in response to another diuretic (5HT) alone or in combination with *Aedae*CAPA-1 treatment (Figure 3-7). Fluid secretion and cation measurements were repeated in saline (unstimulated) conditions and data are consistent with results from Figure 3-6, showing a significant decrease in tubule secretion rate post AaAQP1 knockdown and no change in secreted fluid [Na^+] or [K^+] post dsAQP1-injection (Figure 3-7A-C). Upon 5HT stimulation, dsAQP1-injected MTs experienced a significant decrease in secretion rate (Figure 3-7D), along with significant increases in both [Na^+] and [K^+] (Figure 3-7E, F), relative to MTs from dsβLac-injected mosquitoes. Finally, with the addition of both 5HT and *Aedae*CAPA-1, there was no change in secretion rate post AaAQP1 knockdown (Figure 3-7G); however, significant changes in [Na^+] and [K^+] were measured in dsAQP1-injected MTs (Figure 3-7H, I) with [Na^+] decreasing while [K^+] increased in the secreted fluid.

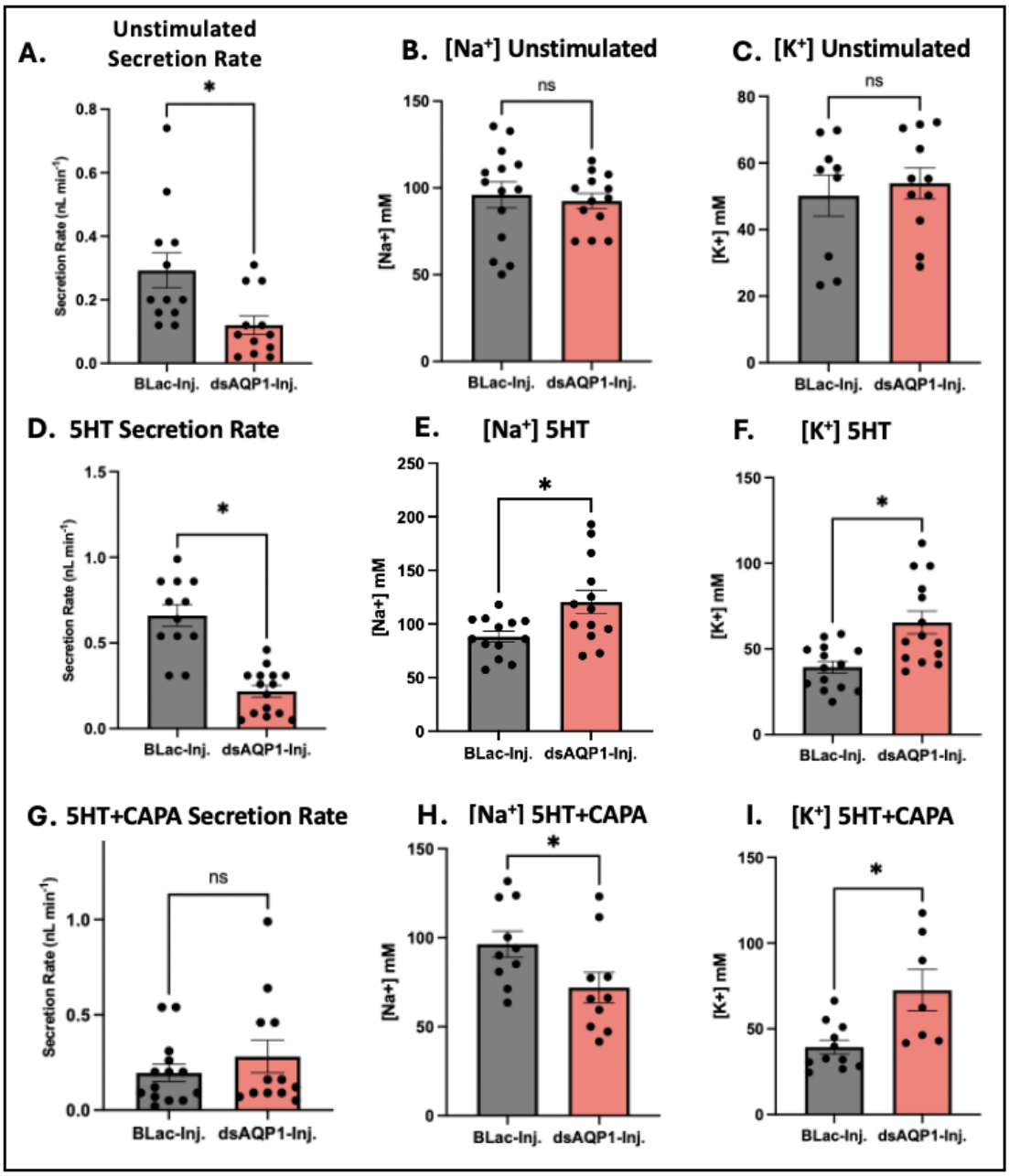

Figure 3-7. The effects of AaAQP1 knockdown on female mosquito Malpighian tubule secretion rate and cation concentration with the addition of 5HT and 5HT with *Aedae*CAPA-1. MTs were injected with dsβLac or dsAQP1 and then treated with physiological saline, 100nmol l^{-1} 5HT, or 100nmol l^{-1} 5HT with 0.1 fmol l^{-1} *Aedae*CAPA-1, each for 1hr at room temperature before measurements were taken. **A, D, G.** Secretion rate measured over 1hr (nL min^{-1}) in each treatment. **B, E, H.** $[Na^+]$ measured in the secreted droplets of female MTs. **C, F, I.** $[K^+]$ measured in the secreted droplets of female MTs. All values presented are mean±SEM and n=7-15. For each panel, an unpaired t-test was used to assess changes between treatments and significant differences are denoted with an * ($p<0.05$).

3.5 Discussion

The rapid production of urine post blood feeding in adult female mosquitoes has been studied extensively and has been largely attributed to the production and release of diuretic neuropeptides including the calcitonin-like hormone DH_{31} and the biogenic amine 5HT (Wheelok et al., 1988; Clark & Bradley, 1998). More recently, the CAPA peptides, specifically *Aedae*CAPA-1 has been shown to inhibit both *Drome*DH_{31}- and 5HT-stimulated secretion in adult female *Ae. aegypti* (Sajadi et al., 2018), while also eliciting its anti-diuretic effects in other hematophagous insects such as *Rhodnius prolixus* (Paluzzi et al., 2008). To date, it has been shown that diuretics including DH_{31} and 5HT cause an increase in fluid and ion secretion by the MTs, while the anti-diuretic *Aedae*CAPA-1 causes a decrease in stimulated fluid and ion secretion by the MTs (Sajadi et al., 2018). However, little is known about how water transport in the tubule epithelium, which is critical for this diuresis, might be regulated by the various neurohormones released post blood feeding in adult female mosquitoes.

This study aimed to understand the role of a water-specific aquaporin (AQP) known as AaAQP1 (DRIP homolog) in the diuretic and anti-diuretic response seen in adult female *Ae. aegypti* MTs. Due to the role of AaAQP1 in water secretion by the MTs (Drake et al., 2010), I predicted that knockdown of the AQP would decrease basal secretion rate by the MTs. In addition, I predicted that *Drome*DH_{31}- and 5HT-stimulated secretion would be decreased following AaAQP1 knockdown, which would be further amplified with the addition of *Aedae*CAPA-1.

AaAQP1 has been identified as one of six AQPs in the adult female mosquito and is one of at least four AQPs that is responsible for water transport across the tubule epithelium (Drake et al., 2010; Drake et al., 2015). In addition, AaAQP1 mRNA in *Ae. aegypti* MTs has been shown to be significantly upregulated ~3hr post blood meal (PBM) but returns to levels observed in non-

blood fed (NBF) MTs at ~24hr PBM (Drake et al., 2010). The diuretic process has been shown to begin while the female mosquito is still engorging on a blood meal, with peak fluid secretion being reached <10 min PBM (Williams et al., 1983; Beyenbach & Piermarini, 2010), indicating that the removal of excess fluid by the MTs is a rapid process following a blood meal. Recently, I showed that in the short-term period following a blood meal (~30 min PBM), AaAQP1 protein levels did not change and remained consistent with levels seen in NBF MTs, as well as MTs 24hr PBM (Picinic et al., 2024). In this study, 5HT stimulation (1hr incubation) of MTs resulted in AaAQP1 protein levels as well as localization in the MTs that did not change and remained consistent with the levels of AaAQP1 in unstimulated MTs. The addition of *Aedae*CAPA-1 to 5HT-stimulated MTs did not alter AaAQP1 protein levels or localization, similar to 5HT-stimulated MTs. It is likely that due to the opportunistic nature of female *Ae. aegypti*, AaAQP1 protein would be readily available in the MTs for the onset of a blood meal. Therefore, the addition of 5HT, one of many diuretics acting in the post-prandial response of female *Ae. aegypti*, did not alter total AaAQP1 protein levels. It has been suggested that AaAQP1 may be regulated by translocation at the membrane of the MTs, as there is evidence that shows blood feeding induces phosphorylation of AaAQP1 (Kandel et al., 2022), which could explain why AaAQP1 protein levels remain constant PBM. Although protein levels of AaAQP1 remained unchanged post 5HT and 5HT with *Aedae*CAPA-1 treatments, I found that knocking down AaAQP1 in female *Ae. aegypti* using dsRNA microinjections caused significant inhibition of the diuretic activity of 5HT on the MTs. AaAQP1 knockdown caused significant decreases in basal as well as 5HT-stimulated secretion rate. Presumably, as a result of decreased fluid in the primary urine, I found $[Na^+]$ and $[K^+]$ to be significantly higher in the secreted fluid of MTs from dsAQP1-injected females. Also, 5HT has been shown to elicit kaliuretic activity through its signalling cascade involving cAMP as a second

messenger, thereby driving the secretion of K^+ across the tubule membrane (Veenstra, 1988; Clark and Bradley, 1995; Sajadi et al., 2018). In the adult mosquito, the diuretic role of 5HT is complicated because although it has been shown to induce increased fluid and ion secretion by the MTs (Sajadi et al., 2018), the magnitude and significance of its effects on rapid urine production is unclear (Cady & Hagedorn, 1999). Cady and Hagedorn (1999) studied the effects of 5HT on urine production in adult *Ae. aegypti* and found that extremely high doses (10^{-3}-10^{-2}nmol l^{-1}) were required to detect changes in percent urine collected, highlighting the question of what exactly the role of 5HT is in the post-prandial diuresis of *Ae. aegypti*. However, it appears that AaAQP1 is important in basal urine secretion as well as in executing the activity of 5HT in increasing fluid secretion as its effects were drastically reduced with AaAQP1 knockdown.

In this study, I also examined the effects of *Aedae*CAPA-1 on 5HT-stimulated secretion and cation concentration in the fluid secreted by MTs post AaAQP1 knockdown in female *Ae. aegypti*. In treatments involving both 5HT and *Aedae*CAPA-1, secretion rate by MTs was similar between the dsβLac-injected MTs and dsAQP1-injected MTs, indicating that the addition of *Aedae*CAPA-1 to 5HT-stimulated MTs abolished the effects of AaAQP1 knockdown on the secretion of primary urine. In addition, [Na^+] was significantly reduced, whereas [K^+] was significantly increased in the secreted fluid following AaAQP1 knockdown. *Aedae*CAPA-1 has been shown to work through the cGMP/PKG signalling pathway to elicit its anti-diuretic effects on both 5HT- and DH_{31}-stimulated tubule secretion (Sajadi et al., 2018). In doing so, *Aedae*CAPA-1 results in inhibition of the apically localized V-type H^+-ATPase in 5HT-stimulated MTs in adult *Ae. aegypti* (Sajadi et al., 2023). It is likely that water transport by AaAQP1 is interrupted upon the addition of *Aedae*CAPA-1 to 5HT-stimulated secretion because of a partially reduced transport gradient where less H^+ ions are moved across the tubule membrane into the lumen (Sajadi et al.,

2023), which could lead to less water being moved via AaAQP1 into the tubule lumen. Thus, with AaAQP1 knockdown, it is possible that *Aedae*CAPA-1-inhibited basal secretion is not affected and decreased [Na^+] with AaAQP1 knockdown post 5HT with *Aedae*CAPA-1 treatment occurs as a result of a reduced H^+ gradient that favours ion secretion. In previous studies, urine secreted by 5HT stimulated MTs (with or without *Aedae*CAPA-1) was found to have increased [K^+] (Sajadi et al., 2018) that aligns with the findings of this study, where [K^+] remained elevated with 5HT and 5HT with *Aedae*CAPA-1, even after AaAQP1 knockdown. In adult female *Ae. aegypti* haemolymph, K^+ levels are kept at ~3.4mM with a considerable amount of K^+ entering the tubule lumen via inward rectifying potassium (Kir) channels, raising K^+ levels to ~65mM inside the lumen (Beyenbach & Piermarini, 2010). Post 5HT stimulation, [K^+] of secreted fluid has been measured ~150mM and ~140mM post 5HT with *Aedae*CAPA-1 stimulation (Sajadi et al., 2018). Based on the current findings on AaAQP1, it is possible that the movement of K^+ via Kir channels is independent of AaAQP1 in 5HT-stimulated secretion, as [K^+] remained at similar levels following AaAQP1 knockdown with 5HT and 5HT with *Aedae*CAPA-1-stimulated secretion.

In this study, I also observed the effects of the mosquito natriuretic peptide *Drome*DH_{31} on AaAQP1 protein abundance and localization in female *Ae. aegypti* MTs. Western blotting showed that AaAQP1 protein abundance was significantly decreased in the membrane fraction of MTs post treatment with *Drome*DH_{31} and *Drome*DH_{31} with *Aedae*CAPA-1, relative to unstimulated MTs. However, based on immunohistochemistry which revealed bright AaAQP1 staining at the apical membrane of the tubules in unstimulated, *Drome*DH_{31}, and *Drome*DH_{31} with *Aedae*CAPA-1 treatments, further investigation of the western blotting results was conducted. A study by Karas et al. (2005) found that β-actin was increased at the apical membrane of female *Ae. aegypti* MTs post blood meal and following stimulation with cAMP. Based on my western blot data, where the

membrane fraction of tubule protein was isolated and analyzed, it is possible that in the presence of *Drome*DH$_{31}$ or *Drome*DH$_{31}$ with *Aedae*CAPA-1, an abundance of actin in the membrane fraction caused a dilution of AaAQP1 protein due to an increase in total protein overall. Western blotting on membrane protein treated with *Drome*DH$_{31}$ and *Drome*DH$_{31}$ with *Aedae*CAPA-1 revealed a trend suggesting an increase in β-actin, relative to the unstimulated control (although the difference at present in not significant). Further replicates are required to confirm whether increased β-actin and total protein in the membrane fraction of *Drome*DH$_{31}$ and *Drome*DH$_{31}$ with *Aedae*CAPA-1-treated MTs could explain the decrease in AaAQP1 protein seen within the same samples.

The role of AaAQP1 was also examined in response to *Drome*DH$_{31}$ and *Drome*DH$_{31}$ with *Aedae*CAPA-1 treatment by observing the effects of AaAQP1 knockdown on female *Ae. aegypti* tubule secretion rate and cation concentration. In comparison to the decrease in secretion rate I observed with AaAQP1 knockdown in unstimulated MTs, AaAQP1 knockdown did not have an effect on *Drome*DH$_{31}$-stimulated secretion rate or cation (Na^+ and K^+) concentration in the secreted fluid. DH$_{31}$ has been identified as the main diuretic hormone released in circulation post blood meal (PBM) for female *Ae. aegypti* (Petzel et al., 1986; Wheelock eet al., 1988; Coast et al., 2005; Sajadi et al., 2018). Specifically, DH$_{31}$ has been shown to increase fluid secretion by the MTs 5-fold relative to basal secretion rates (Coast et al., 2005; Sajadi et al., 2018). In the MTs, five of the six *Ae. aegypti* AQP genes are expressed (Drake et al., 2010; Picinic et al., 2024), with AaAQP1, AaAQP2, AaAQP4, and AaAQP5 mRNA being upregulated 3hr PBM (Drake et al., 2010; 2015). It is likely that in the presence of DH$_{31}$, which would be present in the immediate PBM response, the magnitude of water secretion by the MTs is so great that multiple AQPs, in addition to AaAQP1, facilitate post-prandial transepithelial water secretion. Therefore, upon knockdown of

AaAQP1 in the presence of *Drome*DH$_{31}$, there is no apparent physiological consequence suggesting other AQPs (i.e., AaAQP2, 4, or 5) could play a more prominent role in transporting water in DH$_{31}$-stimulated MTs.

Furthermore, the addition of *Aedae*CAPA-1 to *Drome*DH$_{31}$-stimulated MTs showed similar results to that of unstimulated (basal) secretion rates, where AaAQP1 knockdown caused a significant decrease in secretion rate. *Aedae*CAPA-1 has been shown to inhibit DH$_{31}$-stimulated secretion in *Ae. aegypti* MTs, working through the inhibition of the VA (Sajadi et al., 2018; Sajadi et al., 2023). Therefore, in the presence of the anti-diuretic *Aedae*CAPA-1, secretion rates are lowered being similar to basal secretion rates and thus, AaAQP1 knockdown is once again causing a decrease in fluid secretion rate. When analyzing the cation concentration in secreted fluid post-*Drome*DH$_{31}$ with *Aedae*CAPA-1 treatment, AaAQP1 knockdown tubules secreted less total Na^+, while K^+ levels remained the same. This is likely due to the dissociation of VA in the presence of *Aedae*CAPA-1 which reduces the transport gradient of Na^+ and thus results in less overall Na^+ in the secreted fluid (Beyenbach & Piermarini, 2010; Sajadi et al., 2023). Therefore, with AaAQP1 knockdown, the addition of *Aedae*CAPA-1 caused reduced transepithelial secretion of water, which led to reduced Na^+ secretion which may indicate less Na^+-favoured ion secretion. Interestingly, [K^+] remained unchanged with AaAQP1 knockdown in *Drome*DH$_{31}$ with *Aedae*CAPA-1 treatment. However, this is likely due to *Drome*DH$_{31}$-alone being natriuretic and thus, K^+ transport occurring in spite of *Drome*DH$_{31}$ or *Drome*DH$_{31}$ with *Aedae*CAPA-1 treatment. Although fluid and Na^+ secretion appeared reduced with AaAQP1 knockdown in the presence of *Drome*DH$_{31}$ with *Aedae*CAPA-1, K^+ is likely continuing to move into the tubule lumen via Kir channels and maintain homeostatic K^+ levels (Beyenbach & Piermarini, 2010).

Overall, the post-prandial response of female *Ae. aegypti* MTs is complex and requires the coordinated action of signalling hormones, ion transporters, and water channels to result in control of urine production and secretion. It is evident that AaAQP1 is a key protein in maintaining homeostatic fluid levels during basal secretion by the MTs. Additionally, AaAQP1 appears to be important in the diuretic action of 5HT to increase fluid secretion, as well as in the anti-diuretic response of *Aedae*CAPA-1 on *Drome*DH_{31}-stimulated MTs. However, further investigation of the other AQPs present in *Ae. aegypti* MTs under the influence of diuretic and anti-diuretic factors is required to gain a better understanding of the hormonal control of transepithelial fluid and ion secretion in the MTs.

3.6 References

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Chapter Four

The localization and abundance of AQPs in the adult male disease vector mosquito, *Aedes aegypti*.

This chapter has not yet been submitted for publication.

4.1 Summary

In recent decades, the control of disease vector mosquitoes including *Aedes aegypti* has become a popular topic of study and strategies have included the use of engineered male mosquitoes. An important prerequisite for utilizing male mosquitoes to mitigate disease transmission is a thorough understanding of their physiology. Most of the focus in research is on female *Ae. aegypti* mosquitoes, with much less known about adult male *Ae. aegypti*. Male mosquitoes are unique to their female counterparts, as they do not feed on vertebrate blood, but instead only feed on plant nectar for all of their vital nutrients. Adult mosquitoes possess a set of osmoregulatory organs including the midgut, Malpighian tubules (MTs), and hindgut that function to maintain haemolymph osmolarity, especially in response to blood or nectar feeding. In particular, the MTs produce a primary urine that removes excess salts and some water. The movement of water is made possible by the presence of aquaporins (AQPs) and in *Ae. aegypti*, six AQP genes have been identified (AaAQP1-6) and localized to female osmoregulatory organs. Additionally, AaAQP1, 2, and 6 have been identified as water-specific, whereas AaAQP4 and 5 are characterized as entomoglyceroporins with the ability to transport some solutes in addition to water. In this study, I localized AaAQP1, 2, 4, 5, and 6 in sugar-fed adult male *Ae. aegypti* using immunohistochemistry. I further examined the role of the water-specific AaAQP1 and the entomoglyceroporin AaAQP4 in the MTs of sugar fed and starved males. Immunohistochemistry revealed AaAQP1 and 4 localization to the apical membrane of the MTs under both sugar fed and starved conditions. In addition, western blotting revealed a significant decrease in AaAQP1 and 4 protein abundance in male *Ae. aegypti* MTs following starvation stress, suggesting a role for both of these particular AQPs in water transport post nectar feeding.

4.2 Introduction

The mosquito *Aedes aegypti* is well known for its ability to transmit several deadly arboviral diseases in subtropical and tropical regions around the globe. In recent years, the development of disease transmission prevention strategies has become a primary focus for global communities impacted by *Ae. aegypti*. A successful target to *Ae. aegypti* population control is the sterilization of male mosquitoes known as the sterile insect technique to prevent reproduction within a population which has been shown to work in many places such as Cuba where Dengue fever is a serious threat (Gato et al., 2021). In order to continue improvements towards development of population control mechanisms for *Ae. aegypti*, understanding the physiology of the male mosquito is vital. The osmoregulatory physiology of *Ae. aegypti* is well understood, but a primary focus has been placed on the females of the species. Adult male *Ae. aegypti* solely feed on plant nectar for all of their life-sustaining nutrients and thus, the sugars they ingest from various plant nectars are used for all energy requirements including baseline metabolism, sperm production, and mating behaviour (Barredo & DeGennaro, 2020). Therefore, in ecosystems where sugar-rich plants are minimal or absent, *Aedes* populations of various species have been shown to decline due to lower insemination rates by male mosquitoes (Barredo & DeGennaro, 2020), highlighting the importance of diet in the success of male mosquitoes. A nectar meal in adult mosquitoes introduces both water and sugars that are dealt with by a complex system of osmoregulatory organs. Initially, the nectar meal is delivered directly to the anteriorly located ventral diverticulum (VD) or crop which is primarily responsible for storage of the meal (Calkins et al., 2017). Then, the VD undergoes peristaltic contractions to pass the nectar meal to the midgut (MG) which is the organ responsible for the digestion of the meal (Rudin & Hecker, 1976).

In female mosquitoes, the MG is responsible for some absorption of ions and water post blood meal (Pacey & O'Donnell, 2014). In male mosquitoes, it is likely that the MG functions similarly post nectar meal at a lesser magnitude due to an influx in water by the nectar meal. More posteriorly are the five Malpighian tubules (MTs) which have been shown to produce an ion-rich primary urine in adult *Ae. aegypti*, limiting the removal of water to prevent dehydration when feeding has not yet occurred (Beyenbach et al., 1993; Beyenbach & Piermarini, 2010). The MTs are comprised of two main cell types; the larger and more abundant principal cells and the smaller distally located stellate cells, which are both involved in the production of primary urine that is driven by the gradient established by the apically located V-type H^+ATPase (VA) (Wieczorek et al., 2009). Finally, the hindgut (HG) is the terminal organ of the alimentary canal and is responsible for the reabsorption of water and further removal of ions before excretion (Paluzzi et al., 2014). The coordination of ion transport and the movement of osmotically obliged water is essential to the functioning of these osmoregulatory organs.

In *Ae. aegypti*, six aquaporin (AQP) genes have been identified, denoted AaAQP1-AaAQP6 (Drake et al., 2010). AaAQP1, 2, and 6 have been shown to be water-specific AQPs (Drake et al., 2015; Sreedharan et al., 2016), while comparatively, AaAQP4 and 5 are known as entomoglyceroporins with an ability to transport water and solutes including urea and glycerol (Drake et al., 2015; Misyura et al., 2017). The transcript abundance and localization of AaAQP1,2,4,5, and 6 have been identified in the osmoregulatory organs of female *Ae. aegypti* (Drake et al., 2010; Picinic et al., 2024), however nothing is currently known about AQPs in male *Ae. aegypti*. Therefore, the purpose of this study was to localize each of the AQPs in adult male *Ae. aegypti* and examine the protein abundance of a water selective AQP (AaAQP1) and an

entomoglyceroporin (AaAQP4) in the MTs of adult males under nectar feeding and in starvation conditions.

4.3 Materials and Methods

Mosquito Rearing and Treatments

Animals used in this study were obtained from an established *Aedes aegypti* colony at York University in Toronto, Ontario. Male and female adult mosquitoes were kept in large mosquito cages, where eggs were collected on moist filter paper, air dried, and then hatched as necessary in 1L baths of dechlorinated tap water. The adult female mosquitoes were fed 2-3 times weekly with sheep's blood in Alsever's solution (Cedarlane Laboratories, ON, Canada) via a previously established feeding method (Rocco et al., 2017). The larvae were fed 2-3 times weekly with 1:1 liver powder-yeast mixture in ddH_2O. Pupae were collected from the larval baths in 10mL containers and placed into individual small mosquito cages, where they were left to emerge as adult mosquitoes. Both female and male mosquitoes were kept together in each individual mosquito cage. For the sugar-fed group, a 10% sucrose-soaked cotton ball was provided to the mosquitoes *ad libitum* from the time of pupal isolation and for tissue harvesting, mosquitoes were selected based on an enlarged crop indicating the male mosquito had acquired a nectar meal. For the starved group, no sucrose-soaked cotton ball was provided to the mosquitoes when pupae were isolated. In both sugar-fed and starved conditions, adult male mosquitoes were collected from the cages at 48hr post-emergence and used appropriately for each experiment.

Localization of AaAQPs with Immunohistochemistry

Immunohistochemistry was completed on the abdomen of male *Ae. aegypti* to localize AaAQP1,2,4,5, and 6. Additionally, MTs were isolated from adult male mosquitoes to localize AaAQP1 and 4 under both sugar-fed and starved conditions. MTs were isolated from male mosquitoes under physiological saline (Petzel et al., 1987) and placed in Bouin's fixative for 1hr

at room temperature (RT). For abdominal samples, male mosquitoes were anesthetized with CO_2 and placed directly into Bouin's fixative for 2hr at RT. Abdomen and isolated MTs samples were then dehydrated in a series of ethanol and xylene, following previously established procedures (Akhter et al., 2017; Misyura et al., 2020; Sajadi et al., 2023; Picinic et al., 2024). Briefly, thin paraffin embedded tissue sections were obtained with an Epredia HM325 manual microtome (MI, USA) and placed on Fisherbrand™ ColorFrost™ Plus Adhesion Microscope slides on a slide warmer for 24hr to improve section adhesion. Slides were then processed in a two-day procedure of washes in 1xPBS. For abdominal sections, three biological replicates (with 3-4 sections each) were placed on each slide and 4 technical replicates were completed. For MTs sections, three total biological replicates were completed, with 3-4 sections from one sugar fed and one starved biological replicate placed on each slide to draw comparisons between the groups. A total of 4 technical replicates were also completed. Abdominal sections were probed with each of the following antibodies following similar methods described by Picinic et al., 2024; anti-AaAQP1 affinity-purified primary antibody (1:1000 rabbit polyclonal antibody against CFFKVRKGDEESYDF, Genscript, NJ, USA) (Misyura et al., 2020), Anti-AaAQP2 affinity purified primary antibody (1:50 rabbit polyclonal antibody against CNGLGNTGLKENVQD, Genscript, NJ, USA) (Durant et al., 2021), Anti-AaAQP4 affinity purified primary antibody (1:500 rabbit polyclonal antibody against CPAEQAPSDVGKSNQS, Genscript, NJ, USA) (Misyura et al., 2020), Anti-AaAQP5 affinity purified primary antibody (1:1000 rabbit polyclonal antibody against CFRREVPEPEYNRELT, Genscript, NJ, USA) (Misyura et al., 2020), or Anti-AaAQP6 affinity purified primary antibody (1:50 rabbit polyclonal antibody against CSFRNMFLADKAKAE, Genscript, NJ, USA). For a membrane marker, the sections were also probed with a mouse monoclonal anti-a5 antibody for Na+/K+-ATPase (NKA) (Douglas Fambrough, Developmental Studies Hybridoma Bank, IA,

USA, 1:10 dilution). MTs sections were probed with the same AaAQP1 antibody as described above for sugar-fed vs. starved groups, as well as a guinea pig anti-V1 antibody for V-type H+-ATPase (VA) as an apical membrane marker (Ab 353-2, gifted by H. Wieczorek, Osnabruck, Germany, 1:5000 dilution). To visualize the AaAQPs, a goat anti-rabbit AlexaFluor 594 antibody (Jackson Immunoresearch) was used at 1:400; to visualize NKA a goat anti-mouse antibody conjugated to Cy5 (Jackson Immunoresearch) was used at 1:500; to visualize VA, a goat anti-guinea pig AlexaFluor 488 antibody (Jackson Immunoresearch) was used at 1:500. All slides were sealed with a glass coverslip and mounted with ProLong® Gold antifade reagent with DAPI (Life Technologies, ON, Canada). Imaging was completed with an Olympus IX81 fluorescent microscope (ON, Canada) in combination with CellSense® 1.12 Digital Imaging software. Final immunohistochemical images were overlayed with ImageJ 1.53a Software (USA).

Gel Electrophoresis and Western Blotting for AaAQP1 and AaAQP4

The quantification of AaAQP1 and AaAQP4 were measured in the MTs of 2-day old male *Ae. aegypti* mosquitoes, after a nectar meal or after starvation from emergence. For each biological replicate, MTs were collected and pooled from ~75 male mosquitoes under physiological saline (Petzel et al., 1987), for a total of 4 biological replicates. Extracted protein from MTs was quantified with a Bradford assay and then samples were prepared for SDS-PAGE, as previously described (Picinic et al., 2024). About 10µg of MTs protein was combined with 50mM Tris buffer and 6x loading buffer (225mmol L-1 Tris-HCl, pH 6.8, 3.5% SDS, 35% glycerol, 12.5% ß-mercaptoethanol, and 0.01% bromophenol blue), followed by denaturation for 5min at 100°C. The protein samples were run on a 12% SDS-PAGE gel (~1.5hr) and then wet transferred onto a polyvinyl difluoride membrane at 90V for 2hr on ice. Membrane blocking was completed with 5%

blocking buffer (5g skim milk powder, 100mL Tris-buffered saline) and then membranes were probed with the specific anti-AaAQP1 affinity purified antibody (1:1000 rabbit polyclonal antibody against CFFKVRKGDEESYDF, Genscript, NJ, USA) or anti-AaAQP4 affinity purified antibody (1:500 rabbit polyclonal antibody against CPAEQAPSDVGKSNQS, Genscript, NJ, USA) at 4°C overnight. The following day, membranes were washed in 1xTris-buffered saline and then probed with an HRP-conjugated goat anti-rabbit secondary antibody (1:5000) (BioRad, CA, USA) for 1hr at room temperature. Protein bands were visualized using the chemiluminescent Clarity™ Western ECL substrate kit (BioRad) and then viewed on a Chemi-Doc MP Imaging System (BioRad). Total protein was visualized with Coomassie staining and used as a loading control. The MTs protein bands were analyzed using ImageJ 1.53a Software (USA) to quantify AaAQP1 and AaAQP4 protein abundance against total loaded protein.

Statistics

Western blotting data for normalized protein abundance was displayed as mean values ± SEM for AaAQP1 and AaAQP4 in male *Ae. aegypti* MTs for both sugar fed and starved conditions. An unpaired t-test was completed to determine significant changes ($p<0.05$) between sugar fed and starved AaAQP1 or 4 proteins in the MTs.

4.4 Results

AaAQP Localization in Male *Ae. aegypti*

AaAQP1, 2, 4, 5, and 6 were localized in abdominal (AB) tissue sections of 2-day old adult male *Ae. aegypti* (Figure 4-1A-E), which were provided with sugar water *ad libitum*. In all images, the Na^+/K^+-ATPase (NKA) was used a membrane marker. Figure 4-1F shows a representative brightfield image of an AB male tissue section.

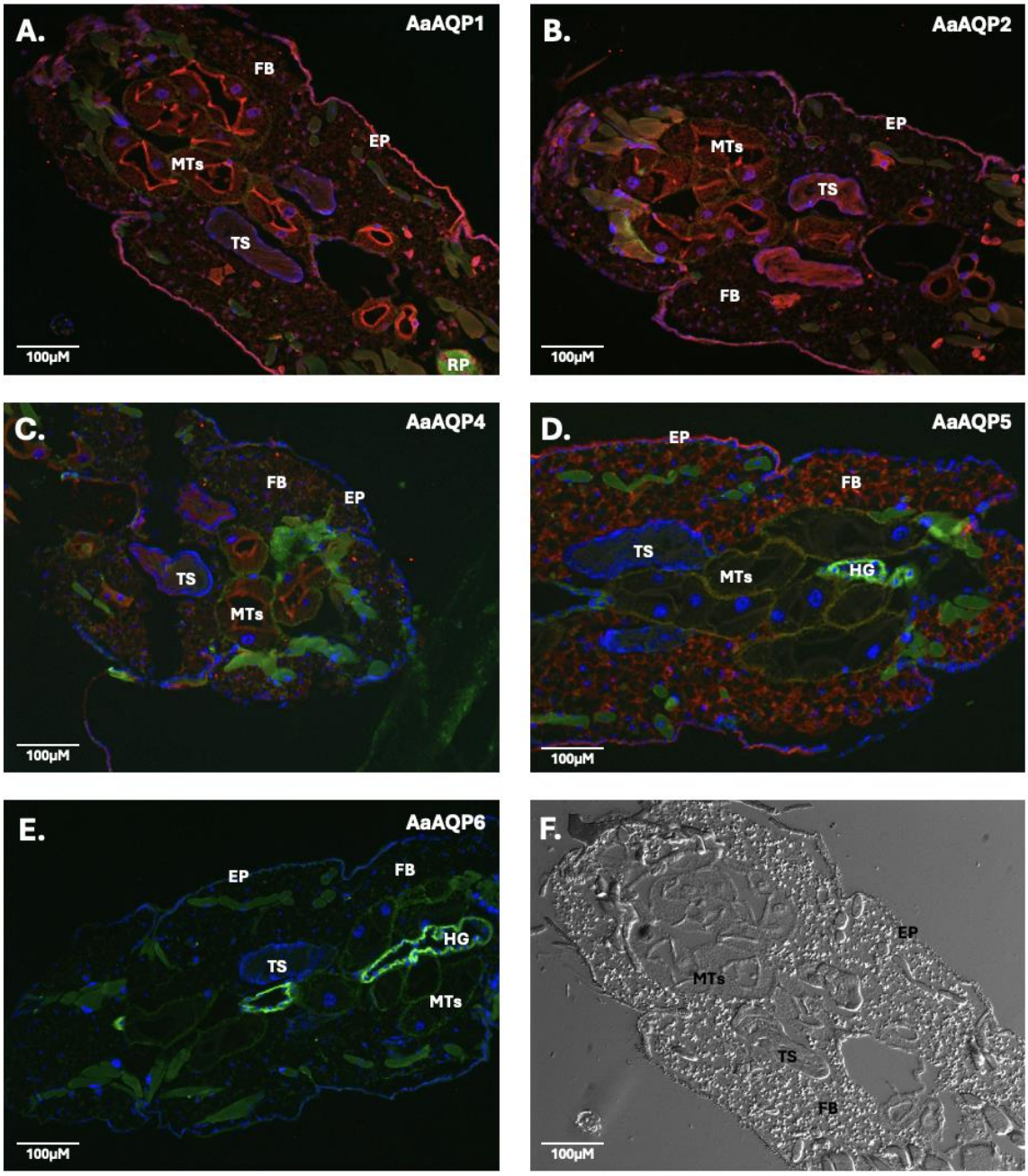

Figure 4-1. Abdominal localization of AaAQP1, 2, 4, 5, and 6 in adult sugar fed male *Aedes aegypti*.

Immunolocalization of aquaporins (AQP) in 2-day old adult male *Ae. aegypti* (n=3), which were fed sugar water *ad libitum*. Images were all captured at 10x magnification. Red staining indicates AQP localization, green staining indicates the localization of the membrane marker used; Na^+/K^+-ATPase (NKA), and blue staining indicates DAPI staining of nuclei. **A.** AaAQP1 localization; **B.** AaAQP2 localization; **C.** AaAQP4 localization; **D.** AaAQP5 localization; **E.** AaAQP6 localization; **F.** representative bright field image. MTs = Malpighian tubules, FB = fat body, EP = epidermis, TS = testes, RP = rectal pads, HG = hindgut.

AaAQP1

Figure 4-1A shows the immunoreactivity of the water-specific AaAQP1 in an AB adult male section. AaAQP1 showed bright and uniform staining at the apical membrane of the MTs, as well as prominent staining in the epidermal tissue (EP). Fat body (FB) tissue showed some staining of AaAQP1, as well as some staining was seen at the outer membrane of the rectal pads (RP). Minimal immunoreactivity of AaAQP1 was seen in the testes (TS), with low intensity staining present throughout the inside of the testes.

AaAQP2

Figure 4-1B shows the immunoreactivity of the water-specific AaAQP2 in an adult male AB section. AaAQP2 immunoreactivity appeared uniformly at the apical membrane of the MTs. Additionally, AaAQP2 staining was seen in the EP tissue and within the TS, with some staining also visible in the FB. It cannot be said if AaAQP2 staining was present in the HG, as no preparations stained with AaAQP2 had clear HG sections.

AaAQP4

Figure 4-1C shows the immunoreactivity of the entomoglyceroporin AaAQP4 in an adult male AB section. Overall, AaAQP4 immunoreactivity in the AB section was moderate, in comparison to the previously discussed AaAQPs. AaAQP4 mainly appeared uniformly at the apical membrane of the MTs. Additionally, some staining of AaAQP4 was visible in the TS and FB. It cannot be said if AaAQP4 staining was present in the HG, as no preparations stained with AaAQP4 had clear HG sections. No AaAQP4 staining was detected in the EP.

AaAQP5

Figure 4-1D shows the immunoreactivity of the entomoglyceroporin AaAQP5 in an adult male AB section. AaAQP5 immunoreactivity was mainly present in the FB tissue, with some staining appearing in the EP. In the MTs, minimal AaAQP5 staining was present at the basolateral membrane of the MTs, which is seen as yellow staining from colocalization with NKA. No AaAQP5 immunoreactivity was detected in the TS or HG.

AaAQP6

Overall, AaAQP6 immunoreactivity appeared absent in the male AB section (Figure 4-1E). No staining was detected in the MTs, FB, TS, EP, or HG.

AaAQP Localization and Abundance in Response to Starvation in Male *Ae. aegypti*

To investigate the role of a water-specific AQP (AaAQP1) and an entomoglyceroporin (AaAQP4) in the response to sugar-feeding, immunohistochemistry and western blotting were completed on adult male MTs. In isolated sections of MTs from sugar-fed mosquitoes, AaAQP1 staining appeared uniform at the apical membrane, which possibly suggests its presence in both the principal and stellate cells (Figure 4-2A). Additionally, AaAQP1 appeared colocalized with the V-type H^+-ATPase (VA), further confirming its localization at the apical membrane of principal cells of the MTs (Figure 4-2B). In the sections of MTs from starved mosquitoes, AaAQP1 staining was similarly localized to the apical membrane of the MTs and staining intensity was also similar in comparison to the sugar fed group (Figure 4-2C). Additionally, staining appeared less uniform and colocalization with VA in the starved sections was less consistent throughout the apical membrane of the MTs (Figure 4-2D) as VA appeared to stain apically and cytosolically. Protein

abundance of AaAQP1 in MTs was examined from sugar-fed versus starved male mosquitoes (Figure 4-2E). With starvation, AaAQP1 abundance was significantly lower in comparison to MTs from sugar fed mosquitoes ($p<0.05$).

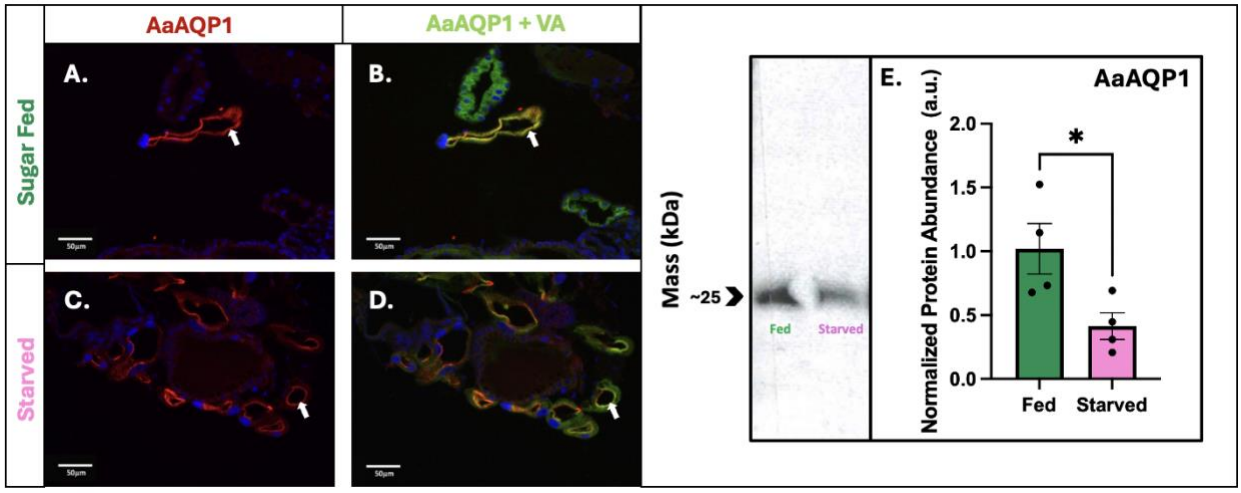

Figure 4-2. Localization and abundance of AaAQP1 in male *Aedes aegypti* Malpighian tubules.

Immunolocalization and protein abundance of AaAQP1 in male MTs, with sugar feeding and also with starvation. The left panel (**A-D**) represents the immunolocalization of AaAQP1, where red staining indicates AaAQP1 presence in the MTs, green staining indicates the apical MT marker; V-type H^+-ATPase (VA), and blue staining representing DAPI staining of tubule nuclei. Presence of yellow-green staining indicates co-localization of AaAQP1 with VA. **A.** AaAQP1 immunoreactivity at apical membrane of sugar fed MTs, **B.** AaAQP1 co-localized with VA at apical membrane of sugar fed MTs. **C.** AaAQP1 immunoreactivity at apical membrane of starved MTs, **D.** AaAQP1 with partial co-localization of VA at apical membrane of starved MTs. The right panel **(E.)** represents the normalized protein abundance of AaAQP1 in male *Ae. aegypti* MTs, post sugar feeding or post starvation. A significant decrease (*) in AaAQP1 protein was observed with starvation, in comparison to the sugar fed group. An unpaired t-test was completed to determine a significant difference ($p<0.05$).

Immunohistochemistry on sections of MTs isolated from sugar fed mosquitoes revealed AaAQP4 staining uniformly at the apical membrane (Figure 4-3A) similar to observations for AaAQP1. The localization of AaAQP4 at the apical membrane in principal cells was confirmed with colocalization with VA in the MTs (Figure 4-3B). In starved mosquitoes, AaAQP4 immunoreactivity appeared less abundant at the apical membrane of MTs; however, staining remained uniform at the membrane (Figure 4-3C). Colocalization with VA appeared to be less pronounced in the MTs from starved mosquitoes, where VA staining was not only found on the apical membrane but also in the cytosolic portion of the principal cells (Figure 4-3D). Furthermore, western blotting revealed a significant decrease in the protein abundance of AaAQP4 in MTs from starved mosquitoes, in comparison to levels in MTs samples from sugar fed mosquitoes (Figure 4-3E).

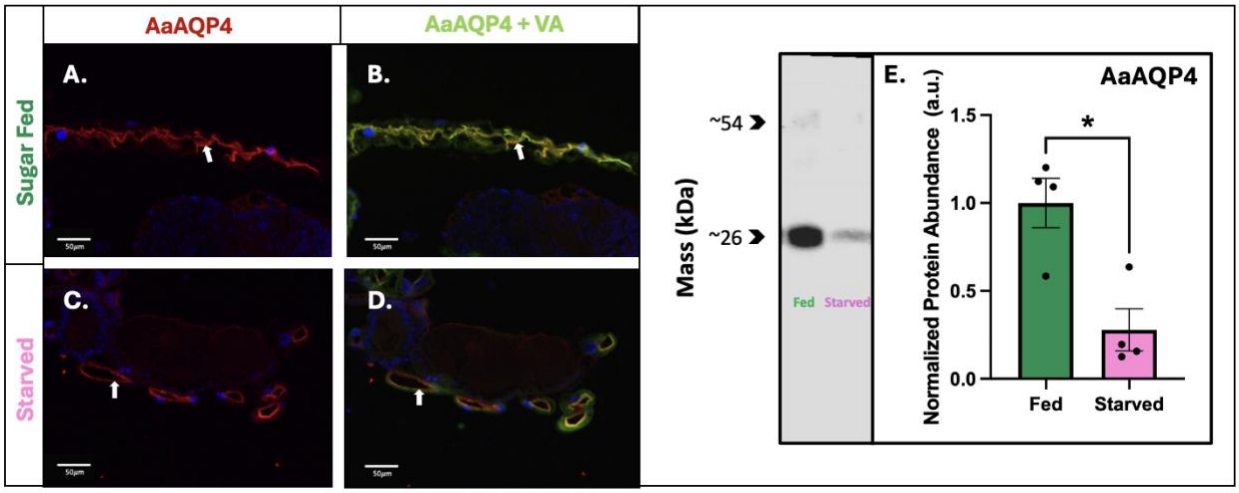

Figure 4-3. Localization and abundance of AaAQP4 in male *Aedes aegypti* Malpighian tubules.

Immunolocalization and protein abundance of AaAQP4 in male MTs, with sugar feeding and also with starvation. The left panel (**A-D**) represents the immunolocalization of AaAQP4, where red staining indicates AaAQP4 presence in the MTs, green staining indicates the apical MT marker; V-type H^+-ATPase (VA), and blue staining representing DAPI staining of tubule nuclei. Presence of yellow-green staining indicates co-localization of AaAQP4 with VA. **A.** AaAQP4 immunoreactivity at apical membrane of sugar fed MTs, **B.** AaAQP4 co-localized with VA at apical membrane of sugar fed MTs. **C.** AaAQP4 immunoreactivity at apical membrane of starved MTs, **D.** AaAQP4 co-localized with VA at apical membrane of starved MTs. The right panel (**E.**) represents the normalized protein abundance of AaAQP4 in male *Ae. aegypti* MTs, post sugar feeding or post starvation. A significant decrease (*) in AaAQP4 protein was observed with starvation, in comparison to the sugar fed group. An unpaired t-test was completed to determine a significant difference ($p<0.05$).

4.5 Discussion

In this study, I localized AaAQP1, 2, 4, 5, and 6 in the adult male *Ae. aegypti* mosquito and examined expression of AaAQP1 (water-specific) and AaAQP4 (entomoglyceroporin) in the main osmoregulatory organ, the Malpighian tubules (MTs) under sugar-feeding and starved conditions. The primary goal of this study was to determine the localization of each AaAQP in the adult male mosquito, while taking a deeper look into the role of a water-specific AaAQP and an entomoglyceroporin in the MTs in response to combined dietary and osmotic stress as a result of starvation.

AaAQP Localization in the Epidermis

The cuticle is an important part of a terrestrial adult mosquito, as it prevents desiccation by providing a hard waxy shield to the body which limits water loss to the environment. The cuticle of adult mosquitoes including *Ae. aegypti* and *Anopheles funestus* has been shown to increase in thickness in response to insecticides, further amplifying the important role the cuticle plays in the success of mosquito populations (Sugumaran & Semensi, 1987; Wood et al., 2010; Samal & Kumar, 2020). In addition, the epidermis is a layer of cells that is closely associated with and lies just beneath the cuticle, with the responsibility of producing chitin and synthesizing the protective cuticle (Farnesi et al., 2012). The expression of AQPs in the epidermis of terrestrial insects is broadly unknown but has been suggested that terrestrial insects can obtain water through active transport from the air during times of need (Beament, 1964). In this study, I detected immunolocalization of AaAQP1, 2, and 5 in the epidermis of sugar-fed adult male *Ae. aegypti*, while comparatively, immunoreactivity for AaAQP4 and 6 was not observed in the epidermis. AaAQP1 and 2 have been identified as water selective AQPs in *Ae. aegypti* (Drake et al., 2015)

and their presence in the male epidermis may be indictive of water transport needed for maintenance of epidermal tissue and thus production of the exoskeleton. In a study by Drake et al. (2010), moderate levels of AaAQP1 mRNA and low levels of AaAQP2 mRNA were detected in the fat body collections which also contained epidermal tissue and cuticle of female *Ae. aegypti*. In addition, studies on the sleeping chironomid *Polypedilum vanderplanki* have found an AQP orthologue (*PvAqp1*) present in the epidermis of the insect, which increased with desiccation stress (Kikawada et al., 2008). The presence of water selective AQPs in terrestrial insect including male *Ae. aegypti* epidermal cells may allow for water permeability when additional absorption of water is required to evade lethal dehydration. Unlike female mosquitoes, the males do not engorge on water-rich blood meals and thus are at a higher risk to desiccation as a result of their smaller surface area to volume ratio. My detection of AaAQP5 immunoreactivity in the epidermis of sugar fed male mosquitoes may be linked to the movement of small molecules across the epidermis of the male mosquito, as it is a known entomoglyceroporin (Drake et al., 2015). My immunohistochemistry was completed on sugar-fed male mosquitoes, therefore AaAQP5 could be involved in sugar transport at the epidermis post-nectar feeding. Using the *Aedes* Atlas (Hixson et al., 2022), epidermal cells were shown to express AaAQP1, AaAQP2, and AaAQP5, which all appeared to increase with blood feeding in female *Ae. aegypti* while there was relatively no detection of AaAQP4 and 6 in the epidermal cells. Therefore, regulation of some AaAQPs such as AaAQP5 may be tightly controlled within the epidermal cells of female *Ae. aegypti* post blood feeding and thus, could be occurring in adult males with nectar feeding.

AaAQP Localization in the Fat Body

In mosquitoes, the fat body (FB) is dispersed throughout the body and is mainly responsible for nutrient storage including lipids and carbohydrates, as well as the production of yolk proteins in adult females following a blood meal. Transcriptome analysis of the female *Ae. aegypti* fat body revealed many genes that are upregulated 24hr post blood meal (Feitosa et al., 2006); however much less is known about the fat body of male mosquitoes. Previously, AaAQP1-6 transcript abundances were detected in the fat body of adult female *Ae. aegypti* (Drake et al., 2010; Price at al., 2011) and later, AaAQP1, 2, 4, and 5 protein localization detected in the fat body of the female mosquito as well (Picinic et al., 2024). In this study on male *Ae. aegypti*, minimal immunoreactivity of AaAQP1, 2, and 4 were detected in the fat body, whereas prominent AaAQP5 immunoreactivity was observed in the fat body. AaAQP1, 2, and 4 likely play a minimal role in water or solute (AaAQP4) transport in the male fat body in light of the current immunohistochemistry results, as staining of all three AQPs appear relatively low. Similarly, AaAQP1, 2, and 4 transcript abundance in non-blood fed female *Ae. aegypti* were low in the FB in comparison to the other organs including the Malpighian tubules and midgut (Drake et al., 2010). My immunohistochemistry results revealed strong staining of AaAQP5 in the male fat body, which is consistent with non-blood fed female *Ae. aegypti* showing prominent AaAQP5 staining in the fat body (Picinic et al., 2024). The entomoglyceroporin AaAQP5 has been shown to transport solutes including the sugar alcohol adonitol and the disaccharide trehalose (Drake et al., 2015). Plant nectar is mainly comprised of glucose and fructose; however, sugar alcohols and other disaccharides like trehalose can also be found in plant nectar (Nicolson & Thornburg, 2007). Therefore, it is reasonable that male *Ae. aegypti* would require an abundance of AaAQP5 in the fat body, as it has an ability to transport important sugars that are taken in by the male upon nectar

feeding for use in daily metabolism and energy storage. Nectar feeding has been demonstrated to occur very shortly after emergence in the adult male mosquito *Aedes provocans* (Smith & Gadawski, 1994), as there is an immediate need for nutrient absorption before gonad maturation. The fat body is an important organ for opportunistic insects including *Ae. aegypti*, because it is able to store energy in the form of trehalose and glycogen, which the latter can be broken down into trehalose and used as energy when needed (Arrese & Soulages, 2010). Trehalose can be transported out of the fat body adipocytes and used for energy through the presence of trehalose membrane transporters (Kikawada et al., 2007; Arrese & Soulages, 2010), but based on the findings in this study, may also be transported by the abundantly expressed AaAQP5 in male *Ae. aegypti*. Additionally, lipid metabolism in the fat body produces an abundance of water as a biproduct, in particular as a result of triglyceride breakdown, and thus AQPs may be localized in the fat body to aid in water removal from the tissue to maintain osmotic balance (Arrese & Soulages, 2010).

AaAQP Localization in the Testes

Male mosquitoes possess a set of paired testes as their primary reproductive organ, which are responsible for the production of sperm through spermatogenesis (Oliva et al., 2014). The process of gamete production in insects involves a complex coordination of hormone signalling and nutrient consumption, which has been shown in some insects including adult *Ae. aegypti* and the red flour beetle *Tribolium castaneum* (Begnum et al., 2009; Sajadi & Paluzzi, 2024). In this study, I detected the presence of AaAQP1, 2, and 4 in the testes of adult male *Ae. aegypti,* with the most prominent staining seen for AaAQP2 in the testes. In female *Ae. aegypti*, low levels of AaAQP1 and 4 mRNA were detected in the ovarian tissue, with the highest expression in the

ovaries being from AaAQP2 (Drake et al., 2010). The role of AQPs in insect spermatogenesis is entirely unknown; however, studies on the male tobacco budworm *Heliothis virescens* have shown that haemolymph osmotic pressure is strongly correlated to the onset and phase of spermatogenesis (Loeb & Birnbaum, 1981). In vertebrate models such as teleost fishes, AQPs have been shown to be involved in early spermatogenesis, sperm health, and sperm motility (Huang et al., 2006; Boj et al., 2015). It is possible that AaAQP2 along with the help of AaAQP1 and 4 are involved in fluid movement in the testes which could be related to sperm survival and motility. Additionally, I used sugar-fed male mosquitoes in this study to mimic a typical scenario in nature, which could have resulted in active spermatogenesis and therefore, a pronounced presence of AaAQP2 in the testes.

AaAQP Localization in the Hindgut

The hindgut is an important osmoregulatory organ in mosquitoes, as it is responsible for alterations to the urine composition before excretion, which can be adjusted based on the osmotic needs of the insect. The hindgut is comprised of two main regions; the ileum which is mainly responsible for moving undigested material and urine to the terminal portion known as the rectum, which is responsible for water and salt reabsorption from the urine (Klowden, 2013; Paluzzi et al., 2014; Lajevardi & Paluzzi, 2020). In this study focusing on adult male *Ae. aegypti*, I only detected the presence of AaAQP1 protein localization in the rectal pads of the rectum. AaAQP1 mRNA has previously been found in the HG tissue of adult female *Ae. aegypti* (Drake et al., 2015), its homolog AgAQP1 found in gut tissue of adult female *Anopheles gambiae* (Liu et al., 2011), and its homolog DRIP in *Drosophila melanogaster* HG tissue (Chintapalli et al., 2013). Although there is no previous research involving AaAQP1 in adult male *Ae. aegypti*, it was earlier established that AaAQP1 mRNA is significantly upregulated 24hr after a blood meal in female *Ae. aegypti* HG

tissue (Drake et al., 2015). Furthermore, AaAQP1 immunoreactivity was also detected in the rectal pads of female *Ae. aegypti* 24hr post blood meal (Picinic et al., 2024). These findings in female *Ae. aegypti* after a blood meal indicates a potential influence of a nectar meal in the adult males on AaAQP1 abundance and localization in the HG. Although the intake of a nectar meal is only a fraction of the osmotic load on the male mosquito, it is the only meal they acquire in the terrestrial stage of their lifetime and thus, may induce changes in regions of the body such as the HG, where AaAQP1 may be involved in increasing the reabsorptive capacity of the HG to reuptake water post sugar-feeding to optimize hydration and maintain osmotic homeostasis.

AaAQP Localization in the Malpighian tubules

The primary osmoregulatory organ in insects is known as the Malpighian tubules (MTs), and in *Ae. aegypti*, there are five structurally and functionally analogous MTs that produce an ion-rich primary urine (Beyenbach et al., 1993; Beyenbach & Piermarini, 2010). The production of primary urine is driven by active transport of ions followed by osmosis of water via AQPs (Wieczorek et al., 2009). In this study, I observed the MTs of sugar fed male *Ae. aegypti* in AB tissue sections and found immunoreactivity of AaAQP1, 2, and 4 at the apical membrane of the tubules, while AaAQP5 was localized at the basolateral membrane of the tubules. As previously mentioned, AaAQP2 is a water-specific AQP (Drake et al., 2015) and is localized at the apical membrane of non-blood fed female *Ae. aegypti* (Picinic et al., 2024). However, in the adult females staining of AaAQP2 appeared punctate in non-blood fed individuals indicative of AaAQP2 localization in stellate cells, which later appeared uniform in staining at the apical membrane following a blood meal (Picinic et al., 2024). In *Drosophila melanogaster*, PRIP (AaAQP2 homolog) was found to be present in the stellate cells of the MTs (Cabrero et al., 2020).

Additionally, AaAQP2 transcript abundance in female *Ae. aegypti* MTs has been shown to increase 3hr post blood meal, indicating its possible role in water secretion post feeding (Drake et al., 2010). With immunohistochemistry, I found AaAQP2 to be evenly distributed at the apical membrane of the male MTs, indicating a presence of the AQP in the principal cells but could also be present in the stellate cells. My studies were completed on sugar-fed males, therefore the localization of AaAQP2 in the MTs appears similar in sugar fed males to its localization in the MTs of blood fed females (Picinic et al., 2024). Furthermore, I found minimal AaAQP5 localization uniformly at the basolateral membrane of the adult male MTs, which aligns with findings on adult female MTs that have similar patterns of basolateral staining of AaAQP5 (Picinic et al., 2024). AaAQP5 is an entomoglyceroporin, but still has a high capacity for water transport (Drake et al., 2015). Its localization potentially in both the principal and stellate cells of the tubules at the basolateral membrane highlights its role in moving water to or from the haemolymph as needed by the insect. In another mosquito species *Culex pipiens*, the mRNA of Eglp4, which is the homolog of AaAQP5 was found in high abundance in the adult males, in comparison to the other AQPs observed (Yang & Piermarini, 2017). Furthermore, in *D. melanogaster* MTs, Eglp4 was also found to be localized at the basolateral membrane of the principal cells (Cabrero et al., 2020). In male *Ae. aegypti*, it is likely that AaAQP5 could play a similar role to that of its female counter part, which is the transport of water and some solutes at the haemolymph-facing surface of the MTs to maintain homeostatic levels of fluids in the haemolymph.

Quantification of AaAQP1 and 4 in Fed Versus Starved Male Malpighian tubules

I wanted to take a deeper look into the potential roles of both the primary water channel AaAQP1 and the main entomoglyceroporin AaAQP4 in relation to diet in the MTs of male *Ae.*

aegypti. To do so, I used immunohistochemistry on isolated male MTs and found AaAQP1 staining to be present uniformly at the apical membrane of MTs in sugar fed male mosquitoes. However, in starved male *Ae. aegypti* I observed AaAQP1 immunoreactive staining to be less uniformly distributed across the tubule membrane. Western blotting showed a decrease in AaAQP1 protein abundance in MTs from male mosquitoes post starvation, relative to sugar fed male MTs. AaAQP1 mRNA has been found to be highly expressed in female *Ae. aegypti* MTs, relative to the other AQPs present and has been shown to increase with a blood meal (Drake et al., 2010). It is possible that in male *Ae. aegypti*, AaAQP1 in the MTs is required to remove excess water post nectar-feeding while, under starvation, would minimally express AaAQP1 to avoid excess water removal that could lead to lethal dehydration.

Finally, I also used immunohistochemistry on isolated male MTs to localize the entomoglyceroporin AaAQP4 and found staining to be present uniformly at the apical membrane of MTs from sugar fed male mosquitoes. With starvation, I found a noticeable decrease in AaAQP4 staining intensity at the apical membrane of the MTs. Supporting this qualitative assessment of reduced immunoreactive staining, western blotting confirmed a decrease in AaAQP4 protein in MTs from male mosquitoes post starvation, relative to MTs from sugar fed male mosquitoes. AaAQP4 mRNA has been detected in female *Ae. aegypti* MTs and, similar to AaAQP1, has been shown to increase with a blood meal (Drake et al., 2010). AaAQP4 protein has also been localized to the apical membrane of female MTs pre- and post-blood feeding (Picinic et al., 2024). Since AaAQP4 is a true entomoglyceroporin and favours the transport of solutes (Drake et al., 2015), its localization in male *Ae. aegypti* MTs is likely similar to its role in female MTs. Nectar feeding introduces an abundance of water and sugars to the male digestive tract and haemolymph (Barredo & DeGennaro, 2020), therefore the MTs may use AaAQP4 to remove some if the carbohydrate

metabolites that are destined for excretion. In *D. melanogaster,* the homolog of AaAQP4 (known as Eglp2) has also been localized to the principal cells of the MTs during their normal feeding conditions (Cabrero et al., 2020). In female *Ae. aegypti* and female *Aedes albopictus*, AQP4 (Eglp2 homolog) is downregulated 24hr post blood meal, which is considered the post-diuretic period of blood feeding (Drake et al., 2010; Drake et al., 2015; Esquival et al., 2014). The downregulation of AaAQP4 in female *Ae. aegypti* 24hr post blood meal has been proposed to be due to tubule secretion rates returning back to homeostatic levels and therefore, AaAQP4 levels must return to its original levels to reduce excess removal of water (Drake et al., 2015). In starved male *Ae. aegypti*, a similar mechanism may be at play where a reduction in AaAQP4 in the MTs help prevent unnecessary and excess water loss, which could increase the risk of desiccation.

In conclusion, this current study localized AaAQP1, 2, 4, 5, and 6 in the adult male *Ae. aegypti* mosquito. I also looked at the effects of diet on AaAQP1 and 4 localization and abundance in the MTs of male *Ae. aegypti*, to understand how feeding status (ie. fed versus starved) may influence a water selective AQP versus an entomoglyceroporin. Overall, I found that AaAQP1 in the MTs appears to be influenced by the presence or absence of a nectar meal. This was confirmed through western blotting which revealed decreases in AaAQP1 abundance in the MTs following starvation of male mosquitoes. Similar results were found with AaAQP4, where its abundance was significantly decreased in the MTs of starved male mosquitoes. The findings of this study are important given the physiology of male *Ae. aegypti* remains an understudied field, despite recent interest in the use of the males for mosquito population control. Advancing our understanding of AQPs, including their roles in male *Ae. aegypti*, can help to identify vital processes that could be altered for population control and thus, preventing the spread of disease.

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Chapter Five

The discovery of an aquaporin (CrAQP2) in the freshwater larval midge, *Chironomus riparius* and its role in response to road de-icers

This chapter has not yet been submitted for publication.

5.1 Summary

Freshwater *Chironomus riparius* midge larvae are ubiquitous in the Northern Hemisphere and have been identified as bioindicators since they can tolerate a range of environmental changes including increased salinity. In recent decades, freshwater ecosystems of temperate regions have experienced an increased threat to salinization due to the use of road de-icers. Traditional road de-icers include NaCl-based pellets or brine which are applied to roadways and walkways during winter months in temperate regions. As ambient temperatures fluctuate, snow and ice melt carry the NaCl into nearby freshwater ecosystems and significantly raise salt levels. Due to the detrimental effects of freshwater salinization on freshwater fauna, organic de-icers including brine beet juice de-icer (BBJD) have been implemented as an "ecofriendly" alternative to traditional road salt. However, not much is known about the effects of BBJD on freshwater invertebrates. For aquatic insects such as *C. riparius* larvae, they can tolerate appreciable changes to water salinity through the use of osmoregulatory organs which are the gastric caeca, midgut, Malpighian tubules, hindgut, and anal papillae. Osmoregulation in each of these organs is driven by the active transport of ions and the movement of osmotically obliged water to maintain internal haemolymph osmolarity. A key component in insect osmoregulation is the presence of water channel proteins known as aquaporins (AQPs). AQPs have been identified and characterized in many insects as transmembrane domain proteins that allow for water to move along its osmotic gradient through a pore formed by the channel. To date, there is limited knowledge on the influence of salinization and specifically road de-icers on AQPs in aquatic insects. In this study, I characterized a water-specific AQP known as CrAQP2 (PRIP homolog) in *C. riparius* larvae, identifying the Malpighian tubules and the anal papillae as the major organs for CrAQP2 immunolocalization. Furthermore, I observed the effects of BBJD and salt-contaminated water (SCW) on CrAQP2 mRNA transcript

abundance in the osmoregulatory organs of midge larvae and monitored survival and body moisture in response to de-icer contaminated water following CrAQP2 knockdown. SCW caused upregulation of CrAQP2 transcript in the midgut and anal papillae while reducing expression in the hindgut. Comparatively, BBJD had little effect on CrAQP2 transcript levels with only reduced abundance in the hindgut. CrAQP2 knockdown resulted in a significant decrease in total body moisture over a 24hr period regardless of treatment and significantly reduced survival of midge larvae transferred into BBJD water or SCW over a 7-day period. Therefore, CrAQP2 appears to be important in maintaining total body water levels stable over the short term and plays a role in the larvae's ability to respond to salinization over the long-term.

5.2 Introduction

The study of freshwater fauna has been a popular topic due to the unique diversity seen in freshwater ecosystems. Inland waters harbour a wide variety of animals, with over 60% of those animals being aquatic insects (Dijkstra et al., 2014). The ecological role of aquatic insects is important as they act as primary consumers, detritivores, and predators (Dijkstra et al., 2014). In particular, the presence of aquatic detritivorous insects is essential to freshwater ecosystems because they feed on decaying matter and work to clean their environment. One of many detritivores and primary consumers is the dipteran Chironomidae species, *Chironomus riparius* which is a non-biting freshwater midge that is widely distributed throughout the Northern Hemisphere (Pinder, 1986; Nemec et al., 2012).

Larval *C. riparius* reside in freshwater aquatic environments as benthic organisms and their post-embryonic development involves four larval instar phases before pupating and emerging as a terrestrial adult. The larvae have been effectively used as bioindicators for freshwater ecosystems in response to changes in water temperature, oxygen levels, and overall water quality (Vogt et al., 2011; Nicacio & Juen, 2015; Manfrin et al., 2018). *C. riparius* larvae have been shown to tolerate moderate increases in salinity (Jonusaite et al., 2010). This is significant because in recent decades, anthropogenic activities including but not limited to, the application of fertilizers, resource mining, and the use of road de-icers have resulted in increased levels of salts in freshwater ecosystems. These activities have thus been identified as deadly threats to freshwater ecosystems (Shuler & Relyea, 2018). In the Northern Hemisphere where *C. riparius* is widely distributed, the use of road de-icing agents through the winter months is important in preventing ice build-up on roadways but leads to freshwater salinization. Typically, the de-icers used are in the form of NaCl pellets or NaCl brine. However, as temperatures fluctuate in these northern regions, the snow and ice melt carries

the NaCl with it into the nearby freshwater ecosystems. It has been shown that road salt runoff and increased salinity can have detrimental effects on freshwater organisms that inhabit these aquatic ecosystems due to elevated levels of Na^+ and Cl^- (Meter et al., 2011; Nowghani et al., 2019; Hintz et al., 2021; Dugan & Arnott, 2022). As a potentially eco-friendly alternative to road salt, brine-beet juice de-icers (BBJD) containing relatively lower levels of NaCl are being implemented in limited amounts as replacement to the traditional NaCl de-icers (Nutile & Sloan, 2019b; Picinic et al., 2022). However, as the BBJD is derived from sugar beets, it is naturally high in K^+-levels, with reportedly 7-fold greater levels of K^+ in comparison to what is found in NaCl de-icers (Fay and Shi, 2012; Wruss et al., 2015; Picinic et al., 2022). The impact of K^+ on freshwater animals warrants further investigation before widespread use of BBJD as a de-icing agent. In previous studies, it has been shown that increasing concentrations of BBJD in the surrounding media of a freshwater bivalve causes body K^+ levels to drastically rise (Gillis et al., 2021). More recently, it was shown that BBJD may be equally harmful compared to the traditional NaCl-based de-icer to the amphipod crustacean, *Hyalella azteca*, due to increasing levels of ions in the water which impacted the activity of important enzymatic ion-transporters (Picinic et al., 2022).

The resilient nature of aquatic insects including *C. riparius* to changing salinity is largely due to the functioning of several specialized osmoregulatory organs found in the alimentary canal that work to regulate salt and water levels. Most anteriorly in the alimentary canal is the foregut (FG) which is responsible for moving food posteriorly and plays a small role in some ion-absorption into the haemolymph (Dow, 1986; Jonusaite et al., 2013; Linser & Dinglasan, 2015). Then, just below the FG are the pouch-like projections of the gut, known as the gastric caeca (GC) which function in some ion and water absorption into the haemolymph (Dow, 1986; Clark et al., 1999; Jonusaite et al., 2013; Linser & Dinglasan, 2015; D'Silva et al., 2017). Connected to the GC

is the largest osmoregulatory organ, the midgut (MG), which is primarily involved in food digestion, nutrient absorption, and some ion and water balance (Dow, 1986; Clark et al., 1999; Jonusaite et al., 2013; Linser & Dinglasan, 2015). Then, located at the junction between the MG and the most posterior portion of the gut, the hindgut (HG), are the Malpighian tubules (MTs). The MTs function in conjunction with the HG to produce urine that favours water secretion in freshwater aquatic insects, driven by the active transport of ions and movement of water via osmosis (Clark & Bradley, 1998; Donini et al., 2006; Zadeh-Tahmasebi et al., 2016). The ion rich primary urine secreted by the MTs is passed to the HG where final alterations of the urine such as the reabsorption of ions can occur, especially in a freshwater environment (Bradley & Phillips, 1977; Jonusaite et al., 2013; Linser & Dinglasan, 2015). Additionally, some larval-stage insects such as *C. riparius* and the mosquito *Aedes aegypti*, possess externally localized osmoregulatory organs known as the anal papillae (AP). The AP are balloon-shaped organs comprised of a one-cell layer thick epithelium that is primarily involved in active ion uptake from the environment in freshwater aquatic insects (Jarial, 1995; Nguyen & Donini, 2010; Surendran et al., 2018). All together, these osmoregulatory organs work to regulate the ionic composition and osmolarity of body fluids and play an important role in the ability of *C. riparius* to tolerate changes in environmental salinity.

To date, the effects of salinization on ion transport in freshwater insects has been studied, however comparatively much less is known about how salinization affects the transport of water. A key component in regulating and maintaining water levels in freshwater insects is the presence of water channel proteins, known as aquaporins (AQPs). AQPs are characterized as transmembrane domain proteins that form a channel in which water can traverse cellular membranes according to the osmotic gradient (Campbell et al., 2008; Spring et al., 2009; Drake et

al., 2015; Picinic et al., 2024). AQPs have been characterized in other dipteran insects such as the fruit fly *Drosophila melanogaster* (Dow & Davies, 2001; Kaufmann et al., 2005; Campbell et al., 2008) and the mosquito *Ae. aegypti* (Duchesne et al., 2003; Drake et al., 2010; Drake et al., 2015; Akhter et al., 2017; Misyura et al., 2017; Misyura et al., 2020; Picinic et al., 2024). Additionally, some AQP genes have been identified in midges such as the sleeping chironomid *Polypedilum vanderplanki* (Kikawada et al., 2008) and the non-flying Antarctic midge *Belgica antarctica* (Yi et al., 2011); however, there is currently no information about AQPs in the freshwater midge, *C. riparius*.

Of the six known insect AQP genes, AQP1 and AQP2, which are named following nomenclature established previously (Drake et al., 2010), have been identified as water-specific AQPs that have a high selectivity for water transport (Drake et al., 2015). AaAQP1 has been localized in larval *Ae. aegypti* to key osmoregulatory organs including the GC, MG, MTs, HG, and AP (Marusalin et al., 2012; Misyura et al., 2020). The AQP1 homolog in *B. antartica* was also found to be localized to the FG, MG, and MTs (Yi et al., 2011), alluding to its important function in water transport in these various osmoregulatory organs. Additionally, AaAQP2 has also been localized to the GC, MG, MT, HG, and AP of larval *Ae. aegypti* (Marusalin et al., 2012; Misyura et al., 2020) and its homolog has been localized with immunohistochemistry in the FG, MG, and HG of the midge *B. antarctica* (Yi et al., 2011). Furthermore, AaAQP2 has been shown to be salinity responsive in the AP of larval *Ae. aegypti*, where increased salinity in brackish water conditions caused a significant increase in AaAQP2 transcript and protein levels relative to freshwater conditions (Durant et al., 2021).

In order to begin to understand how both NaCl and BBJD may affect the regulation of water transport in freshwater insects I identified an important putative water channel protein

CrAQP2, a homolog of the insect AQP2, and studied its expression in the osmoregulatory organs of *C. riparius* exposed to NaCl or BBJD. I hypothesized that CrAQP2 transcript and protein would be abundant in each of the osmoregulatory organs of *C. riparius*, with highest expression expected in the MTs and AP where ion and water transport are constant in hypoosmotic conditions. Additionally, I predicted that in the presence of the de-icers, CrAQP2 would be upregulated in the organs that typically function in water absorption such as the MG; whereas CrAQP2 was predicted to be downregulated in the organs that typically function in water secretion such as the GC and MTs. Additionally, I predicted that de-icer contamination would result in increased CrAQP2 in the AP as a result of increased external osmolarity relative to FW. Furthermore, I predicted that there would be no change in expression of CrAQP2 in the HG, as expression levels were predicted to be low in this organ based on previous findings with *Ae. aegypti* larvae (Marusalin et al., 2012). Finally, I hypothesized that the reduction of CrAQP2 function through dsRNA knockdown in the midge larvae would result in increased mortality when larvae were faced with abrupt transfer to salinity.

5.3 Materials and Methods

Animal Rearing

Chironomus riparius larvae were taken from a lab established colony that is maintained at York University (Jonusaite et al., 2013). Larvae developed in ~6L tanks containing ~3cm of sand (K&E Industrial Sand, ON, Canada) and 3-4L of artificial fresh water (AFW: 300μM NaCl, 300μM $NaHCO_3$, 380μM $CaCl_2$, 43μM KCl; pH 7.6) that mimics their typical freshwater environment. The tanks were aerated with air stones connected to a constant air supply. The larvae were fed every 2-3 days with crushed TetraFin Goldfish Flake Food (Tetra Holding US, VA, USA) and kept at room temperature on a 12h:12h light: dark schedule. The water in the tanks was topped up once a week and water changes were completed every 3-4 weeks. For treatment with de-icers, 2% brine beet juice de-icer (BBJD) and 7g/L NaCl-contaminated water (SCW) were chosen based on previously established LC_{50} curves, which determined 50% survival at each of those concentrations for late-stage *C. riparius* larvae (Reinsborough & Donini, 2025 pending revision; Jonusaite et al., 2013).

Identification of CrAQP2, Tissue Collection, and Quantification of *CrAQP2* Expression with qPCR

To identify CrAQP2 in *C. riparius* larvae, the *C. riparius* reference genome PGI_CHIRRI_v4 (GCA_917627325.4; Schmidt et al., 2020) was searched by tblastn operation using the AQP2 (PRIP) protein sequences of *Aedes aegypti* (AY433444), *Polypedilum vanderplanki* (AB281619), and *Belgica antarctica* (AB602340) through the National Center for Biotechnology Information (NCBI) database. Primers were then designed to target a large portion of the putative CrAQP2 gene (Table 5-1). To obtain total RNA samples, 3-4 fourth instar larval *C.*

riparius were taken from the lab colony and transferred to a chilled 300µl aliquot of lysis buffer, prepared from the PureLink RNA minikit (Invitrogen, MA, USA). RNA extraction on whole body samples was completed following the protocol provided in the PureLink RNA minikit (Invitrogen). RNA samples were purified using the TURBO DNAfree™ Kit (Applied Biosystems, ON, Canada) and cDNA was then synthesized using the iScript cDNA synthesis kit (BioRad, CA, USA) consisting of both oligo(dT) nucleotides and random hexamer primers. The cDNA template was then amplified with Q5® High-Fidelity DNA Polymerase (M0491 - NEB, ON, Canada) using specific *Craqp2* primers (Table 5-1) and amplicon band size was confirmed on an agarose gel. The amplified product was then purified using the Monarch PCR Purification Kit (T1030L NEB, ON, Canada), concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE, USA) and then samples were sent for Sanger sequencing (The Centre for Applied Genomics, Sick Kids, ON, Canada). Sequencing quality and base accuracy was analyzed using Geneious Software (Dotmatics, MA, USA). Following this, quantitative real time PCR (qPCR) primers were designed from the confirmed partial gene sequence, targeting exon-exon boundaries to ensure cDNA amplicon specificity (Table 5-1).

Table 5-1. Oligonucleotides used for *Chironomus riparius* AQP2 gene amplification.
Each oligo name is listed with the corresponding forward and reverse primer sequences used to produce each amplicon. Oligonucleotides used for AQP2 gene identification and amplification are shown and were used for PCR, qPCR (RT-PCR), and dsRNA synthesis.

Oligo Name	Forward Primer Sequence	Reverse Primer Sequence	Expected Band Size	Experiment Used For
CrAQP2	CACTGGGTGTTGACGAGATC	TAAAGAGATGGGCGCACTTC	780bp	PCR
***Craqp2* qPCR**	TGCTATGACTATTGGACATG	ACATGTAGTGCTGCAGTTCC	153bp	qPCR
40S Reference Gene	TGTACAGAAGAAGAGGTCGAGAAA	GCATACCACGATGAGCACG	230bp	qPCR
RP11 Reference Gene	CCGTACATTGCACAGTTCGT	CATTATAGCCAGGTCTTCCC	216bp	qPCR
Elf1 Reference Gene	GCTCCTGGACATCGTGATTT	TGACTCCCAATGTGAAAGCC	159bp	qPCR
Tubulin Reference Gene	ACTTCTGCTTCAAAATGCGTG	CTACGGTTGGTTCCAGATCG	237bp	qPCR
CrAQP2 dsRNA	CACTGGGTGTTGACGAGATC	GCAACACTTCCAAAACATTGAGC	304bp	dsRNA Synthesis
dsAQP2 Reverse T7	CACTGGGTGTTGACGAGATC	TAATACGACTCACTATAGGGGCAACACTTCCAAAACATTGAGC	320bp	dsRNA Synthesis
dsAQP2 Forward T7	TAATACGACTCACTATAGGGCACTGGGTGTTGACGAGATC	GCAACACTTCCAAAACATTGAGC	320bp	dsRNA Synthesis
BLac dsRNA	ATTTCCGTGTCGCCCTTATTC	CGTTCATCCATAGTTGCCTGAC	800bp	dsRNA Synthesis

CrAQP2 transcript levels were then measured in the alimentary canal of AFW-reared larval *C. riparius* including the foregut with gastric caeca (FG+GC), midgut (MG), Malpighian tubules (MTs), hindgut (HG), and anal papillae (AP). The organs were isolated and pooled from ~40 larvae on ice in 200µl of chironomid saline (5 mM KCl, 74mM NaCl, 1mM $CaCl_2$, 8.5 mM $MgCl_2$, 10.2 mM $NaHCO_3$, 8.6 mM HEPES, 20 mM glucose, 10 mM glutamine), which were dissected in a Sylgard®-lined petri dish filled with the same saline. For each organ, a total of four biological replicates were dissected. Following collection, organ samples were centrifuged at 10,000 *g* for 1 min at RT, the saline was removed, and 1mL of TRIzol® reagent (Invitrogen, ON, Canada) was added. Samples were sonicated on ice using an XL 2000 Ultrasonic Liquid Processor (QSONICA - LLC, CT, USA) and total RNA was isolated from the lysed organ samples following the manufacturer's guidelines. The resulting RNA samples were treated with the TURBO DNAfree™ Kit for removal of any potential genomic DNA (Applied Biosystems, ON, Canada). RNA sample concentrations and purity values were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE, USA). cDNA for each organ was synthesized using the iScript cDNA synthesis kit (BioRad, CA, USA), as previously stated and following the manufacturer's instructions. For the study of the effects of de-icers on CrAQP2 transcript abundance in each of the osmoregulatory organs previously listed, midge larvae were taken from the lab colony and placed in 100mL of either BBJD, SCW or AFW (control) for 24hr acute exposures. Following that, all samples were treated identically for RNA isolation and cDNA synthesis (as described above), and for qPCR. CrAQP2 transcript abundance in AFW, BBJD, and SCW-treated midge larvae was determined with qPCR conducted using a CFX Duet Real-Time PCR System (Bio-Rad Laboratories, ON, Canada) in combination with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, ON, Canada), following the manufacturer's instructions.

Relative CrAQP2 transcript abundance was calculated using the previously established $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001) and values were normalized to the geometric mean of Rp11 and 40S reference genes for all organs, aside from the MTs where values were normalized to the geometric mean of tubulin and Elf1 (Table 5-1). All reference genes were confirmed to show no changes in transcript abundance with each treatment including AFW, BBJD, and SCW, in each of the organs studied with the respective sets of reference genes. The expression profile across the osmoregulatory organs in freshwater involved 4 biological replicates, while the individual CrAQP2 expression analyses for each organ in response to road de-icers involved 4-6 biological replicates, all loaded as triplicates for quantitative accuracy.

Immunohistochemistry

Immunohistochemistry (IHC) was carried out on whole body (WB) *C. riparius* midge larvae to localize CrAQP2 in the alimentary canal. Midges were taken from the lab established colony and placed in 100mL of AFW, BBJD, or SCW for 24hr acute exposures. Midges were placed in Bouin's fixative for 2hr at room temperature followed by a replacement of fixative with 70% ethanol and kept at 4°C until ready for dehydration. For each treatment, 3-4 biological replicates were used, in addition to ~4 technical replicates for confirmation of immunostaining consistency. The IHC procedures carried out in this study followed previously established protocols (Akhter et al., 2017; Misyura et al., 2020; Sajadi et al., 2023; Picinic et al., 2024). Briefly, fixed WB samples were embedded in paraffin wax and then ~5μm sections were cut using an Epredia HM325 manual microtome (Epredia, MI, USA). Sectioned tissues were then placed on Fisherbrand™ ColorFrost™ Plus Adhesion Microscope slides, with each slide having tissue

sections from the AFW, BBJD, and SCW samples, adjacent to each other. Slides were then processed in a two-day procedure where tissue sections were rehydrated and repeatedly washed with 1xPBS. All slides were probed with an affinity purified polyclonal antibody raised against a peptide representing a small fragment of the *Aedes aegypti* AQP2 homolog (AaAQP2) (1:50 rabbit polyclonal antibody raised against the peptide CNGLGNTGLKENVQD, Genscript, NJ, USA) (Durant et al., 2021; Picinic et al., 2024). An alignment between the custom AaAQP2 antigen sequence and the putative CrAQP2 antigen sequence showed ~50% similarity and can be seen in Supplementary Figures (5-S1). The mouse monoclonal anti-a5 antibody for Na^+/K^+-ATPase (NKA) (Douglas Fambrough, Developmental Studies Hybridoma Bank, IA, USA, 1:5 dilution) was used as a membrane marker for all tissue sections (Picinic et al., 2024). The secondary antibody used to detect AaAQP2-like protein was a goat anti-rabbit AlexaFluor 594 conjugated (Jackson Immunoresearch) antibody used at a 1:400 dilution and the secondary antibody used to detect NKA was a goat anti-mouse secondary antibody conjugated to Cy2 (Jackson Immunoresearch) used at a 1:500 dilution. To determine antibody specificity, control slides were prepared, where the AaAQP2 primary antibody was omitted from the processing step and only secondary antibody was added. Control and experimental slides were treated identically, where tissue sections from AFW, BBJD, and SCW samples were placed adjacent to each other on each slide and ran through the same IHC processing protocol together. Slides were then mounted with 1:1 of glycerol:1xPBS mounting media containing 4μg/mL DAPI and left to dry for at least 24hr before imaging. Fluorescent microscopy was completed using an Olympus IX81 fluorescent microscope (Olympus Canada, ON, Canada) and images were captured using CellSense® 1.12 Digital Imaging software (Olympus Canada). Identical acquisition settings were utilized for all

tissue sections on each individual slide, to ensure comparisons could be made between AFW, BBJD, and SCW tissue samples. Images were overlayed using ImageJ 1.53k software.

dsRNA Synthesis and Confirmation of CrAQP2 Knockdown

To obtain whole body RNA samples, 3-4 fourth instar larval midges were transferred to a chilled 300µl aliquot of lysis buffer, prepared from the PureLink RNA minikit (Invitrogen, MA, USA) and RNA extraction, purification, and cDNA synthesis was completed as described above. To obtain a large amount of *aqp2* cDNA serving as template for dsRNA synthesis, PCR reactions with the Q5 Polymerase (NEB, ON, Canada) using specific *Craqp2* primers designed to target a relatively large portion of the gene (Table 5-1). The amplified product was then purified using the Monarch PCR Purification Kit (T1030L NEB, ON, Canada) and concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE, USA). dsRNA synthesis was completed following a previously established protocol (Chasiotis et al., 2016; Misyura et al., 2017). The T7 promoter sequence was added to the 5' end of forward and reverse primers used to amplify a portion of the CrAQP2 gene. In addition, as a knockdown control gene, a fragment of the bacterial β-lactamase (βLac) gene was amplified from a pGEM-T-Easy vector using previously established primers (Chasiotis et al., 2016) and βLac primers with T7 promoter sequence were used (Table 4-1). Separate reactions were run which included the dsRNA cDNA template with either the appropriate forward primer and the reverse primer with a T7 promoter sequence or the forward primer with T7 promoter sequence and the appropriate reverse primer. Following amplification, matching reactions were pooled and then purified with the Monarch PCR Purification kit (T1030L NEB). The amplicons containing T7 promoter sequences were then used to make dsRNA for CrAQP2 and βLac with the Promega T7 RiboMAX Express RNAi System Kit

(Promega, WI, USA), following the manufacturer's instructions. Delivery of dsRNA for CrAQP2 or βLac involved feeding larval midges following a protocol previously established for larval *Ae. aegypti* knockdown (Singh et al., 2013; Chasiotis et al., 2016; Durant & Donini, 2018) with minor modifications. Specifically, for each biological replicate, ~10-12 third and fourth instar larvae were taken from the lab colony and placed in 150µl of RNAse-free water containing 0.5µg/µl dsRNA (CrAQP2 or βLac), left to incubate in the open microcentrifuge tube for 2hr at room temperature, before being transferred to 100mL of aerated AFW. Larvae were left in the aerated AFW containers for 48hr and 72hr post dsRNA feeding before 4-5 whole body larvae were sonicated in chilled lysis buffer, total RNA was extracted, and cDNA synthesized as described above. To confirm knockdown, qPCR on the cDNA samples was performed as described above and the relative transcript abundance in 48hr and 72hr post CrAQP2 dsRNA feeding was normalized to the dsβLac treatment groups.

Midge Survival

To assess the survivability of larval *C. riparius* under osmotic challenges post CrAQP2 knockdown, three rounds of survival assays were completed. For each round, 30 larvae were placed in microcentrifuge tubes at a density of ~10 larvae/150µl of 0.5µg/µl dsAQP2 or dsβLac. The microcentrifuge tubes were kept open at room temperature for 2hr. Midges were transferred to containers with 100mL of aerated AFW, for dsAQP2 and dsβLac individuals, respectively and held for 48hrs. Then, the group of larvae were evenly distributed into to 200mL of AFW as the control, or BBJD or SCW as the treatment groups, all with constant aeration. The midge larvae were then left in their treatments for 7 days, with daily survival counts completed. For dsβLac-treated individuals, the total number of larvae assessed was: n=17 for AFW, n=18 for BBJD, and

n=17 for SCW. For dsAQP2-treated individuals, the total number of larvae assessed was, n=18 for AFW, n=18 for BBJD, and n=19 for SCW. All treatment groups were fed identically, with each container receiving 0.1g of crushed TetraFin Goldfish Flake Food (Tetra Holding US, VA, USA) ~48hr post transfer into AFW, BBJD, or SCW.

Body Moisture

Potential effects of CrAQP2 knockdown on total body moisture of *C. riparius* larvae under osmotic challenges was measured following a previously established protocol (Nguyen & Donini, 2010). Larvae were placed in microcentrifuge tubes at a density of ~10 larvae/150µl of 0.5µg/µl dsAQP2 or dsβLac for 2hr at room temperature, as described above and then transferred into aerated containers of AFW for 48hr. Larvae were equally distributed to containers containing 100mL of AFW, BBJD, or SCW for a 24hr acute exposure. Following this exposure, individual midges were dried on a KimTech wipe and placed into individual pre-weighed PCR tubes where their wet weight was measured using a microbalance (model UMXP; Mettler-Toledo International, ON, Canada). Larvae in their individual PCR tubes were placed in an oven set to 60°C for ~5 days. Larvae were weighed again in their individual PCR tubes to obtain their dry weight and the difference between wet weight and dry weight was calculated to determine total body moisture. For AFW n=6-8; BBJD n=7-10; SCW n=7-10.

Statistics

All data sets were analyzed using Prism® 9 software (GraphPad Software Inc., CA, USA). Each qPCR data set, including the CrAQP2 expression profile and effects of de-icers on CrAQP2 transcript in each organ, was analyzed with a one-way ANOVA with multiple comparisons prior

to log-transformation. An unpaired t-test was used to determine significance between dsβLac-treated and dsAQP2-treated midges to determine knockdown efficiency of CrAQP2 transcript. For survival data and body moisture data, a one-way ANOVA with multiple comparisons was completed to determine significant differences between dsβLac-treated and dsAQP2-treated midge larvae.

5.4 Results

CrAQP2 Transcript Abundance in Osmoregulatory Organs

CrAQP2 transcript was detectable in the osmoregulatory organs examined (Figure 5-1). The MTs had the highest levels of CrAQP2 transcript, which was significantly higher than all other osmoregulatory organs analyzed with at least 10 times greater abundance. Comparatively, CrAQP2 transcript abundance in all other examined osmoregulatory organs showed no statistical difference, although the trend indicated moderate levels in the AP and MG, while HG and GC/FG had the lowest abundance.

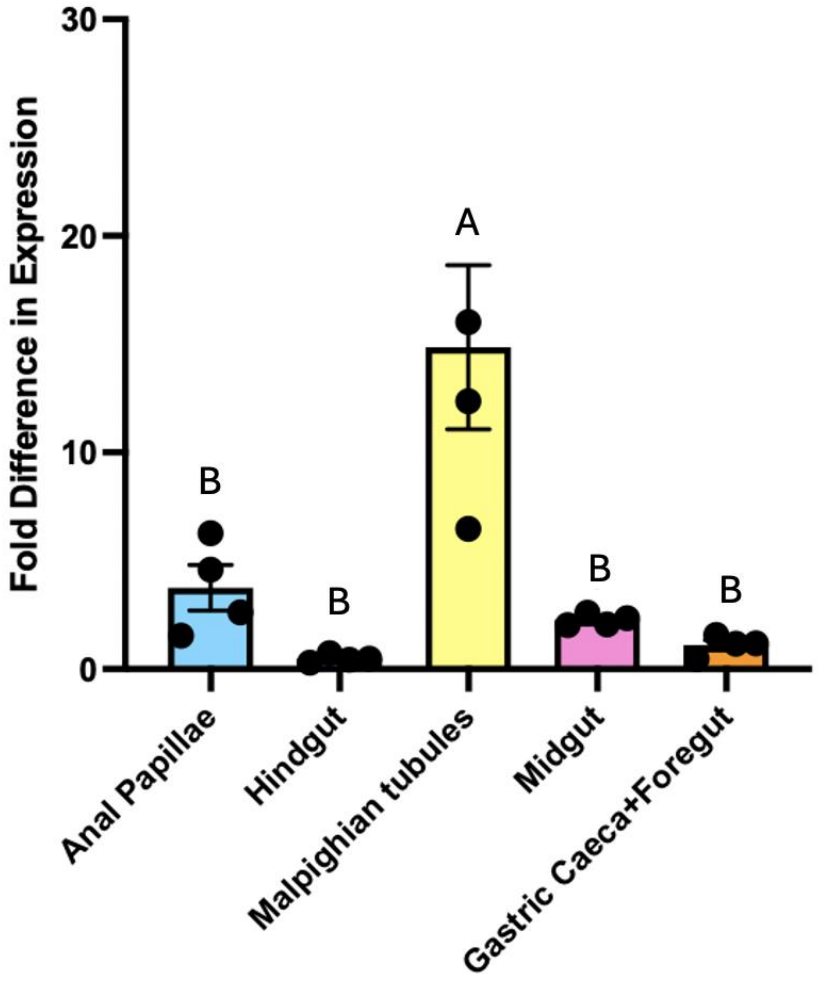

Figure 5-1. CrAQP2 expression profile in the osmoregulatory organs of *Chironomus riparius* larvae in freshwater.

Larval freshwater *Chironomus riparius* aquaporin-2 (CrAQP2) transcript abundance in the osmoregulatory organs, including the gastric caeca with foregut (GC+FG), midgut (MG), Malpighian tubules (MTs), hindgut (HG), and anal papillae (AP). CrAQP2 was detected in each of the organs studied, with highest transcript abundance seen in the MTs and lowest abundance seen in the HG tissue. Different letters between mean (± SEM) values show significantly different values between organs and their CrAQP2 transcript abundances. A one-way ANOVA with Tukey's multiple comparison's test was run (different letters = $p<0.05$), n=4-5.

Immunolocalization of CrAQP2 in Larval *C. riparius*

I utilized a custom antibody raised against a peptide representing a small portion of AaAQP2, the AQP2 homolog of *Aedes aegypti*. This antigen sequence is similar to the putative CrAQP2 sequence (see Supplementary Figures; 5-S1). Using this antibody, AaAQP2-like immunoreactivity was detected in the osmoregulatory organs, including the GC+FG, MG, MTs, and AP (Figure 5-2). Although immunohistochemistry was completed for organs of larvae exposed to AFW, BBJD and SCW, no qualitative differences appeared in AaAQP2-like staining between treatments, therefore representative images from AFW larvae were chosen to summarize the localization of CrAQP2 in organs [See Supplementary Figure 5-S2 for BBJD and SCW-treated midge organs and the relative AaAQP2-like immunoreactivity]. For all tissue sections, NKA was used as a membrane marker (green), whereas DAPI (blue) was used to stain nuclei in each image (Figure 5-2). Beginning with the anterior portion of the alimentary canal, AaAQP2-like immunoreactivity was detected in the GC but not in the FG region, with staining appearing uniform within the basolateral membrane of the GC (Figure 5-2A). AaAQP2-like immunolocalization was also visible in the MG tissue, with a majority of staining appearing at the apical or lumen facing membrane of the gut cells (Figure 5-2B). Transverse cross sections of the MTs show mainly cytosolic as well as apical staining of AaAQP2-like protein (Figure 5-2C). In the HG, there was no AaAQP2-like immunoreactivity detected (Figure 5-2D). Finally, AaAQP2-like immunoreactivity was detected within the basolateral membrane of the externally located AP organ, with no staining visible at the apical membrane (Figure 5-2E). The negative control, which excludes the primary antibody, had no AaAQP2-like immunoreactivity in any region of the whole-body midge (Figure 5-2F).

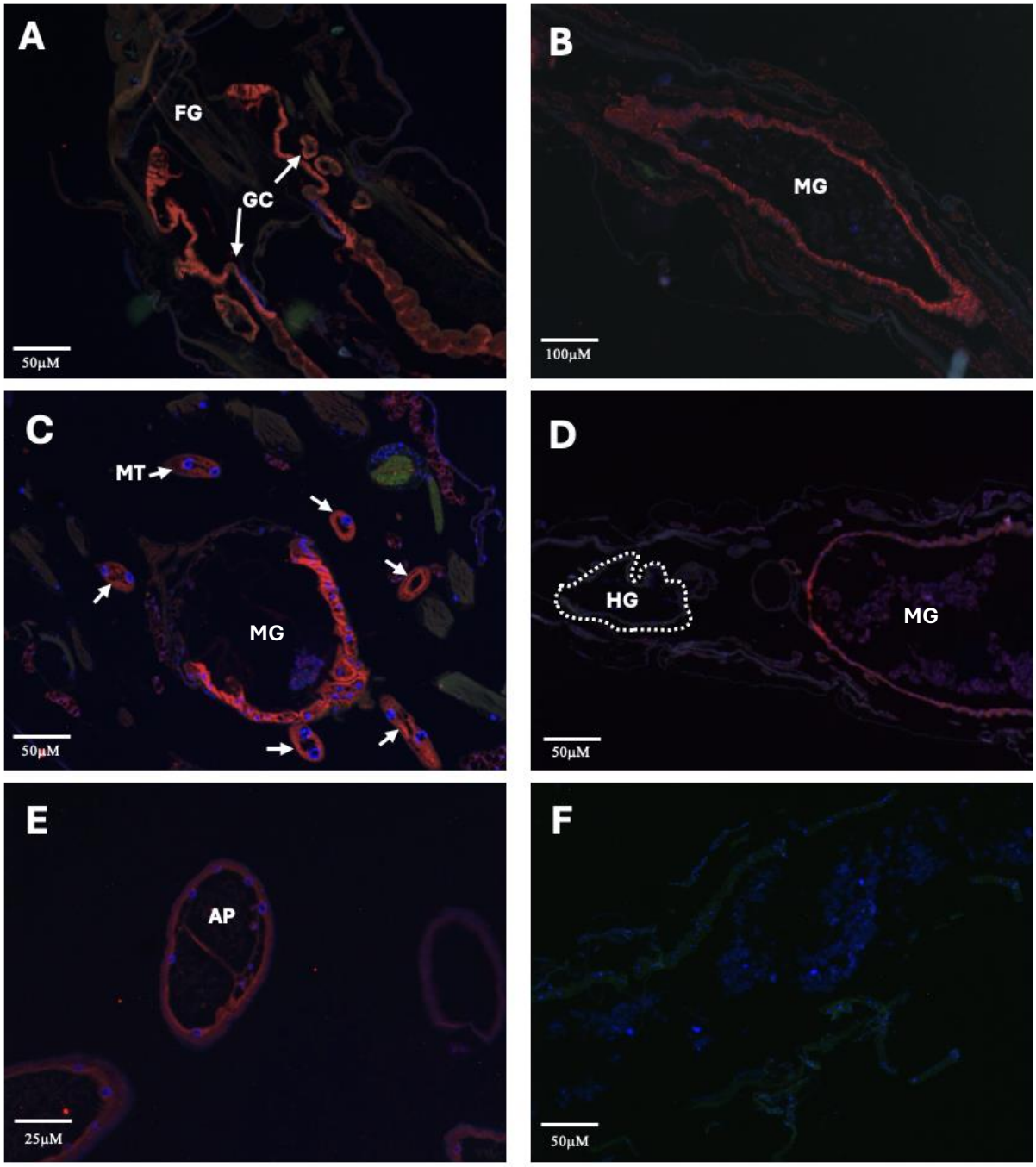

Figure 5-2. Immunolocalization of AQP2 in the osmoregulatory organs of 4th instar larval *Chironomus riparius* midges. A. Gastric caeca (GC) and foregut (FG), **B.** midgut (MG), **C.** Malpighian tubules (MT), **D.** hindgut (HG), and **E.** anal papillae (AP). Panel **F.** shows the negative control with primary antibody omitted, where no AQP2 staining was observed over the whole-body midge section. For each organ, at least 3-4 biological replicates were completed each with 4-5 technical replicates run. Red staining indicates AQP2 immunoreactivity; green staining indicates the Na^+/K^+-ATPase (NKA) membrane marker immunoreactivity; and blue (DAPI) staining for nuclei.

Effects of De-Icers on CrAQP2 Transcript Abundance in Midge Osmoregulatory Organs

Acute exposure (24 hr) to BBJD or SCW did not alter the CrAQP2 transcript abundance in the GC+FG and MTs (Figure 5-3A & 5-3C). In the MG and AP, SCW treatment significantly increased the CrAQP2 transcript abundance relative to AFW; however, BBJD did not affect CrAQP2 transcript levels (Figure 5-3B & E). In the HG, CrAQP2 transcript abundance was significantly reduced with both BBJD and SCW treatment compared to AFW (Figure 5-3D).

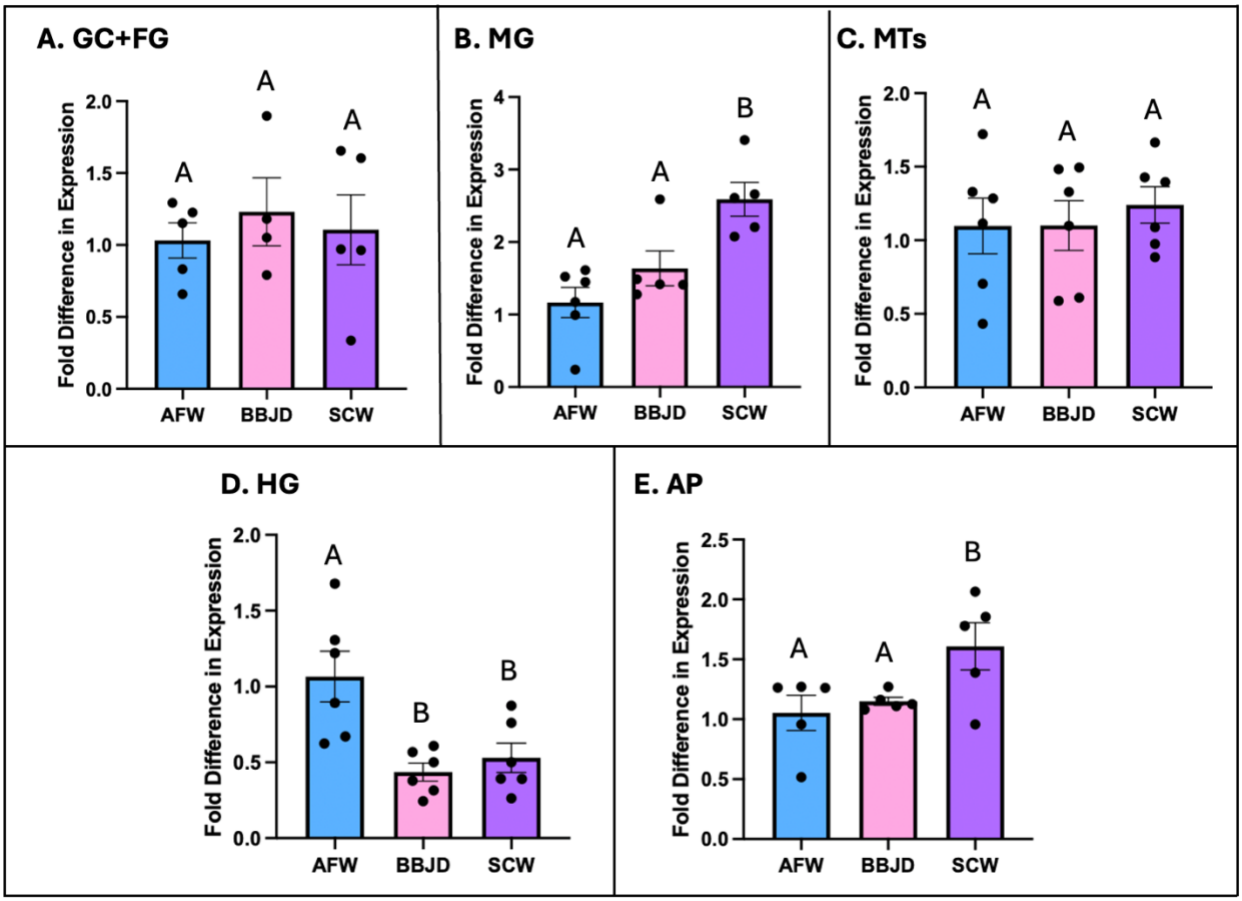

Figure 5-3. Analysis of CrAQP2 expression in the osmoregulatory organs of larval *Chironomus riparius* in response to road de-icers.
Transcript abundance of CrAQP2 in the osmoregulatory organs of the midge following acute (24hr) exposures to artificial freshwater (AFW), 2% brine beet juice de-icer (BBJD), and 7g/L NaCl-contaminated water (SCW). For each graph, values are displayed as mean±SEM and a one-way ANOVA with Tukey's multiple comparison's test was run (different letters = $p<0.05$). **A.** CrAQP2 abundance in the gastric caeca with foregut (GC+FG) tissue, showing no changes in transcript abundance with de-icer treatment (n=4-5). **B.** CrAQP2 abundance in the midgut (MG), which increased in SCW relative to the AFW and BBJD groups (n=5-6). **C.** CrAQP2 abundance in the Malpighian tubules (MTs), showing no changes in transcript abundance with de-icer treatment (n=6). **D.** CrAQP2 abundance in the hindgut (HG), which decreased in BBJD and SCW relative to the AFW group (n=6). **E.** CrAQP2 abundance in the anal papillae (AP), which increased in SCW relative to the AFW group (n=5).

Influence of CrAQP2 Knockdown on Midge Survival Under Prolonged Exposure to De-Icers

The survival of *C. riparius* larvae was assessed in CrAQP2 knockdown larvae under prolonged exposure to de-icers. Larvae exposed to CrAQP2 dsRNA showed significantly reduced transcript abundance (~90%) after 48 hr exposure to dsRNA where levels were just 10% of those in the ßlac dsRNA control group (Figure 5-4A). Survivability was assessed over one week post CrAQP2 knockdown. When maintained in AFW, midge survival decreased over the course of 7 days in both dsßLac-treated and dsCrAQP2-treated larvae, with no significant differences observed between the two groups (Figure 5-4B). When midges were transferred from AFW into BBJD, survival of dsCrAQP2-treated larvae was stable for the first 2-3 days post transfer, but significantly decreased relative to the dsßLac-treated larvae 4 days post transfer, before no survival was seen by day 5 (Figure 5-4C). Similarly, in the SCW exposure group, dsCrAQP2-treated larvae had survival comparable to dsßLac-treated larvae for the first 2 days; however, a rapid decline in survivability was seen at 3-days post transfer with ~50% surviving, followed by <10% survival at 4-days post transfer and no survival by day 5 post transfer (Figure 5-4D).

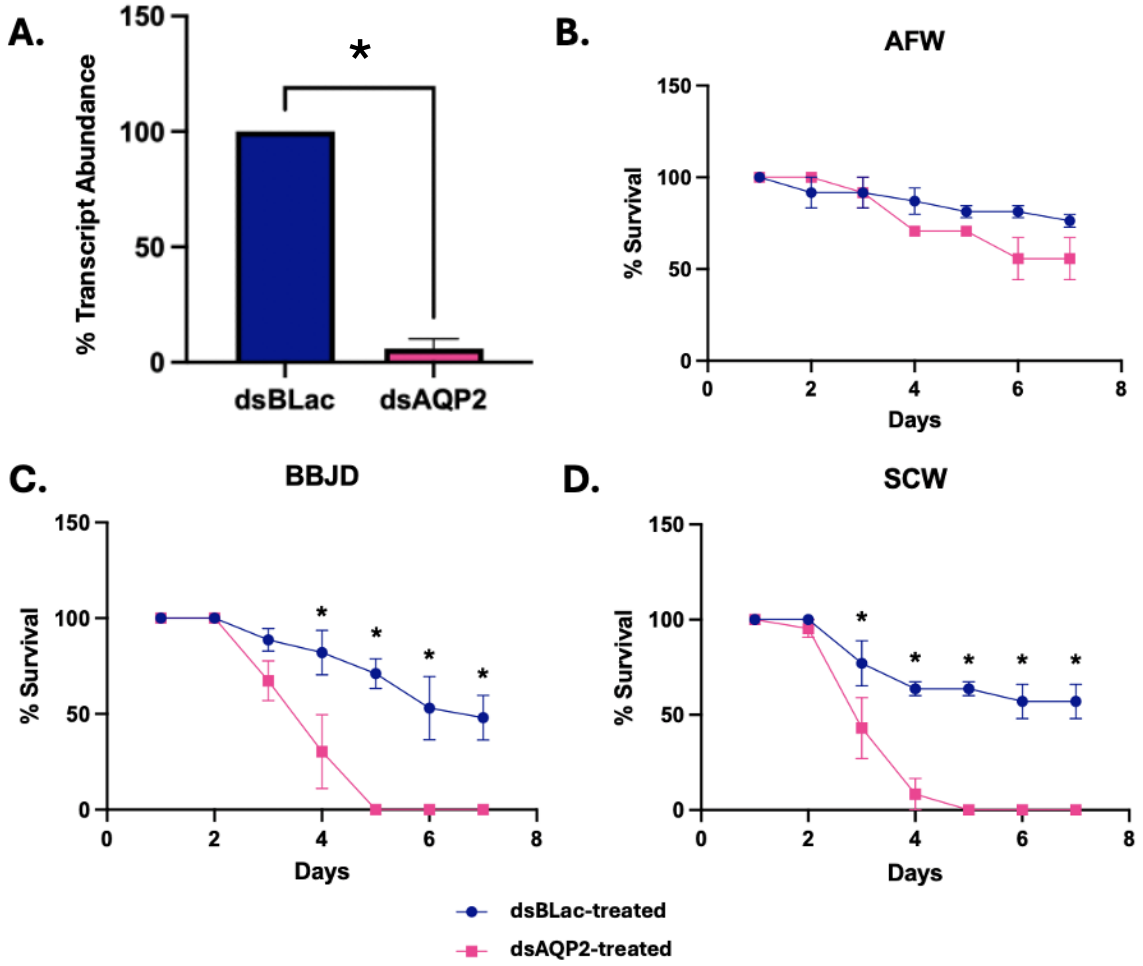

Figure 5-4. *Chironomus riparius* survival post CrAQP2 knockdown and following prolonged exposure to road de-icers.

Midge larvae survival was observed over a 7-day period, following AQP2 knockdown and exposure to artificial freshwater (AFW), 2% brine beet juice de-icer (BBJD), or 7g/L NaCl-contaminated water (SCW), with mean±SEM values reported. All treatments were completed on both dsAQP2-treated and dsβLac-treated larvae, as a knockdown control and were treated identically. **A.** Verification of CrAQP2 gene knockdown in midge larvae, 48hr post dsRNA treatment. **B.** Graph showing % survival of midge larvae in AFW for dsβLac-treated (n=17) and dsAQP2-treated (n=18) individuals. Survival between control and knockdown larvae was statistically similar over the 7-day period. **C.** Graph showing % survival of midge larvae in BBJD for dsβLac-treated (n=18) and dsAQP2-treated (n=18) individuals. Survival in the AQP2 knockdown group was significantly less than the control by 4-days post transfer to BBJD (>50% survival), with 0% survival by day 5. **D.** Graph showing % survival of midge larvae in SCW for dsβLac-treated (n=17) and dsAQP2-treated (n=19) individuals. Survival in the AQP2 knockdown group was significantly less than the control by 3-days post transfer to SCW (~50% survival), with 0% survival by day 5. A one-way ANOVA with multiple comparisons was run for each set of values and data points with an asterisk (*) denote statistically significant differences ($p<0.05$) between the dsβLac-treated and dsAQP2-treated larvae at each time point.

Influence of CrAQP2 Knockdown on Total Body Water Content Under Exposure to De-Icers

Total body water following CrAQP2 knockdown in *C. riparius* larvae exposed to de-icers was measured. Body moisture percentage was determined following CrAQP2 knockdown with acute (24hr) exposures to AFW, BBJD, or SCW. Larvae exposed to AFW with CrAQP2 knockdown experienced a significant decrease in body moisture in comparison to the dsßLac-treated individuals (Figure 5-5A). Similarly, both BBJD and SCW exposure following CrAQP2 knockdown resulted in a significant decrease in body moisture in comparison to ßLac-treated controls (Figure 5-5B, 5C).

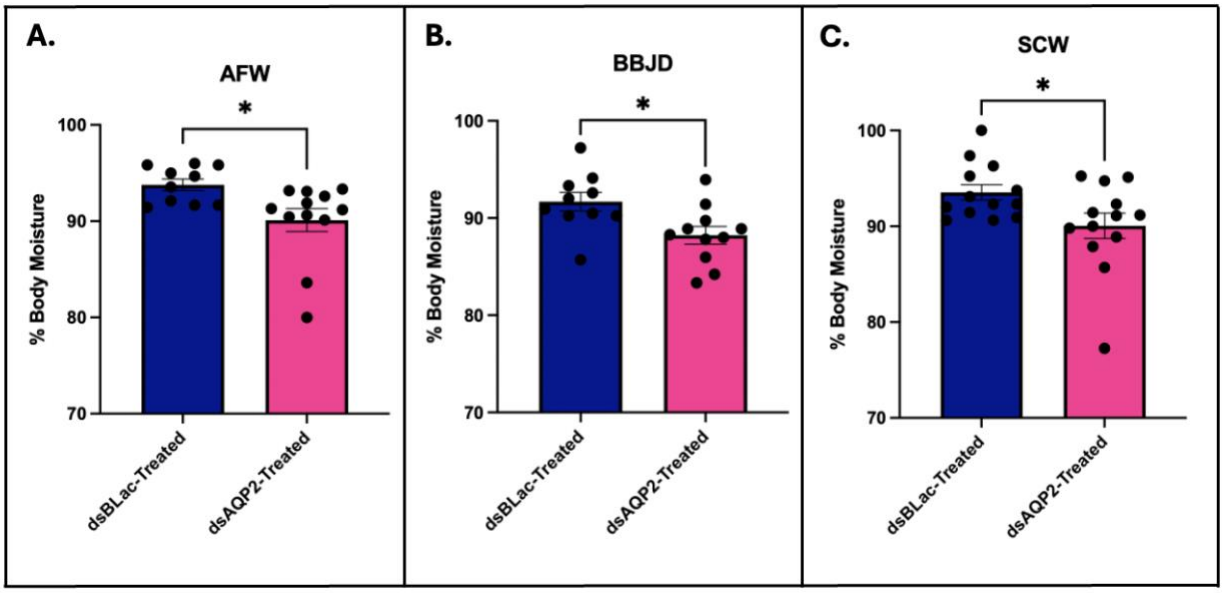

Figure 5-5. Total body moisture post CrAQP2 knockdown in larval *Chironomus riparius* post transfer to AFW, BBJD, and SCW.
Body moisture percentage (%) was measured after CrAQP2 knockdown was induced via dsRNA treatment and a subsequent acute (24hr) exposure to artificial freshwater (AFW), 2% brine beet juice de-icer (BBJD), or 7g/L NaCl-contaminated water (SCW). As a knockdown control, dsβLac-treatment was used for all conditions. **A**. Body moisture % post AFW transfer, showing significant decrease in body moisture with CrAQP2 knockdown. **B**. Body moisture % post BBJD transfer, showing a significant decrease in body moisture with CrAQP2 knockdown. **C**. Body moisture % post SCW transfer, showing significant decrease in body moisture with CrAQP2 knockdown. For all panels (**A-C**), individual unpaired t-tests were completed and an * represents $p<0.05$

5.5 Discussion

Salinization of freshwater is a serious global issue particularly in temperate climates due to the use of de-icing agents during winter season which is a contributing factor on these ecosystems with the melting ice and snow. This could have detrimental consequences to organisms living in freshwater, including aquatic insects, which are important in maintaining a healthy ecosystem. Although a number of studies have examined the effect of salinization on ion transport in freshwater insects (Jonusaite et al., 2011; Jonusaite et al., 2013; Scheibener et al., 2016; Jonusaite et al., 2017; Nowghani et al., 2019; Lambret et al., 2021; Picinic et al., 2022), comparatively little work has been done on water transport. In this study, I identified a putative water selective aquaporin (AQP) in *C. riparius* larvae which I named CrAQP2 and examined the effects of de-icing agents including NaCl and brine-beet juice de-icer (BBJD) on CrAQP2 expression and function.

CrAQP2 Localization and Effects of De-Icers in *C. riparius* Osmoregulatory Organs

I found CrAQP2 gene transcript and protein to be present in almost all of the osmoregulatory organs of midge larvae in their typical freshwater environment simulated in the laboratory by artificial freshwater (AFW). CrAQP2 is a homolog of the AQP channel PRIP in *Drosophila melanogaster* (Cabrero et al., 2020), as well as AaAQP2 in the mosquito *Aedes aegypti* (Drake et al., 2010). Homologs of PRIP have also been identified in other insects such as the northern house mosquito *Culex pipiens* (Yang & Piermarini, 2017) and the weevil *Rhyzopertha dominica* (Tan & Chen, 2023).

Malpighian tubules (MTs)

The highest abundance of CrAQP2 transcript was observed in the MTs of larval *C. riparius*. With immunohistochemistry, I found putative CrAQP2 (AaAQP2-like staining) localization dispersed throughout the cytosol as well as the apical membrane of the MT cells, visible in transverse cross sections of the tubules. The observed staining pattern is consistent with CrAQP2 localization in the larger more abundant principal cells of MTs. In *D. melanogaster,* PRIP is highly expressed in the stellate cells of the MTs, to aid in transepithelial water entry into the lumen (Cabrero et al., 2020); whereas in adult *A. aegypti*, AaAQP2 is expressed in both the principal and stellate cells of the MTs (Drake et al., 2010; Picinic et al., 2024). The MTs play an important role in both aquatic and terrestrial insects to produce primary urine, while maintaining haemolymph osmolarity. For *C. riparius* larvae, the MTs must consistently produce urine to rid of excess water as a result of their hypoosmotic environment. It is assumed that CrAQP2 in midge larva follows the previously shown preference for water transport seen with AaAQP2 (PRIP) (Drake et al., 2015), however further evidence on its selectivity is necessary to draw conclusions. With de-icer treatment (BBJD and SCW), I found no changes to CrAQP2 transcript abundance nor did I find any qualitative differences in AaAQP2-like staining in the MTs. Similar to these results, it was shown earlier in *Ae. aegypti* larvae that rearing in brackish water (increased salinity) did not affect AaAQP2 transcript abundance relative to levels in freshwater reared larvae (Misyura et al., 2020). Interestingly, MTs from *C. riparius* larvae exposed to BBJD were shown to secrete fluid at rates 1.5 times those of MTs from AFW and SCW exposed larvae (Reinsborough & Donini, 2025 pending revision). Therefore, it seems that the underlying expression of CrAQP2 is sufficient to support different rates of fluid secretion. Furthermore, the role of other AQPs that have yet to be discovered in the MTs needs to be studied.

Anal Papillae (AP)

In this study, the gene expression profile revealed CrAQP2 transcript as well as AaAQP2-like immunoreactive staining in the AP of midge larvae. The AP are important osmoregulatory organs in midge larvae, responsible for the active uptake of ions from the external freshwater environment (Jarial, 1995; Nguyen & Donini, 2010). The AP epithelium also allows water to move bidirectionally depending on the external media (Marusalin et al., 2012; Akhter et al., 2017). In freshwater, *Ae. aegypti* AP have been shown to move water into the haemolymph as it is osmotically obliged to follow active ion uptake and it is likely that AaAQP2 plays an important role in the water uptake at the AP (Marusalin et al., 2012). Under long-term brackish water conditions, the AP of *Ae. aegypti* are structurally different than in FW, as early studies on the AP show a decrease in papillae size in response to 0.9% NaCl (Wigglesworth, 1938) and then later, showed a decrease in surface area of the papillae in response to salinity (Sohal & Copeland, 1966). AaAQP2 (AaAQP1b) transcript was found to be highly expressed in the AP in their typical freshwater environment (Marusalin et al., 2012). It has been proposed that AaAQP2 (AaAQP1b) is responsible for water transport across the AP membrane due to its high transcript and protein levels (Marusalin et al., 2012; Durant et al., 2021); therefore, CrAQP2 could likely also be an important AQP in mediating water transport at the AP epithelium of *C. riparius*, since I found CrAQP2 immunolocalization on the basolateral membrane of the AP.

With respect to the influence of de-icers, exposure to SCW caused a significant increase in CrAQP2 transcript abundance in midge AP while BBJD exposure did not change CrAQP2 transcript levels. The effects of increased salinity such as brackish water exposure has caused similar findings in *Ae. aegypti* larvae, where a significant increase in AaAQP2 protein was determined in mosquito AP, relative to the freshwater control (Durant et al., 2021). The

haemolymph of *C. riparius* remains hyperosmotic to the surrounding SCW (haemolymph: ~ 270 mOsmol versus SCW: ~ 225 mOsmol); however, the gradient at which water entry is favoured is decreased with the increased external osmolarity from a difference of ~ 160 mOsmol in AFW to ~45 mOsmol in SCW (see Reinsborough & Donini, 2025 pending revision). In BBJD, the difference between haemolymph (hyperosmostic to BBJD) and the BBJD is ~ 80 mOsmol (Reinsborough & Donini, 2025 pending revision). Therefore, it stands to reason that a greater influx of water across the AP is occurring in BBJD relative to SCW conditions, and the level of water influx in BBJD may be sufficient to deal with the increased fluid secretion by the MTs in BBJD (Reinsborough & Donini, 2025 pending revision) In contrast, the lower influx of water at the AP that would be caused by the reduced osmotic gradient in SCW may not be sufficient to avoid net water loss with the MTs maintaining rates of fluid secretion similar with those in AFW. This may be the reason why larvae in SCW have increased expression of CrAQP2 in the AP, so as to increase the influx of water when faced with a lower osmotic gradient in an attempt to avoid dehydration.

The Midgut (MG)

CrAQP2 transcript was also detected in the MG where AaAQP2-like immunoreactivity was localized to the apical membrane (lumen-facing side) of MG epithelium. In previous studies with *A. aegypti*, AaAQP2 transcript was detected in the midgut of larvae and adults, and AaAQP2 was immunolocalized to the basolateral epithelial membrane of the posterior midgut in adults (Drake et al., 2010; Marusalin et al., 2012; Picinic et al., 2024). In some adult mosquitoes such as *Ae. aegypti* (Drake et al., 2010; 2015) and *Anopheles sinensis* (Zhao et al., 2024), entomoglyceroporins that transport some solutes are more highly expressed in MG tissues, aiding

in absorption of some solutes into the haemolymph. In the Antarctic midge *B. antarctica*, a DRIP-like AQP was localized to various organs including the MG (Yi et al., 2011). It has been suggested that in freshwater aquatic insects such as larval *Ae. aegypti*, imbibed water is absorbed into the haemolymph following the active uptake of ions (D'Silva et al., 2017; Misyura et al., 2020). Since *C. riparius* larvae inhabit similar aquatic environments to that of *Ae. aegypti* larvae, CrAQP2 in the midgut could be important in water absorption. In the midgut, CrAQP2 protein is located on the apical, lumen facing membrane of the MG epithelial cells where it may absorb water from the lumen into the epithelia for subsequent absorption into the hemolymph. I also found increases in CrAQP2 transcript abundance in the MG with SCW treatment relative to AFW; however, no changes were seen with BBJD. If CrAQP2 is involved in water uptake across the MG in the midge, it is possible that in response to SCW exposure, the larval midges upregulate CrAQP2 levels in the MG to increase the uptake of water into the haemolymph to avoid dehydration caused by excess ion uptake. In larval *C. riparius*, the concentration of Na^+ and Cl^- in the haemolymph were increased with transfer to brackish water (Jonusaite et al., 2010), which could result in increased water movement into the haemolymph due to the greater osmotic gradient. Additionally, brackish water rearing increased the MG permeability of larval *Ae. aegypti* by increasing the presence of septate junction proteins (Jonusaite et al., 2017). Increased permeability of the gut could result in increased water absorption, which may explain the increase in CrAQP2 I observed in the MG with SCW. Interestingly, no change in CrAQP2 transcript was seen with BBJD in the MG. The ingredients of most beet juice-based de-icers are not entirely disclosed, however K^+ levels in BBJD are much higher than the typical NaCl-based de-icer, as it is derived from K^+-rich sugar beets. BBJD might not alter CrAQP2 levels because although it has relatively high levels of NaCl (~12% w/v), its effects on K^+ levels are much greater, which may not be connected to the driving force of

water across the MG epithelium (Picinic et al., 2024). Overall, the current data indicates that CrAQP2 is present on the lumen facing (apical) membrane of the MG and thus could play a role in water uptake from imbibed water to the haemolymph.

The Gastric Caeca and Foregut (GC+FG)

In this study, I detected CrAQP2 transcript expression and immunoreactivity in the GC+FG of *C. riparius* in freshwater. As the FG mainly serves as a conduit for food passage to the midgut, the GC function more in osmoregulation by utilizing active ion transport that drives water secretion via aquaporins into the luminal space (Misyura et al., 2020). It has been shown in larval *Ae. aegypti* that the distal versus proximal cells of the GC play different roles in proton secretion versus absorption, which is linked to the activity of the V-type H^+-ATPase (D'Silva et al., 2017). Additionally, overall, the GC typically function in moving Na^+ and K^+ from the haemolymph into the GC lumen in freshwater reared larval *Ae. aegypti* (D'Silva et al., 2017). Along with active ion transporters, AaAQP2 transcript (and other AQPs) is expressed in the GC of freshwater larval *Ae. aegypti* (Misyura et al., 2020). In light of these facts, water will move based on concentration gradient and osmotically follow ion transport from the haemolymph into the GC lumen to aid in water secretion in a dilute environment. Due to their shared freshwater environments, the GC in midge larvae are likely to function in a similar manner to those of larval *Ae. aegypti*. Therefore, CrAQP2 is likely present in the GC of *C. riparius* larvae to aid in the secretion of water from the haemolymph in avoidance of over-diluting the haemolymph in a freshwater environment (Misyura et al., 2020). When looking at the effects of BBJD and SCW on CrAQP2 in the combined GC+FG sample, gene transcript did not change with de-icer treatment. It is plausible that the GC play a role in consistently secreting ions and water into their lumen and despite changes in external

salinity, their role does not change. It has been shown that when *Ae. aegypti* larvae are reared in brackish water, there is a reduction in ATPase activity and overall differential pattern of Na^+ and K^+ flux across the caecal epithelium (D'Silva et al., 2017). However, it was demonstrated earlier that the transepithelial potential, as well as the sum of cation concentrations, does not change in the caeca, which suggests that total water moved across the membrane is likely consistent (D'Silva et al., 2017). Relatedly, it was also reported that AaAQP2 mRNA abundance in larval *Ae. aegypti* gastric caeca did not change following exposure to brackish water (Misyura et al., 2020). Therefore, these findings appear to follow a similar trend likely due to *C. riparius* and *Ae. aegypti* sharing a common freshwater environment. I propose that increased rearing salinity caused by BBJD and SCW does not affect CrAQP2 in the GC+FG since total water movement across the caecal membrane is likely not affected and instead, continues to move into the lumen based on total ion transport. Therefore, CrAQP2 is likely involved in water secretion from the haemolymph to the GC lumen, as it is present uniformly throughout the GC and did not change with increased external salinity.

The Hindgut (HG)

In this study, I did not detect high abundance of CrAQP2 in the HG, which is consistent with previous studies on *Ae. aegypti* larvae that have found extremely low levels of AaAQP2 in the HG, relative to the other AQP isoforms expressed in this organ (Marusalin et al., 2012). Due to their freshwater environment, the HG is likely working to actively re-uptake ions while leaving water in the final urine destined for excretion (Bradley & Phillips, 1977; Jonusaite et al., 2013); therefore, it suggests that CrAQP2 holds a less important role in the HG of *C. riparius* larvae. Similar to treatments on the other organs examined in this study, I measured the effects of BBJD

and SCW on CrAQP2 in the HG of *C. riparius*. De-icer treatment significantly decreased the already very low levels of CrAQP2 in the HG, relative to AFW and immunohistochemistry revealed little to no presence of CrAQP2 in the HG. Therefore, the HG may only play a minor role in regulating body water levels with its main function keeping water in the gut lumen for excretion.

Role of CrAQP2 in Total Body Water Content with De-Icer Treatment

To understand the role of CrAQP2 in the response of *C. riparius* larvae to de-icer treatment, I treated larvae with CrAQP2 dsRNA and was able to achieve significant knockdown based on transcript abundance measured 2-days following dsRNA treatment. Total body moisture was measured with de-icer treatment as a proxy for the overall contribution of CrAQP2 to osmotic regulation in midge larvae; however, this was measured after 1-day abrupt exposure to SCW and BBJD to understand the effects of CrAQP2 knockdown on acute exposure to both de-icers which is normally what the animals will be exposed to in their natural habitat. Overall, total body moisture was significantly reduced with CrAQP2 knockdown in response to abrupt transfer into all three conditions including the control AFW, SCW, and BBJD. A major reason for the reduction I've seen in body moisture with CrAQP2 knockdown likely involves both the MTs and the AP. Under control conditions (AFW), CrAQP2 knockdown resulted in reduced total body water content in *C. riparius* larvae. It is likely that a reduction in the functioning of CrAQP2 caused reduced water entry at the AP, where they have been shown to heavily favour water uptake in their freshwater environment (Marusalin et al., 2012). In addition, it is possible that the MTs would continue to produce primary urine at a consistent rate even with CrAQP2 knockdown, as in other insect species such as *Ae. aegypti*, four of six AQP genes have been shown to be localized in the MTs (Drake et al., 2010; Marusalin et al., 2012; Misyura et al., 2020; Picinic et al., 2024). This provides a model

where water transport can occur through many AQPs simultaneously and therefore the reduction in CrAQP2 functioning may not have a significant effect on the functioning of the MTs. As a result, CrAQP2 knockdown results in decreased body moisture likely due to decreased capacity for water uptake at the AP.

A similar explanation for reduced body moisture with CrAQP2 knockdown is plausible with de-icer treatment. As I previously saw with transcript abundance in SCW, CrAQP2 transcript was upregulated in the AP but remained stable in the MTs. This is likely due to an increased need for water uptake in a hyperosmotic environment, while still needing to rid of excess ions and water by the MTs to maintain homeostatic osmolality. As seen in larval *Ae. aegypti*, transfer into saltwater conditions causes increases in haemolymph ion levels and altered ion transport at the AP, that must be dealt with by the animal through ion excretion (Donini et al., 2007). Additionally, *C. riparius* haemolymph osmolarity increases with exposure to SCW relative to exposure to AFW (Reinsborough & Donini, 2025 pending revision). Therefore, upon CrAQP2 knockdown, blunted expression of CrAQP2 occurs in the AP, resulting in insufficient water being taken up across AP and, as the MTs continue to produce primary urine to rid of excess ions in the haemolymph, the midge larvae experience dehydration leading to significantly lower total body water content. Similarly, when *C. riparius* larvae were abruptly transferred to BBJD, a significant reduction in body moisture occurred. It has been shown that relative to AFW treatment, BBJD results in significantly increased haemolymph osmolarity similar to that of SCW in larval *C. riparius* (Reinsborough & Donini, 2025 pending revision). Therefore, a similar outcome with the MTs may occur in response to BBJD where they continue to produce primary urine in an attempt to rid of excess ions resulting in an overall loss of water, as no change was observed in CrAQP2 transcript abundance in the MTs with BBJD treatment. However, BBJD did not alter CrAQP2 levels in the

AP either relative to AFW which may be because although BBJD does cause increase external osmolarity relative to AFW, it does so at a lower magnitude than SCW (Reinsborough & Donini, 2025 pending revision), which may not be enough of a driving force to result in changes in transcript abundance at the AP. Therefore, decreased body moisture with acute BBJD exposure is likely a result of continued urine production by the MTs where dehydration inevitably occurs with constant urine production in combination with potentially reduced water uptake from the surrounding environment.

In addition, I also completed survival studies over 7 days (long-term) post CrAQP2 knockdown in AFW, BBJD, and SCW, to understand the role of CrAQP2 in long-term survival post de-icer contamination. When *C. riparius* larvae were kept in AFW for 7-days, no significant differences in survival were found between the CrAQP2-knockdown individuals and the βLac-knockdown individuals. As previously mentioned, in *Ae. aegypti* mosquitoes, six AQP genes have been identified, with a majority of them having a large capacity for water transport (Drake et al., 2010; Drake et al., 2015). It is likely that although acute exposure to AFW post CrAQP2 knockdown resulted in reduced body moisture, survival over the long-term period was not affected because there may be other AQPs at play, mitigating water movement and preventing lethal dehydration. Additionally, the external osmolarity of AFW (~1mOsm) favours the osmotic gradient that brings water into the haemolymph via the AP (Marusalin et al., 2012; Reinsborough & Donini, 2025 pending revision) and although CrAQP2 is comprised with knockdown, it is possible that water may still enter via remaining functioning CrAQP2 or through other AQPs, if present in the AP.

Finally, I examined the effects of CrAQP2 knockdown on midge survival (7 days) in the presence of BBJD and SCW. I found that CrAQP2-knockdown midges experienced a significant

increase in mortality by 4 days post-transfer to BBJD and 3 days post-transfer to SCW from AFW. It has been shown that SCW and BBJD treatments result in significantly higher osmolarity in comparison to AFW osmolarity (Reinsborough & Donini, 2025 pending revision), thereby reducing the osmotic gradient for water entry at the AP into the haemolymph. Consequently, knockdown of CrAQP2 may compromise the ability of important osmoregulatory organs such as the AP to allow for the already limited absorption of water to occur when exposure to SCW and BBJD occurs, thus resulting in death after long term exposure to each treatment. With acute exposure to SCW and BBJD following CrAQP2 knockdown, reduced body moisture had occurred and although I did not measure the resulting body moisture post-7-day exposure to each treatment, the abrupt dehydration resulting in significantly lower total body water content may have aided in the lack of recovery when exposed to the salt stress and thus a greater loss of water resulted in death.

In conclusion, this study identified the first AQP in *Chironomus riparius* which I named CrAQP2, and also measured its transcript abundance and distribution at the protein level using immunohistochemistry in the alimentary canal of the midge larvae. Furthermore, the influence of common road de-icers (BBJD and NaCl) on CrAQP2 expression in each of the osmoregulatory organs was observed and knockdown of CrAQP2 by dsRNA treatment revealed the role of this water channel in mitigating osmotic stress associated with salinization. I found that CrAQP2 is expressed in most areas of the midge alimentary canal and overall, NaCl-contaminated water caused differential gene expression in a few of the osmoregulatory organs while BBJD only affected CrAQP2 expression in the HG. However, with CrAQP2 knockdown, treatment with either BBJD or NaCl on midge larvae was lethal after only a few days. A reduction in CrAQP2 functioning associated with knockdown results in reduced body moisture for midge larvae in

freshwater, SCW, and BBJD. From this study, it is evident that CrAQP2 is an important water channel protein in *C. riparius*, with a role in homeostatic water transport. Furthermore, salinization of freshwater influences CrAQP2 expression and its role in osmoregulation. CrAQP2 appears to be vital in the ability of *C. riparius* to adapt to and survive in increased salinity caused by road de-icer contamination.

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5.7 Supplementary Figures

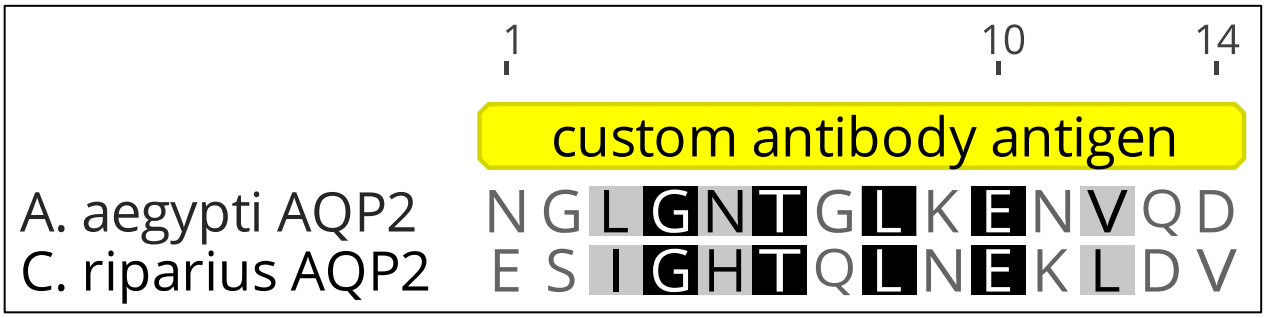

Figure 5-S1. *Aedes aegypti* AaAQP2 epitope alignment with the putative CrAQP2 epitope in *Chironomus riparius*.
The custom AaAQP2 antibody antigen from *Ae. aegypti* is shown in an alignment with the deduced CrAQP2 epitope of *C. riparius*. The alignment was done using Geneious Software (Dotmatics, MA, USA) demonstrating ~50% similarity over the antigen spanning residues.

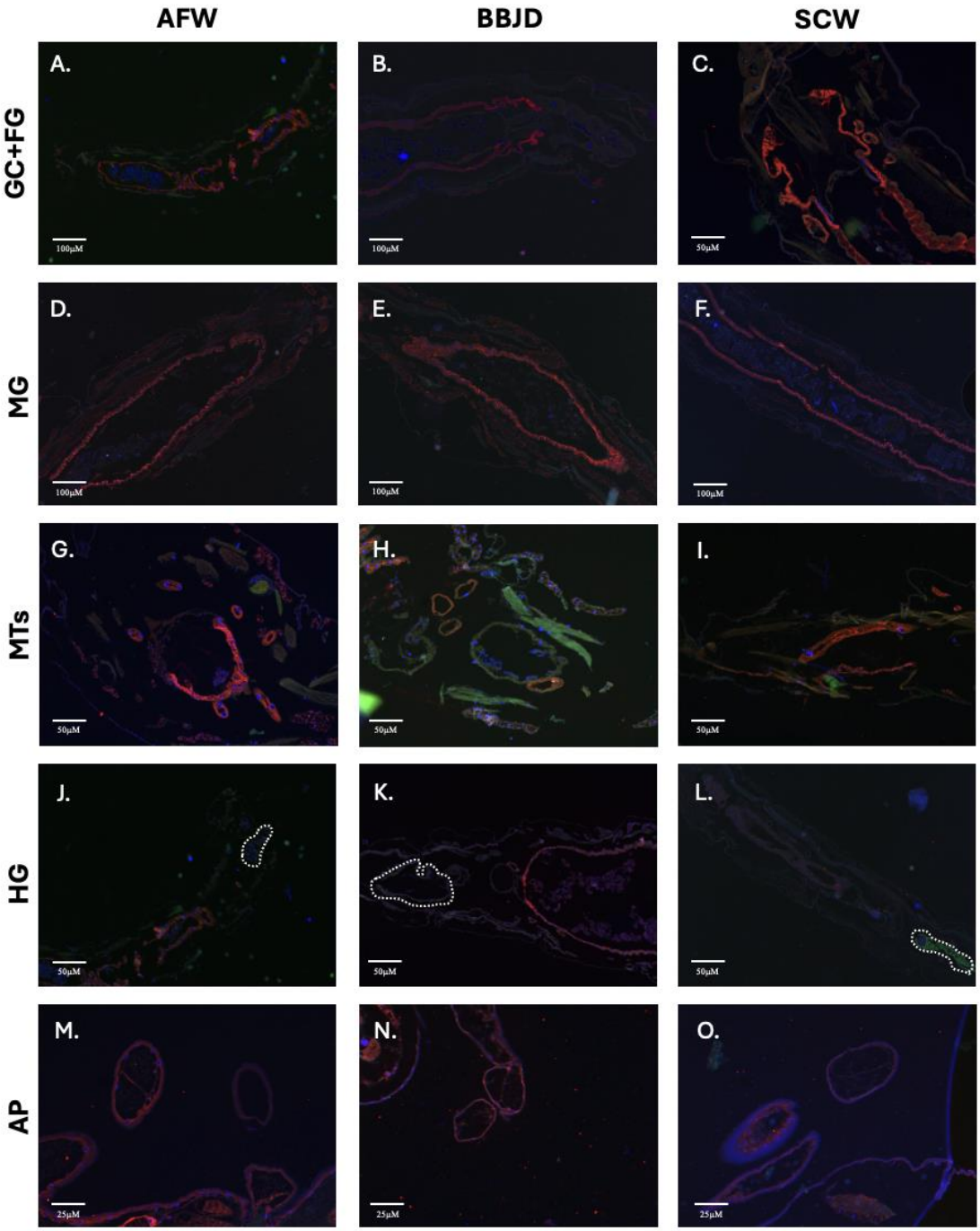

Figure 5-S2. Immunolocalization of CrAQP2 in osmoregulatory organs of larval *Chironomus riparius* in AFW, BBJD, and SCW.

Localization of AQP2 in tissue sections of the osmoregulatory organs of 4th Instar larval *C. riparius* midges in AFW, BBJD, and SCW including **A-C.** gastric caeca (GC) with foregut (FG), **D-F.** midgut (MG), **G-I.** Malpighian tubules (MTs), **J-L.** hindgut (HG), and **M-O.** anal papillae (AP). n=3-4 biological replicates for each organ, in addition to 4-5 technical replicates each. Red staining indicates AQP2 immunoreactivity; green staining indicates the Na^+/K^+-ATPase (NKA) membrane marker immunoreactivity; blue staining indicates the presence of individual nuclei.

Chapter Six

Effect of salt and brine-beet juice de-icer on osmoregulatory physiology of the freshwater amphipod *Hyalella azteca* (Saussure, 1858) (Amphipoda: Hyalellidae)

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Picinic, B., Durant, A.C., and Donini, A. (2022). Effect of salt and brine-beet juice de-icer on osmoregulatory physiology of the freshwater amphipod *Hyalella azteca* (Saussure, 1858) (Amphipoda: Hyalellidae). *Journal of Crustacean Biology,* **42**(2), 1-11.

6.1 Summary

The anthropogenic salinization of freshwater is concerning because it can negatively impact the success and survival of freshwater animals. Road salt (NaCl) in cold climates contributes to salinization and organic based de-icers have been developed to mitigate the effects of NaCl on freshwater. One of these de-icers is sugar beet juice, and few studies have examined its effects on freshwater animals. I exposed *Hyalella azteca* (Saussure, 1858), a freshwater amphipod, to different concentrations of NaCl (salt-contaminated water or SCW) and a NaCl brine and beet-juice mixture used as a de-icing product (brine-beet juice de-icer, BBJD). The LC_{50} of NaCl on *H. azteca* was 12.8 g l^{-1} and for BBJD was 4.6% (which at that percentage contained ~ 4.2 g l^{-1} Na^+). Sub-lethal doses of SCW elevated haemolymph Na^+ and BBJD exposure resulted in elevated K^+ concentration as well as acidification of the haemolymph. Both Na^+/K^+ ATPase (NKA) and V-type H^+-ATPase (VA) were localized to the coxal gills, whereas only NKA was found in the sternal gills. There was a qualitatively apparent decrease in expression of NKA in the gills of SCW-treated amphipods. NKA and VA expression qualitatively increased with BBJD exposure in the gut. The NKA and VA activity in whole-body homogenates was lower in BBJD and SCW. Results show that *Hyalella azteca* responds to SCW and BBJD by altering parameters of ionoregulatory physiology in different ways.

6.2 Introduction

The epibenthic hyalellid scud, *Hyalella azteca* (Saussure, 1858), is regarded as the most broadly distributed amphipod in freshwater ecosystems of North America (Witt & Hebert, 2000). *Hyalella azteca* is used here to refer to a cryptic species complex consisting of at least seven very closely related species (Witt & Hebert, 2000; Gonzalez & Watling, 2002). Amphipods are a predominant food source of other freshwater animals, including many species of fishes (Dermott et al., 2005; Camacho & Thacker, 2013). *Hyalella azteca* is also important for the coupling of nutrient movement from the benthic community in which it dominates, to higher trophic levels such as those comprised of fishes (Hargrave, 1971; Mathias, 1971). This amphipod has evolved to be an important model for ecotoxicology studies, particularly as an indicator for water and sediment quality and more broadly, for ecosystem health (Moore & Farrar, 1996; Kennedy et al., 2016; Hasenbein et al., 2018). Knowledge of the basic processes underlying ion and water regulation of *H. azteca,* however, is in its infancy (Hasenbein et al., 2018), particularly in relation to changes in freshwater composition from anthropogenic activities. Declines in abundances of *H. azteca* have been reported in shallow freshwater lakes as a consequence of predation from increasing numbers of fishes and lower water quality due to agricultural activities (Anteau & Afton, 2008; Anteau et al., 2011).

The salinization of freshwater caused by industrial effluent, mining, agricultural runoff, and road salt (i.e., NaCl) is a recognized threat to freshwater organisms (Kaushal et al., 2005; Kaushal et al., 2017; Arnott et al., 2020). The application of salt (predominantly NaCl) during winter months to prevent the formation of ice and mitigate hazardous driving conditions has increased with urbanization and transportation infrastructure (Kaushal et al., 2017). Alternative de-icers that contain beet juice have been developed more recently to reduce the impact of NaCl on

freshwater ecosystems. An example are products that are composed of a NaCl brine-beet juice de-icer mixture (BBJD) that contain smaller amounts of NaCl (12–23 % w/v) compared to traditional road salt or salt brines (Nutile & Solan, 2019). Nevertheless, there has been an increase in the run-off of road salt into freshwater and this is concerning because salt has been shown to impact the physiology and survival of a variety of freshwater aquatic animals including insects (Bervoets et al., 1996; Hassell et al., 2006; Jonusaite et al., 2011; Scheibener et al., 2016; Nowghani et al., 2019), zooplankton (Arnott et al., 2020), molluscs (Gillis et al., 2021), crustaceans (Brooks & Mills, 2006; Piscart et al., 2007; Henry et al., 2012; Castaño-Sánchez et al., 2020), and fishes (Griffith, 2017). An increase in the salinity is unnatural to freshwater animals, including freshwater amphipods, because their osmoregulatory physiology is adapted to actively sequester salts from a dilute habitat (Sutcliffe, 1971; Brooks & Mills, 2006). A detrimental build-up of salts in the body fluids of these animals may occur if the animals cannot limit salt uptake or switch to secreting salts when salinization of their habitat occurs. This added osmotic stress from salt-contamination of freshwater can lead to species declines which have already been demonstrated for other small freshwater crustaceans (Arnott et al., 2020; Valleau et al., 2020).

The gills of amphipods, like many crustaceans, are considered to be principal sites of ion and water exchange between the internal (i.e., intracellular and/or the haemolymph) and external environments (Spicer & McMahon, 1994; Brooks & Mills, 2006; Griffith, 2017). Freshwater amphipods possess trichobranchiate gills which are comprised of filamentous branches giving them a "feather-like" appearance (McLaughlin, 1982; Covich et al., 2010). *Hyalella azteca* possess five pairs of sac-like coxal gills on segments 2–6 of the pereon, and five pairs of tubular sternal gills on segments 3–7 (Gonzalez & Watling, 2002). Gill function of freshwater amphipods has been of particular interest in ecotoxicology studies, particularly those examining the impacts of

freshwater salinization. As important sites of ion exchange, the gills of freshwater amphipods utilize integral active transport enzymes, mainly Na^+- K^+-ATPase (NKA) and V-type H^+-ATPase (VA), to energize transcellular ion transport mechanisms. NKA in the gills of the freshwater amphipod *Gammarus pulex* (Linnaeus, 1758), the euryhaline *G. duebeni* (Lilljeborg, 1852), and the brackish water amphipod *G. tigrinus* (Sexton, 1939), showed maximum activity when individuals were placed in freshwater in comparison to 10% seawater, demonstrating the importance of gill NKA activity in active Na^+ uptake from dilute freshwater (Brooks & Mills, 2006). Hasenbein et al., (2018) identified a number of ion transporters in whole-body homogenates of *H. azteca*, including VA and NKA for ion uptake, and Na^+-H^+-antiporter along with carbonic anhydrase (cah1) for pH regulation. There is currently no information, however, regarding specific transepithelial ion transport mechanisms and general function of the distinct gill pairs of *H. azteca*. Furthermore, it is not clear if ion transport functions in *H. azteca* can be adapted when faced with increased salinity, whether caused by NaCl or products containing beet juice.

In particular, the impacts of BBJD on aquatic communities as it leeches into freshwater ecosystems are relatively unknown. This proprietary agriculturally derived de-icer may pose a unique challenge to freshwater animals as BBJD liquid contains comparatively high levels of K^+ (2.83 ± 0.3 g l^{-1}), almost seven-fold greater than the Na^+ levels (Fay & Shi, 2012; Wruss et al., 2015). Gillis et al. (2021) noted that BBJD is more toxic to freshwater larval mussels than salt brine, and this toxicity was attributed to the high K^+ levels in BBJD. With this information in mind, there is a need for further investigation into the effects of organic de-icers such as beet juice as well as traditional road salts on the physiology of aquatic animals. To address this, I examined the survival and parameters of osmoregulatory physiology of *H. azteca* exposed to BBJD- or NaCl-contaminated water (SCW). It was hypothesised that SCW and BBJD would result in similar

effects on survival and that key ionomotive enzyme expression and activity would be altered with exposure to SCW and BBJD.

6.3 Materials and Methods

Animal Maintenance and Exposure to SCW and BBJD for LC_{50} Determination

Adult male and female (≥ 5mm) individuals of *H. azteca* were selected for studies. Amphipods were obtained from clean field-collected sediment from Lake Erie, Ontario, Canada supplied by the Ontario Ministry of the Environment, Conservation, and Parks. Amphipods were isolated in a 10-l tank with sediment collected from Lake Erie, Ontario, Canada and maintained at room temperature in a 3:1 ratio of aerated artificial freshwater (FW), 18.2 MΩ H_2O with the addition of salts resulting in ion concentrations of 600µM Na^+, 1100µM Cl^- 380µM Ca^{2+}, 43µM K^+ as previously described for *H. rigida* (Nowghani et al., 2017, Nowghani et al., 2019). Amphipods were fed twice weekly with ground TetraFin Goldfish flake food (0.5 grams per feed) (Tetra Spectrum Brands Pet LLC, Blacksburg, VA, USA). For exposures to salt-contaminated water (SCW), NaCl (BioShop Canada, Burlington, ON, Canada) was dissolved in FW (5, 8, 10, 12, 15, 18 g l^{-1} treatments) and 300 ml of each was added to individual aerated containers with 100 g of moist sediment. The containers were covered with a lid to prevent evaporation and kept at room temperature (~ 22 °C). Similarly, for brine-beet juice de-icer (BBJD) (~12% NaCl brine and ~50% beet juice), BBJD liquid was mixed with FW (1.6, 3.2, 3.8, 4.4, 5.0, 5.7, 6.3%, v/v) and 300 ml of each was added to 100 g of moist sediment in individual aerated containers as described above for SCW treatments. Ten amphipods were added to each container (N = 180 amphipods for SCW, N = 190 amphipods for BBJD, N = 30 amphipods for FW controls) and each exposure treatment and concentration was run at least twice. For determination of toxicity levels of SCW and BBJD, amphipods were left undisturbed until 7-d post-exposure at which time individuals were carefully sifted from the liquid/sediment and counted in clean FW. Amphipod survival and LC_{50} estimation was determined by inspection of individuals under a Zeiss stereo dissecting

microscope (Carl Zeiss, Oberkochen, Germany) to confirm normal ventilatory and locomotory movements. After the LC_{50} levels for SCW and BBJD were determined, sublethal doses (> 50 % survival) of SCW and BBJD were selected for physiological studies to ensure that individuals sampled had been challenged by the treatment but were not near death.

Ion-Selective Microelectrode (ISME) Technique

The ISME technique was used to measure ion levels in the haemolymph of amphipods, as well as the ion content of FW, SCW, and BBJD media at the sublethal concentrations that amphipods were reared in for physiological experiments. Amphipods were collected at 7 d of exposure to SCW, BBJD, and FW, and each individual transferred for 30 s to clean artificial freshwater to avoid external ions from the treatment conditions contaminating the haemolymph. Individual amphipods were then briefly transferred onto a Kimwipe (Kimtech Science, Concord, ON, Canada) using a plastic transfer pipette to blot dry any excess water. Using forceps, each amphipod was placed in a Petri dish containing hydrated mineral oil and positioned with a fine insect pin. A second pin was used to puncture the pereon region dorsal to the heart, whereby a droplet of haemolymph formed at the surface of the cuticle. Individual haemolymph droplets were then collected with a micropipette and transferred to a clean dish of hydrated mineral oil for the measurements of ion activity. Ion-selective microelectrodes for the measurement of haemolymph and treatment media Na^+, K^+, NH_4^+, and H^+ (pH) levels were constructed following Donini & O'Donnell (2005). The following calibration solution concentrations were used: Na^+, 30 and 300 mmol l^{-1} NaCl; K^+, 1.5, 15, and 150 mmol l^{-1} KCl; H^+. 100 mmol l^{-1} NaCl and 100 mmol l^{-1} $Na_3C_6H_5O_7$ at pH 7 and pH 10, and NH_4^+; 0.2, 2, and 20 mmol l^{-1} NH_4Cl. Microelectrode slopes (in mV) for a ten-fold difference in ion concentrations of calibration solutions were as follows

(mean ± SEM): 52.5 ± 1.04 for Na^+ (N = 3), 56 ± 1.09 for K^+ (N = 4), 55.6 ± 4.39 for NH_4^+ (N = 3), and 63.46 ± 1.94 for H^+ (N = 3). Voltages were recorded in LabChart 8 Pro Software (AD Instruments, Colorado Springs, CO, USA) and were converted to ion activities utilizing methods described by Donini & O'Donnell (2005).

ATPase Activity Assay

The enzyme activities of ATPases NKA and VA were assessed in whole individuals of *H. azteca* following 7-d exposures to SCW, BBJD, and FW. Between 5–7 amphipods per replicate (N = 4 for FW, N = 3 for SCW, and N = 4 for BBJD) were briefly rinsed in clean fresh water then pooled together in 1.5 ml tubes and flash-frozen using liquid nitrogen. Tubes containing frozen samples were stored at –80ºC until processing. The activities of NKA and VA were established relative to their inhibition by ouabain (5 mmol l^{-1}) and bafilomycin (10 µmol l^{-1}), respectively, following methods detailed previously for arthropod gills and other organs (Jonusaite et al., 2011). Activities were standardized using total protein content of samples which were determined using a modified Bradford method as described by Jonusaite et al. (2011).

Immunohistochemistry

Immunohistochemistry was used to localize NKA and VA to the epithelia of osmoregulatory organs (e.g., gills and gastrointestinal tract) of amphipods. Whole individuals were placed in Bouin's fixative solution for 3 h at room temperature. The fixed amphipods were rinsed in phosphate-buffered saline (PBS), dehydrated in a series of ethanol solutions, embedded in paraffin wax, and sectioned (6 µm thick sections) using a Leica RM 2125RT manual rotary microtome (Leica Microscopy, Concord, ON, Canada) as detailed by Chasiotis & Kelly (2008).

Amphipods were sectioned longitudinally in the sagittal plane to obtain sections of the paired gills. Tissue sections from three to four individuals from each treatment (FW, SCW, and BBJD) were placed on VistaVision Histobond microscope slides (VWR International, Mississauga, ON, Canada). Immunolocalization of NKA and VA in amphipod tissue sections was undertaken as described by Chasiotis & Kelly (2008). Expression of NKA protein was detected using a 1:10 concentration of a mouse monoclonal anti-a5 antibody (Douglas Fambrough, Developmental Studies Hybridoma Bank, IA, USA). A goat anti-mouse secondary antibody conjugated to Cy5 (Jackson ImmunoResearch, West Grove, PA, USA) at a 1:500 dilution. Immunolocalization of VA was undertaken using an antibody generated in guinea pig against the V_1 complex of the VA at a concentration of 1:5000 (Ab 353-2, gifted by H. Wieczorek, Osnabruck, Germany). To visualize VA, an AlexaFlour 488 AffiniPure goat anti-guinea pig secondary antibody was used (Jackson ImmunoResearch) at a 1:500 dilution. Control slides with sections of gill and gastrointestinal tract were processed following the same procedure, except for the omission of primary antibodies against NKA and VA being applied to these slides. The final slides were mounted using ProLong Gold antifade reagent with DAPI (Invitrogen, Burlington, ON, Canada) and viewed using fluorescent microscopy. Images were merged using ImageJ 1.53k software.

Statistical Analyses

Data were analyzed and plotted using GraphPad Prism 9.1.2 software. Statistical tests, sample sizes, and p-values used to assess changes between FW, SCW, and BBJD groups for each experiment are detailed in the captions of Figures 6-1 through 6-4.

6.4 Results

Survival After Chronic Exposure to SCW and BBJD

The survival (%) of *H. azteca* exposed to increasing concentrations of SCW was used to determine the concentration of NaCl that caused 50% mortality (LC_{50}) of amphipods (Figure 6-1). The amphipods (N = 10 per replicate) were exposed to SCW for 7 d at concentrations of 5, 8, 10, 12, 15, and 18 g l^{-1} NaCl dissolved in freshwater. The LC_{50} of SCW was 12.8 g l^{-1} . The sub-lethal dose of SCW used for physiological studies was 12 g l^{-1}. The survival (%) of *H. azteca* exposed to different concentrations of BBJD liquid each for 7 d was used to determine the LC_{50} (Figure 6-2). Amphipods (N = 10 per replicate) were exposed to BBJD liquid for 7 d at concentrations (% v/v) of 1.6%, 3.2%, 3.8%, 4.4%, 5.0%, 5.7%, and 6.3% BBJD diluted in FW. The LC_{50} was 4.6% in the BBJD treatment group. A sublethal dose of BBJD liquid for *H. azteca* resulting in ~25% mortality on average was 3.8% (~8.69 ppt NaCl) BBJD liquid, which was the concentration used for physiological studies.

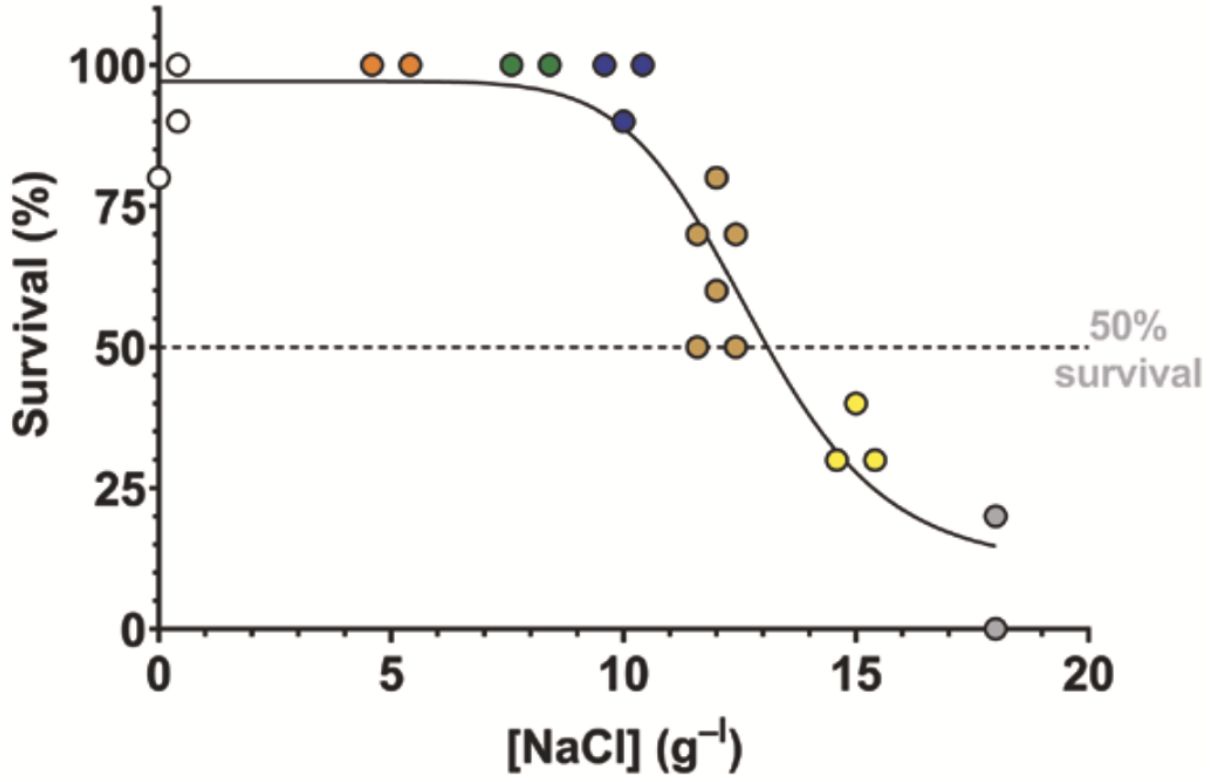

Figure 6-1. Determination of LC_{50} for NaCl (i.e., salt contaminated water, SCW) on individuals of *Hyalella azteca.* Best-fit curve (least squares fit) of percent survival of amphipods at 7-d post exposure to 0, 5, 8, 10, 12, 15, and 18 g l^{-1} NaCl diluted in freshwater. The concentration of NaCl that results in 50% amphipod survival (LC_{50}) was determined to be 12.8 g l^{-1}. Each point illustrates a single replicate (*N*=10 amphipods per replicate point).

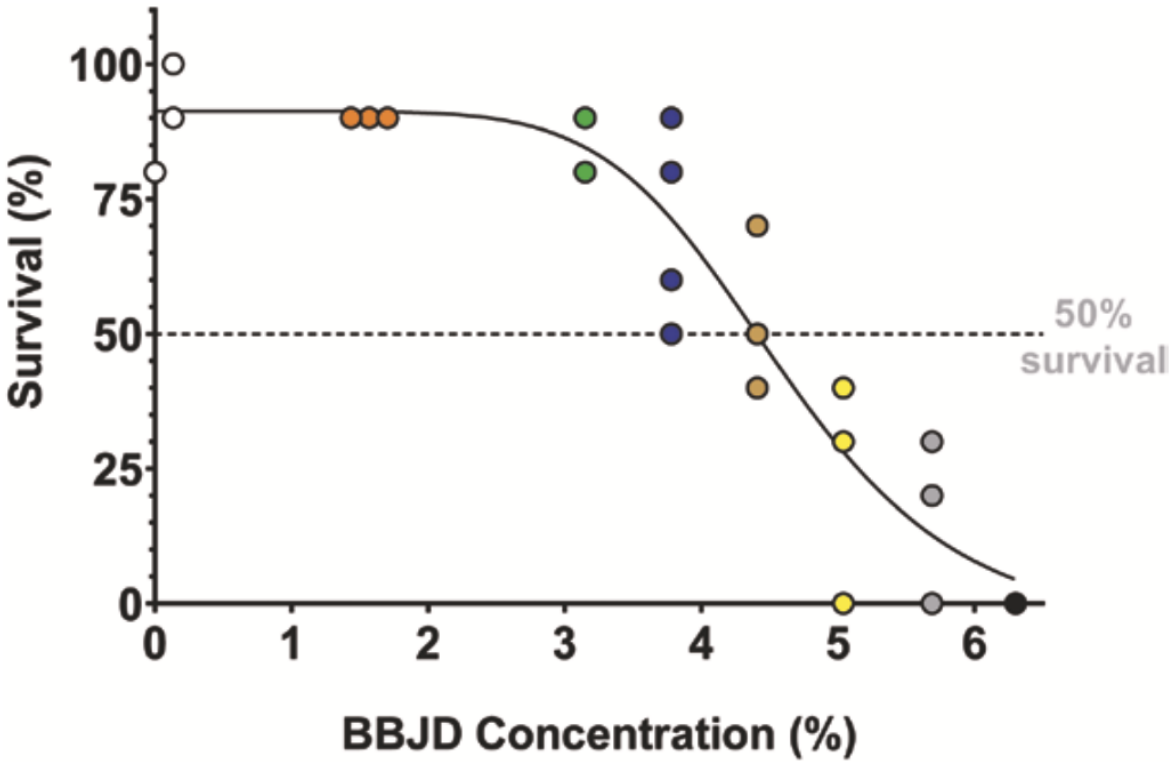

Figure 6-2. Determination of LC_{50} for brine-beet juice de-icer (BBJD) on individuals of *Hyalella azteca.* Best-fit curve (least squares fit) of percent survival of amphipods at 7-d post exposure to 0%, 1.6%, 3.2%, 3.8%, 4.4%, 5.0%, 5.7%, and 6.3% BBJD diluted in freshwater. The concentration of BBJD that resulted in 50% amphipod survival (LC_{50}) was determined to be 4.6% BBJD. Each point illustrates a single replicate (*N*=10 amphipods per replicate point).

Ion Composition and pH Vary Between Freshwater (FW), Brine-Beet Juice De-Icer (BBJD), and Salt-Contaminated Water (SCW) Media

BBJD (3.8%) and SCW (12 g l^{-1}) solutions had similarly elevated concentrations of Na^+ relative to FW at 153.95 ± 14.83 (mmol l^{-1}) and 132.96 ± 7.21, respectively (Table 6-1). The concentration of K^+ was highest in the BBJD conditions (19.61 ± 2.04) compared to both SCW (0.21± 0.02) and FW (0.05 ± 0.01). The concentration of NH_4^+ was higher in both SCW (0.53 ± 0.07) and BBJD (0.49 ± 0.08) compared to FW (0.15 ± 0.01). BBJD liquid alkalinized the water to pH 7.73 ± 0.038, whereas SCW acidified the water to pH 7.03 ± 0.025 compared to FW (7.14 ± 0.024).

Table 6-1. Ion concentrations (mmol l^{-1}) and pH of freshwater (FW), salt-contaminated water (SCW), and brine-beet juice de-icer (BBJD) media used to expose *Hyalella azteca*. Data from FW and sublethal doses of SCW (12 g^{-1} NaCl) and BBJD (3.78%) shows mean ± SEM. (*N*=3) values of water samples collected prior to the addition of sediment and individuals to the media. Different letters indicate a significant difference in ion concentration compared to control FW levels (One-way ANOVA with Tukey's multiple comparisons, $p<0.05$). Letters a-c denote statistical differences between FW, SCW, and BBJD.

	Na^+	K^+	pH	NH_4^+
FW	1.34±0.04[a]	0.05±0.01[a]	7.14±0.02[a]	0.15±0.01[a]
SCW	132.9±7.2[b]	0.21±0.02[a]	7.03±0.03[b]	0.53±0.07[b]
BBJD	153.9±14.8[b]	19.6±2.04[b]	7.73±0.04[c]	0.49±0.08[b]

Levels of Na^+, K^+, NH_4^+, and pH in the Haemolymph of Amphipods

In comparison to the FW group, the haemolymph Na^+ concentration during 7 d of exposure was significantly elevated ($p = 0.0002$) in the SCW group of *H. azteca* (Figure 6-3A). Haemolymph Na^+ concentration exposed to BBJD were similar to the FW group ($p = 0.999$) (Figure 6-3A). The haemolymph K^+ concentration was higher in the BBJD-exposed amphipods in comparison to the FW group ($p = 0.0017$), whereas the K^+ concentration of SCW-exposed individuals did not change in comparison to the FW group ($p = 0.1212$) (Figure 6-3B). The haemolymph NH_4^+ concentration of amphipods exposed to either SCW or BBJD were unaltered in comparison to the FW group ($p = 0.5289$ and $p = 0.0982$, respectively) (Figure 6-3C). Amphipods exposed to BBJD for 7 d showed a significantly decreased haemolymph pH ($p < 0.0001$) in comparison to the FW group, with no difference in haemolymph pH of SCW-exposed individuals (Figure 6-3D).

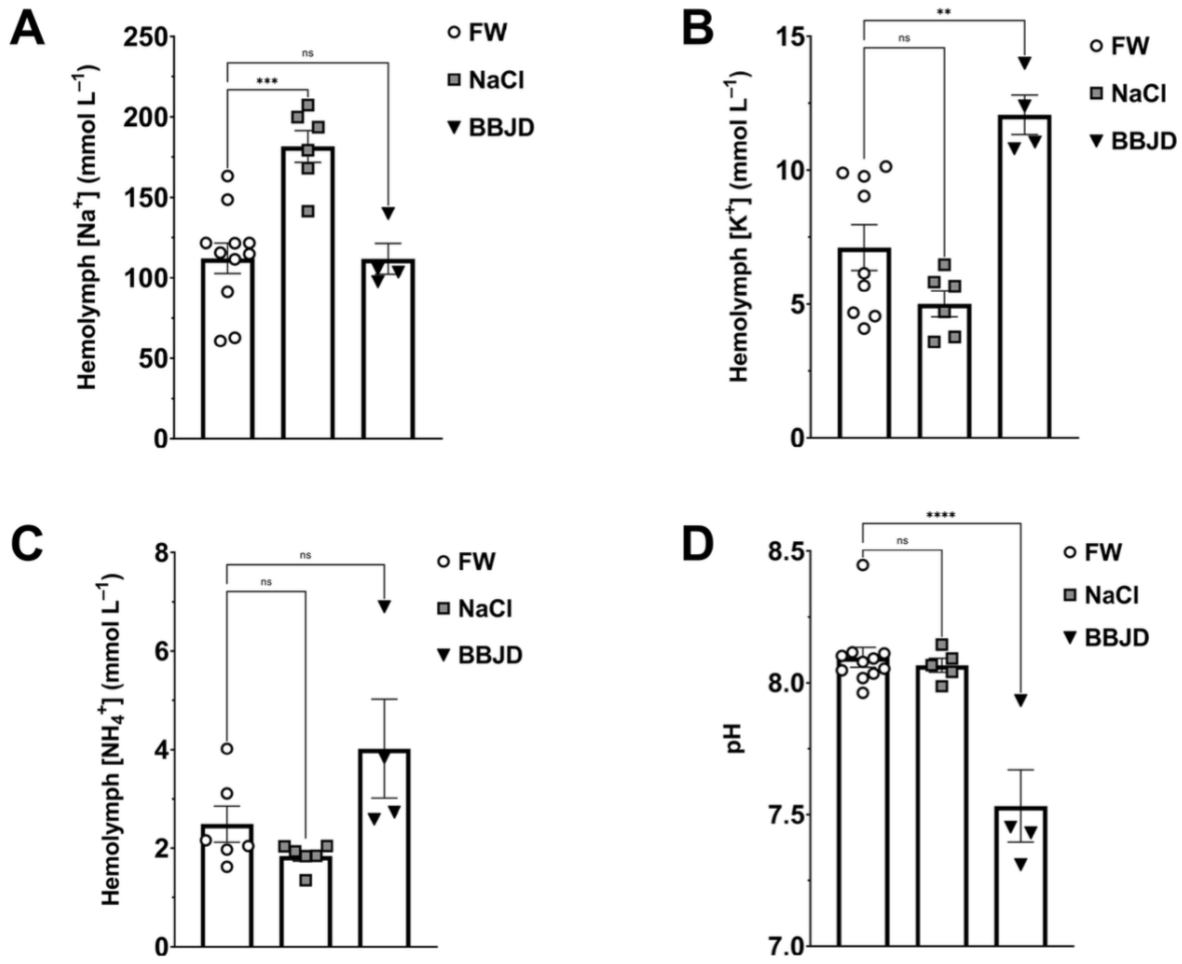

Figure 6-3. Haemolymph ion levels of individuals of *Hyalella azteca* exposed chronically to freshwater (FW), salt-contaminated water (NaCl), and brine-beet juice de-icer (BBJD) media. Levels of haemolymph Na^+ (N =11 for FW, N = 6 for NaCl, N = 4 for BJD) **(A)**. K^+ (N =9 for FW, N = 6 for NaCl, N = 4 for BBJD) **(B)**. $NH4^+$ (N =6 for FW, N = 6 for NaCl, N = 4 for BBJD) **(C)**. pH (N =11 for FW, N = 5 for NaCl, N = 4 for BBJD) **(D)**. Data are shown as mean ± SEM (one way ANOVA, Dunnett's multiple comparisons test). Asterisks indicate significant different to FW control (**, p = 0.0017; ***, p = 0.0002; ****, p < 0.0001).

Activity of ATPases Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (VA)

In comparison to FW individuals of *H. azteca*, the NKA activity was significantly decreased in the whole-body tissues of amphipods exposed for 7 d to SCW and BBJD ($p = 0.0006$ and $p = 0.0001$, respectively) (Figure 6-4). No significant difference in the NKA activity between the SCW and BBJD treatment groups was observed ($p = 0.9764$). The activity of VA was also found to significantly decrease in whole-body tissues of amphipods exposed to SCW and BBJD in comparison to individuals exposed to FW ($p = 0.0006$ and $p = 0.001$, respectively) (Figure 6-4). No significant difference in VA activity between the SCW and BBJD groups was observed ($p = 0.2937$).

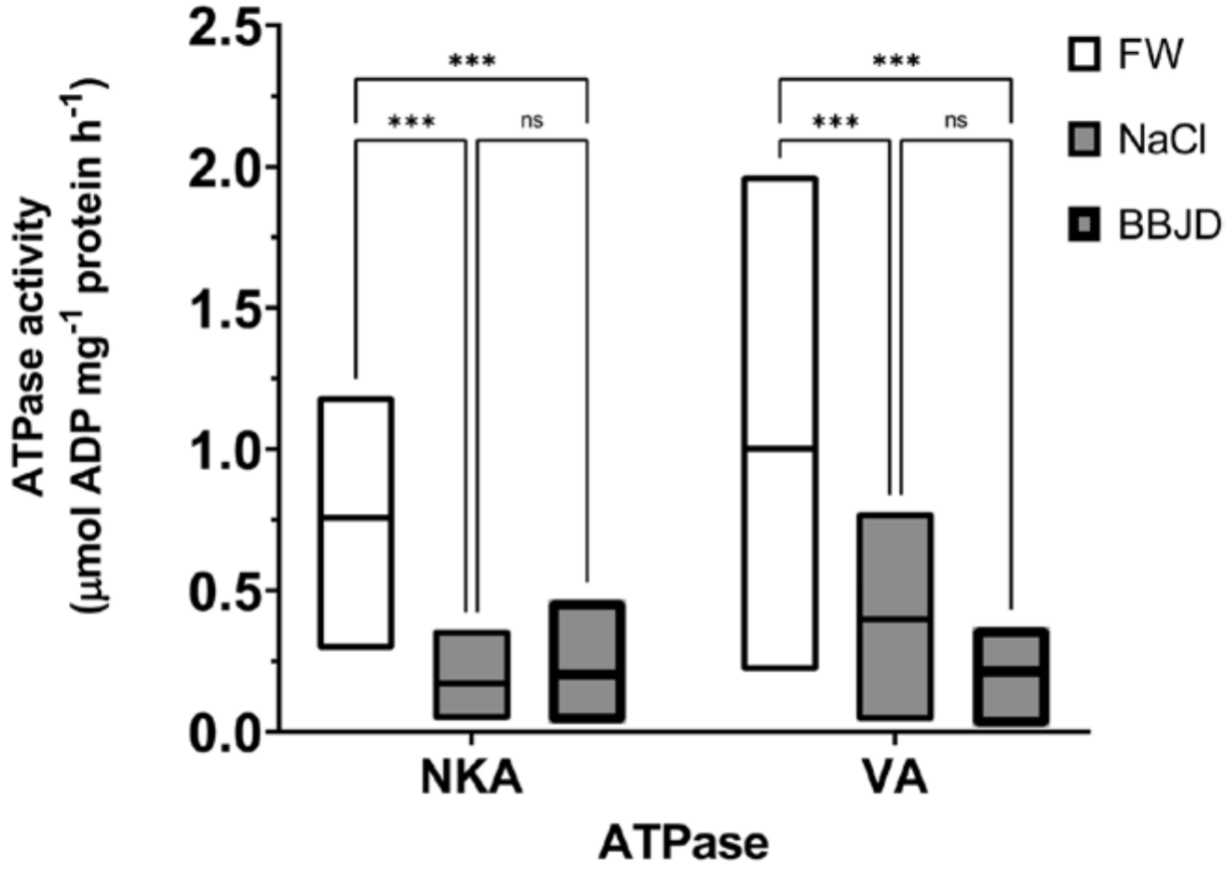

Figure 6-4. Whole-body ATPase activities of individuals of *Hyalella azteca* exposed chronically to freshwater (FW), salt-contaminated water (NaCl), and brine-beet juice de-icer (BBJD) media. Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (VA) activities in whole-animal tissues at 7-d post-exposure. Box plot shows mean (and minimum to maximum values) with a sample size of $N = 4$ replicates for FW, $N = 9$ replicates for NaCl, and $N = 8$ replicates for BBJD with each replicate having 5-7 animals. Data were analyzed using a two-ANOVA (Tukey post hoc test; ***, $p = <0.001$).

Localization of NKA and VA in the Gills and Gastrointestinal Tract

Hyalella azteca has five paired coxal gills on pereon segments 2–6 and five paired sternal gills on segments 3-7 (Figure 6-S1). NKA and VA of FW amphipods were localized in the basolateral and apical membranes of epithelial cells of the larger coxal gills, respectively, whereas it appears that only NKA immunostaining is present in the sternal gills (Figure 6-5A–C). Similar observations in NKA and VA immunolocalization in the gills of SCW (Figure 6-5D, E) and BBJD (Figure 6-5G, H) in exposed amphipods were evident, whereby NKA and VA localized in the basolateral and apical membranes of epithelial cells of the coxal gills; only NKA immunostaining was observed at the basolateral membrane of the epithelium in the sternal gills. NKA and VA immunostaining of the gills was not observed in control slides (Figure 6-5F, I). Separate images of NKA or VA immunostaining only within the coxal and sternal gills of amphipods from FW are provided in Supplementary Figures, Figure 6-S2.

Both NKA and VA were also immunolocalized in the alimentary canal (Figure 6-6). The presence of basolateral NKA and apical VA immunostaining was localized within the midgut epithelium of FW amphipods, which is comparatively lower than the observed NKA and VA staining in the gills (Figure 6-6A, D). In amphipods exposed to SCW for 7 d, NKA immunolocalization in the basolateral membrane of the midgut epithelium was detected, but VA staining within the midgut epithelium of SCW-exposed individuals was not observed (Figure 6-6B). Distinct immunolocalization of NKA in the basolateral membranes and VA localization to the apical membranes of the midgut and hindgut epithelia of BBJD-exposed individuals, comparable to immunostaining levels observed in the gills (Figure 6-6C, F). NKA and VA immunostaining were absent in the alimentary canal of control slides (Figure 6-6E).

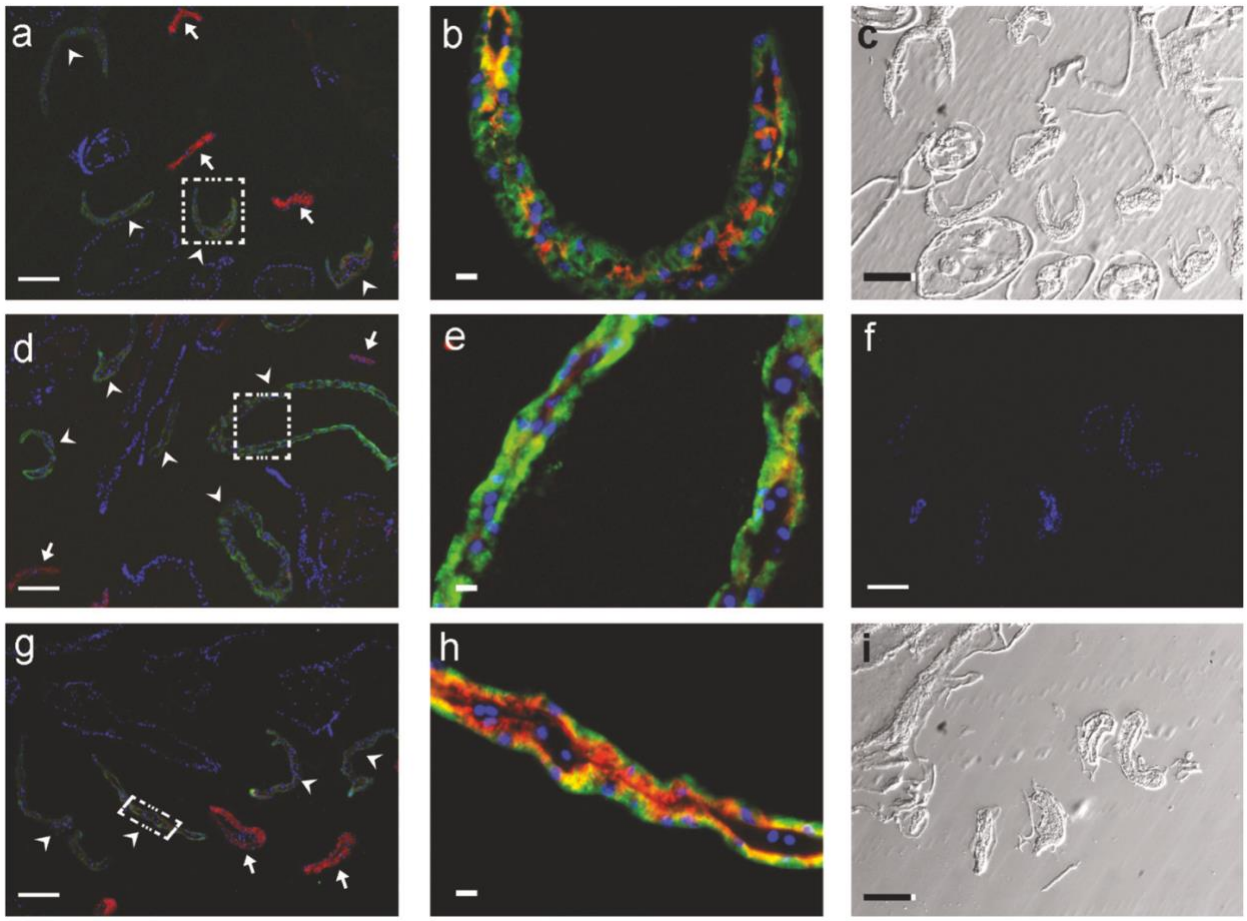

Figure 6-5. Immunolocalization of Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (VA) in cross sections through the coxal and sternal gills of *Hyalella azteca*. NKA (red), VA (green), and nuclear staining (DAPI, blue) in the sternal gills (solid white) and coxal gills (arrowheads) of individuals (**A, B**). A bright field image corresponding to panel (**C**). Amphipods exposed to salt-contaminated water (SCW) for 7 d (**D, E**). Amphipods exposed to brine-beet juice de-icer (BBJD) for 7 d (**G, H**). Dashed white boxes in **A, D,** and **G** indicate regions that are shown in higher magnification in **B, E, H**. Representative control image showing nuclear staining (DAPI, blue) and secondary antibody staining (AlexaFluor 488, AlexaFluor 594) in the gills of freshwater amphipods (**F**). Bright field image corresponding to F (**I**). Scale bars =100 µm in **A, D, G, C, F, I**; 10 µm in **B, E, H**.

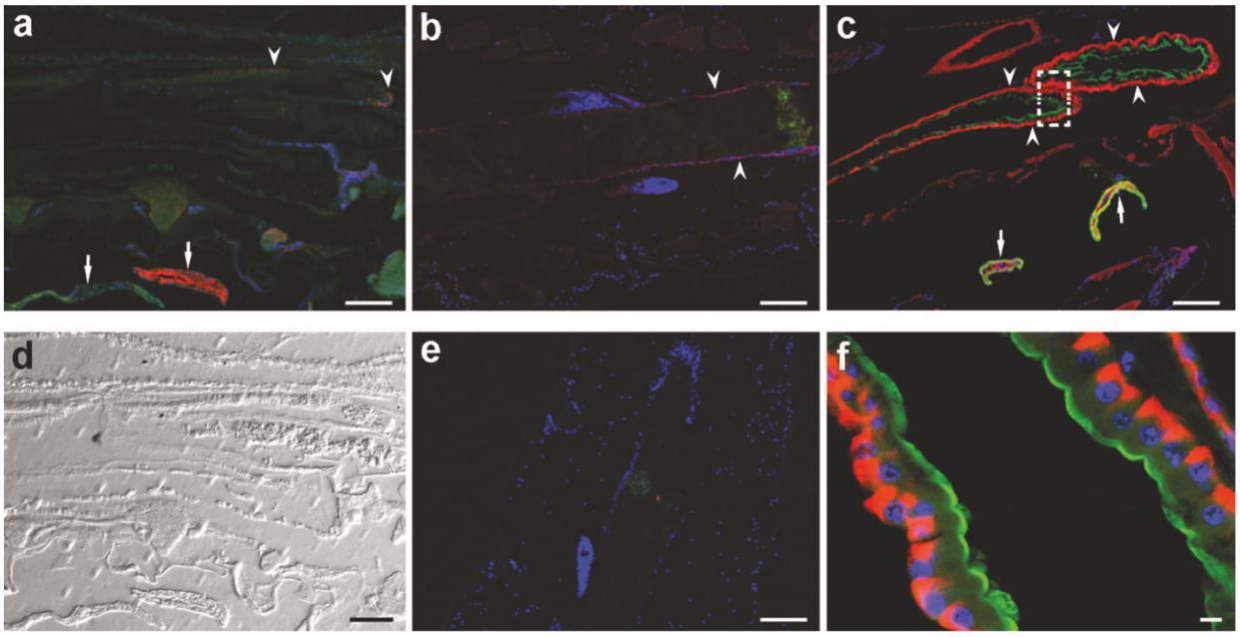

Figure 6-6. Immunolocalization of Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (VA) in cross sections through the alimentary canal of *Hyalella azteca*. NKA (red), VA (green), and nuclear staining (DAPI, blue) localized to the digestive tract epithelia (solid arrowheads) of individuals **(A)**. Amphipods exposed to salt contaminated water (SCW) for 7 d **(B)**. Amphipods exposed to brine-beet juice de-icer (BBJD) liquid for 7 d **(C)**. Bright-field image corresponding to A **(D)**. Gill sections, if present, are indicated by the white dashed arrows. Representative control image showing nuclear staining (DAPI, blue) and secondary antibody staining (AlexaFluor 488, AlexaFluor 594) in the digestive tract of individuals **(E)**. Higher-magnification image of the gut epithelium corresponding to C **(F)**. Dashed white box in **C** indicates the region that is shown in higher magnification in **F**. Scale bars = 100 µm in **A–E**; 10 µm in **F**.

6.5 Discussion

Tolerance of Amphipods to SCW and BBJD

The threshold tolerances of chronic exposure to SCW (LC_{50} of 12.8 g l^{-1} NaCl) of *H. azteca* were in line with semi-acute tolerances of the same species reported previously (96-h LC_{50} >10 g l^{-1} road salt (Benbow & Merritt, 2004). Individuals also showed similar tolerances to SCW that has been documented for other freshwater amphipods such as *Gammarus roeseli* (Gervais, 1835) with a 72-h LC_{50} of 12.6 g l^{-1} NaCl (Sornom et al., 2010) and *Gmelinoides fasciatus* (Stebbing, 1899) with a 15-d LC_{10} of 8.1 g l^{-1} salinity (sea salt in natural river water; Berezina, 2003). Tolerance to salinity of *H. azteca* was also generally similar to that of other crustaceans; for example, juveniles of the freshwater prawn *Macrobrachium rosenbergii* (De Man, 1879) (7-d LC_{50} of 5 or 10 ppt salinity Cheng & Chen, 2000) and the isopod *Asellus aquaticus* (Linnaeus, 1758) (15-d LC_{10} of 8.1 ppt salinity; Berezina, 2003). *Hyalella azteca* has higher tolerances to elevated external ion levels in comparison to other freshwater invertebrates, a finding that appears to be ubiquitous among freshwater amphipods. For example, some cladocerans considered to have high tolerances to salt compared to other cladocerans, such as *Daphnia magna* (Straus, 1820) and *D. pulex* (Leydig, 1860), have LC_{50} thresholds of 6–7 g l^{-1} NaCl and 2.0 g l^{-1} NaCl, respectively (Schuytema et al., 1997; Sarma et al., 2006; Martínez-Jerónimo & Martínez-Jerónimo, 2007). My findings indicate that *H. azteca* can tolerate present salinity levels in urban freshwater bodies of North America, as ambient chloride (Cl^-) concentrations, which remains high in surface waters after dissociating from Na^+, are below 0.01 g/l for inland lakes in Canada, with levels typically not exceeding 0.03 g l^{-1} of Cl^- in the lower Great Lakes (Evans, M. & Frick, C. 2001; Anonymous, 2011). The levels of NaCl tested in this study are not unprecedented, and in fact, are now ecologically relevant, as Cl^- concentrations in some small urban watersheds were recorded to be

well above chronic freshwater Cl^- concentration guidelines and as high as 20 g l^{-1} following periods of snowmelt (Anonymous, 2009; Corsi et al., 2015; Helmueller et al., 2020). Salt contamination of freshwater by means other than road salting, as in agricultural practices involving the clearing of land, irrigation, and grazing, has led to rapid salinization of Australian groundwaters with the influx of saline (3.7-6.4 g l^{-1} Cl^-) soil water (Bennetts et al., 2006).

Hyalella azteca was tolerant (LC_{50}) to a concentration of BBJD containing higher levels of both Na^+ and K^+ than SCW. Little is known about the toxicity of organic de-icing agents, such as BBJD, to aquatic animals although, organic de-icer was demonstrated to limit dissolved oxygen levels and alter zooplankton abundances in freshwater at environmentally relevant concentrations (Schuler et al., 2017). A beet extract-enhanced de-icing formulation resulted in a 10-d LC_{50} of 6.32 g l^{-1} for aquatic larvae of the midge *Chironomus dilutus* (Shobanov, Kiknadze & Butler, 1999), however, the product used was comprised of up to 85%–99% NaCl (Nutile & Solan, 2019), which is much greater than the formulation used in this study (~12% NaCl w/v; MSDS (Eco Solutions, Milton, ON, Canada)). The toxicity of specific ions and the lethal level of salinity varies among taxa and highlights the necessity of comparisons among groups of aquatic animals. Potassium was shown to be the most toxic among the major ions in freshwater to the survival of daphnids and fishes (Mount et al., 1997, 2016). Potassium was also implicated in driving toxicity of larval stage mussels to beet juice-containing solutions (Gillis et al., 2021). Beet juice extracted from sugar beets contains high amounts of K^+ (Wruss et al., 2015), and I measured K^+ concentrations in sublethal levels of BBJD liquid that were greater than 360-fold that of freshwater levels.

Regulation of Haemolymph Ion Levels in Relation to Elevated Ions in SCW and BBJD

Hyalella azteca like many freshwater animals, maintain blood/haemolymph ion concentrations (Na^+ ~112 in mmol l^{-1}, K^+ ~ 7.11 in mmol l^{-1}, NH_4^+ ~2.49 in mmol l^{-1}, and pH ~8.0) at levels much higher than that found in the external freshwater. The haemolymph ion levels of *H. azteca* are similar to levels reported for other freshwater amphipods like *Dikerogammarus villosus* (Sowinsky, 1894; Brooks et al., 2008), *Gammarus pulex* (Brooks & Mills, 2003), and *G. roeseli* (Sornom et al., 2010), as well as freshwater crabs (Onken & Mcnamara, 2002). *Hyalella azteca* responds to sublethal levels of SCW with a corresponding elevation in haemolymph Na^+ levels, which seems to be a common response among freshwater amphipods to increases in environmental salinity (NaCl) (Brooks & Mills, 2006; Sornom et al., 2010). Freshwater amphipods face dilution of body fluids and actively take up ions but in SCW, the high Na^+ concentration (~133 mmol l^{-1}; Table 6-1) likely lead to excessive salt gain through these same ion uptake mechanisms. Sudden exposure to higher levels of salts results in a corresponding increase in ion uptake in aquatic insects (Scheibener et al., 2016; Orr et al., 2021). Acclimation to sublethal levels of SCW did not alter the levels of other major ions (K^+, NH_4^+, H^+) in *H. azteca*; however, Cl^- concentration in the haemolymph were not measured in this study, and Cl^- is typically elevated in freshwater invertebrates acutely exposed to chloride-based salts (Donini et al., 2007; Sornom et al., 2010; Jonusaite et al., 2011; Griffith, 2017; Nowghani et al., 2019).

Haemolymph K^+ concentration in *H. azteca* in freshwater (~7 mmol l^{-1}) were comparable to levels found in the freshwater amphipod *G. pulex* (~5–6 mmol l^{-1}) and were unaffected by exposure to SCW and up to 50% seawater, respectively (Sutcliffe, 1971). A significant increase in haemolymph K^+ concentration in amphipods exposed to BBJD, of which BBJD itself contains high levels of free K^+, was observed in this study. Crustaceans generally lack efficient mechanisms

for potassium regulation by the osmoregulatory organs (i.e., gills) (Griffith, 2017). Freshwater crustaceans are apparently more capable of taking up K^+ from the external medium (Krogh, 2015), unlike their vertebrate counterparts, which have a lower capacity for epithelial K^+ uptake (Sanders & Kirschnerf, 1983). Freshwater bivalves also have high-affinity K^+ transport, presumably across the gills, which is optimal for the uptake of K^+ from naturally dilute (≤ 0.05 mmol l^{-1} K^+) freshwater (Dietz & Byrne, 1990; Wilcox & Dietz, 1995). Consequently, these high affinities for K^+ absorption become a physiological detriment to freshwater bivalves when external K^+ level increases in the aquatic environment (Gillis et al., 2021), and this may be true for many animals that are equipped for high-capacity K^+ uptake in dilute freshwater. Here, *H. azteca* exposed to BBJD liquid seem to tolerate haemolymph K^+ concentrations that are approximately double that of freshwater-exposed amphipods and almost mirrors external K^+ concentrations in the BBJD medium. It is also possible that elevated Na^+ concentration in BBJD liquid offset K^+-mediated toxicity, as shown for the cladocerans *Ceriodaphnia dubia* (Richard, 1894) and *Daphnia magna*, explaining the tolerance to high haemolymph K^+ concentration of *H. azteca* (Mount et al., 2016; Morris et al., 2021).

Acidification of the haemolymph in response to elevated external salt levels has been demonstrated in aquatic invertebrates (Truchot, 1981; Henry & Cameron, 1982; Jonusaite et al., 2011). I found that *H. azteca* exposed to a BBJD medium also showed haemolymph acidification. Chloride has been linked to acid-base balance mechanisms through HCO_3^-/Cl^- exchange in aquatic animals (Griffith, 2017). While I did not measure haemolymph Cl^- concentration nor Cl^- concentration in exposure media, I presume Cl^- concentrations are significantly elevated in BBJD and SCW in comparison to freshwater based on measurements of treatment media from previous work (Nowghani et al., 2019). Alterations in haemolymph ammonium (NH_4^+) concentrations were

examined particularly for BBJD liquid-exposed amphipods due to the possibility of ammonia production during the decomposition of organic matter of the BBJD medium by bacteria (Bushaw et al., 1996). Comparatively low concentrations of NH_4^+ were measured for freshwater, SCW, and BBJD along with a similar observation for haemolymph NH_4^+ concentration. Ion concentrations in the treatment media were measured after 2–3 h of aeration and before the addition of amphipods and may not be reflective of NH_4^+ concentrations after 7-d exposure.

NKA and VA Expression Highlights Differences in Osmoregulatory Strategies in SCW and BBJD

Whole-body NKA and VA activities of individuals decreased after 7 d in SCW and BBJD compared to freshwater. Maximal NKA activity is typically observed in other crustaceans upon exposure to dilute media which is consistent with my results (Siebers et al., 1983; Holliday, 1985, 1988; Brooks & Mills, 2006; Chaudhari et al., 2015). Since NKA and VA are the pumps that energise ion uptake in freshwater conditions, the decrease in NKA and VA activities is likely a response to decrease the rate of ion uptake and limit the concentration of salts in body fluids. Based on my measurements in both SCW and BBJD, there is a reversal of the Na^+ gradient across the body wall of *H. azteca* compared to freshwater such that, at least in the short term, the individuals would face diffusive influx of Na^+ (see Table 6-1 and Figure 6-3A). After 7 d of exposure in SCW, the accumulation of Na^+ in the haemolymph resulted in a slight outward gradient for Na^+, whereas individuals continue to face an inward gradient in BBJD. The gills are the primary site of ion uptake and play an important role in counteracting passive salt loss in many freshwater crustaceans (Pequeux, 1995). Ideally, the NKA and VA activities in the gills could be measured; however, I was limited in the number of *H. azteca* individuals available and therefore carried out a qualitative

immunohistochemical assessment of NKA and VA expression in the gills and gastrointestinal tract instead.

Hyalella azteca is similar to other freshwater amphipods in that they possess both the ordinary coxal as well as sternal gills. The ultrastructure of the sternal gills is distinct from the coxal gills of the freshwater amphipod, *Sternomoera yezoensis* (Ueno, 1933) (Kikuchi et al., 1993). The epithelia of sternal gills are much thicker in *H. azteca* and possess deep basolateral infoldings as opposed to the thinner epithelium of the coxal gill with extensive apical folds, but the specific function of each gill type is unknown. I showed that NKA and VA in *H. azteca* are expressed in the basolateral and apical membranes of the coxal gill epithelium, respectively. In the sternal gills, NKA is located in what appears to be extensive basolateral infolds of the epithelium, which is consistent with the description of *S. yezoensis* sternal gills (Kikuchi et al., 1993), whereas VA is seemingly absent in the sternal gills. Based on these immunohistochemical findings, it is likely that the sternal gills of *H. azteca* have a different function compared to the coxal gills. Qualitatively, VA expression is not affected by 7-d exposure to BBJD or SCW. The NKA expression is also unaffected by BBJD; however, NKA expression appears reduced in the gills of SCW exposed amphipods (see Figure 6-5 A–F). Although I measured NKA and VA activity of whole-body amphipods, I present some hypotheses that can be tested with further work at the organ level. Collectively, results suggest that VA activity is reduced with BBJD and SCW exposure through a similar mechanism that, at least in the gills, does not involve altering the expression of the transporter. On the other hand, NKA activity may be reduced by decreasing the expression of NKA in the gills when amphipods are exposed to SCW, the decrease in NKA activity in BBJD cannot be attributed to expression suggesting different mechanisms are used to control NKA activity in SCW and BBJD.

The use of immunohistochemistry on the gastrointestinal tract showed that NKA and VA were located on the basolateral and apical sides of the gut epithelium, respectively. Exposure to SCW did not qualitatively alter NKA and VA expression; however, both NKA and VA expression appeared to increase with exposure to BBJD (Figure 6-6). This apparent increase in expression does not correlate to whole-body NKA and VA activities and future studies should focus on measuring NKA and VA activities at the organ level. The hindgut plays an integral role in ion (e.g., K^+) reabsorption in aquatic invertebrates (Palackal et al., 1984; Jonusaite et al., 2013; Nowghani et al., 2017), and I hypothesise that elevated NKA and VA expression within this organ equips the amphipod with favorable electrochemical gradients along the gut to combat high concentrations of K^+ in the haemolymph when exposed to a BBJD medium. In this manner, enhanced K^+ reabsorption by the hindgut, from the gut lumen to the haemolymph, will diminish the K^+ gradient across the integument and other ion-permeable epithelia to mitigate passive K^+ influx.

Hyalella azteca is tolerant to levels of salinity from SCW (NaCl) and a brine-beet juice de-icer that are higher than levels tolerated by many other freshwater invertebrates. Differences were observed in some parameters that are indicators of ionoregulatory physiology between amphipods exposed to NaCl and BBJD. Individuals in BBJD accumulated K^+ in the haemolymph and had a visually higher expression of NKA and VA in the epithelium of the gastrointestinal tract, whereas those in SCW had an apparent decrease in NKA expression in the gills. Results suggest that *H. azteca* tolerate SCW and BBJD by altering their ionoregulatory physiology in different ways and that this may be rooted in the high K^+ concentration of BBJD. Since K^+ is more toxic than Na^+ to freshwater invertebrates, BBJD may not be a safer alternative to NaCl de-icers.

6.6 References

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6.7 Supplementary Figures

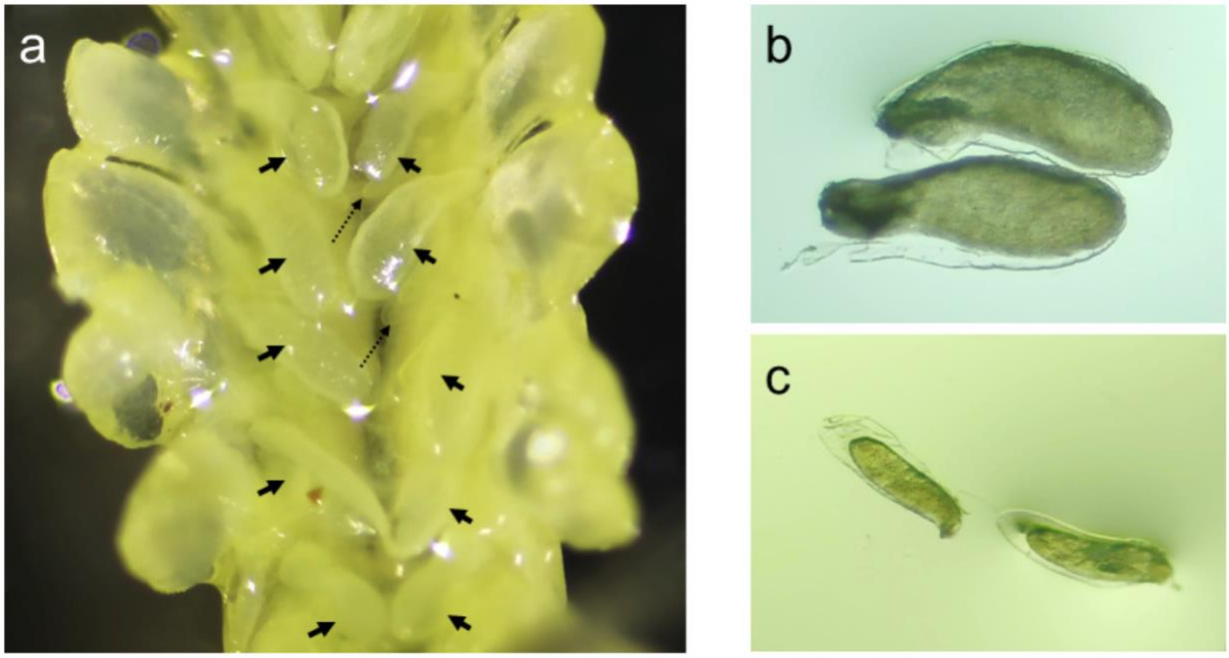

Figure 6-S1. Light microscope image of the coxal and sternal gills of amphipods (*Hyalella azteca*). (a) Formalin-fixed amphipod with solid black arrows illustrating the larger, coxal gills [shown in panel **(b)**] and dashed black arrows indicating the smaller sternal gills [shown in panel **(c)**].

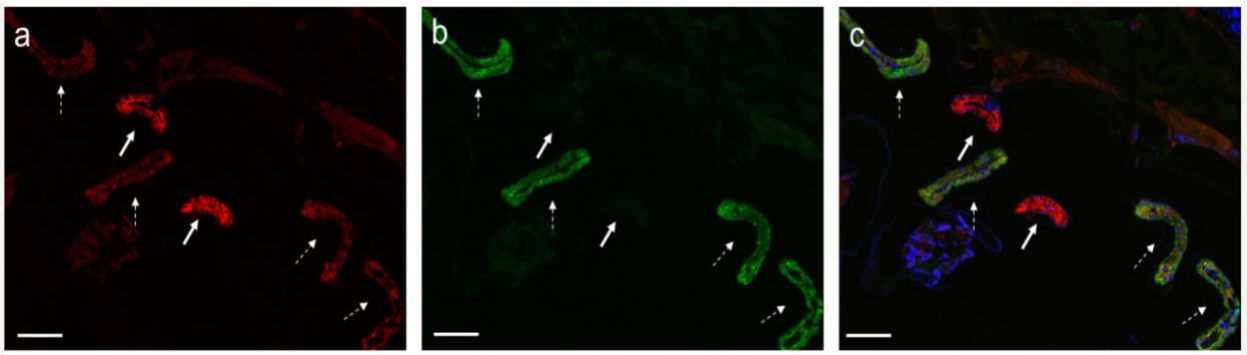

Figure 6-S2. Distinct immunolocalization of Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (VA) in the paired coxal and sternal gills of *Hyalella azteca.* (a) NKA (red), **(b)** VA (green), and **(c)** merge of NKA and VA, and nuclear staining (DAPI, blue) in the sternal gills (solid white arrows) and coxal gills (white dashed arrows) of freshwater amphipods. Scale bars are 100μM.

Chapter Seven

Conclusions and Future Directions

7.1 Summary

At the beginning of this research, the majority of studies on osmoregulatory stress in terrestrial adult *Aedes aegypti* mosquitoes focused on ion transport in important organs such as the Malpighian tubules (MTs) of adult females (Beyenbach et al., 1993; Beyenbach et al., 2010). Although it is well known that aquaporins (AQPs) are important integral membrane proteins that allow for passive diffusion of water through the cell membranes (Agre, 2005), there were gaps in understanding the involvement of AQPs in water excretion from female *Ae. aegypti* post blood meal and in male *Ae. aegypti* post nectar feeding. A deeper understanding has been developed on the localization of AQPs in female and male *Ae. aegypti*, as well as the role of select AQPs in the MTs of both sexes. This research is important because it provides fundamental insight into the function and regulation of aquaporins as well as the osmoregulatory physiology of the disease vector mosquito *Ae. aegypti*, which can lead to development of strategies to reduce disease transmission to humans.

Furthermore, prior to the research described in this dissertation, osmoregulatory stress on aquatic freshwater invertebrates primarily focused on the effects of increased salinity on osmoregulatory physiology (Gillis, 2011; Timpano et al., 2018; Dugan & Arnott, 2022), with little to no understanding on how alternative road de-icers such as brine-beet juice de-icer (BBJD) affected freshwater fauna including larval insects such as *Chironomus riparius* and crustaceans such as *Hyalella azteca*. There is now a better understanding of the effects of BBJD on important freshwater invertebrates and their osmoregulatory physiology, which can provide insight into the development of appropriate eco-friendly road de-icers. The collection of studies presented in this dissertation provides a comprehensive understanding into the role of select AQPs post feeding in adult *Ae. aegypti*, as well as the role of an AQP in relation to road de-icer contamination in larval

C. riparius. Additionally, a further understanding is also concurred in relation to the effects of road de-icers on ion transport and composition of the freshwater amphipod *H. azteca*. This is important because this insight can be utilized to inform policy on the use of de-icing agents. A summary of the major findings and discoveries related to the objectives of this research presented in Chapter 1 of this dissertation are described below.

7.1.1 Characterization of AQPs in Female *Ae. aegypti* and Their Role in Post-Prandial Diuresis

In adult female *Ae. aegypti* MTs, a blood meal was previously shown to increase select AQP gene transcript levels ~3hr post feeding (Drake et al., 2010), however nothing was known about AaAQP protein levels post blood feeding. Using immunohistochemistry, water selective AQPs (AaAQP1 and 2) as well as the entomoglyceroporins (AaAQP4 and 5) were localized to various osmoregulatory organs of adult female *Ae. aegypti* including the midgut (MG), the MTs, and the hindgut (HG), in addition to the energy storing fat body (FB) tissue and the ovaries (OV) pre- and post-blood feeding (Chapter 2; Picinic et al., 2024). A further investigation was completed on AaAQP1 in the female MTs to better understand its role in fluid secretion post blood meal. Tissue sections of isolated MTs revealed apical staining of AaAQP1 that was sustained in non-blood fed, 0.5hr post blood meal, and 24hr post blood meal female mosquitoes (Chapter 2; Picinic et al., 2024). Utilizing immunogold staining as well as *in situ* hybridization, it was determined that AaAQP1 protein and mRNA is mainly localized to the abundant principal cells, with some presence in the smaller and less abundant stellate cells (Chapter 2; Picinic et al., 2024), see Figure 7-1. Interestingly, AaAQP1 protein post blood feeding revealed no change in abundance with a blood meal as determined by western blotting (Chapter 2; Picinic et al., 2024). This finding may

support the hypothesis previously developed which suggested AQPs in insects are likely to be translocated to the membrane during periods of increased need for water transport, which was demonstrated in mammalian kidneys (Campbell et al., 2008). Furthermore, the role of AaAQP1 was investigated in response to select diuretic and anti-diuretic hormones applied to the MTs of female *Ae. aegypti*. Similar to the approach taken in understanding the role of AaAQP1 with blood feeding, immunohistochemistry was used to localize AaAQP1 in female MTs post-stimulation with the diuretic *Drome*DH_{31} or with the diuretic 5HT, as well as each diuretic in combination with the anti-diuretic *Aedae*CAPA-1 (Chapter 3). Although immunolocalization showed brighter staining at the apical membrane with DH_{31}+CAPA relative to the unstimulated (saline) control and DH_{31} alone, AaAQP1 in the membrane protein fraction showed no change in abundance in relation to actin protein abundance (Chapter 3). With 5HT and 5HT+CAPA stimulation, localization and abundance of AaAQP1 remained unchanged in the female MTs (Chapter 3). To gain better insight into the role of AaAQP1 post neurohormone stimulation, AaAQP1 knockdown via dsRNA microinjections was achieved in adult female *Ae. aegypti*. Through functional assessment of knockdown on the MTs, it was found that AaAQP1 knockdown in female MTs significantly reduced basal secretion rates by the tubules, which falls in line with previous studies that have shown a significant reduction in tubule secretion rate post AaAQP5 knockdown in larval *Aedes* mosquitoes (Misyura et al., 2017). Furthermore, it was determined that AaAQP1 is essential in the role of fluid secretion mediated by 5HT, as knockdown of AaAQP1 significantly reduced its diuretic effects (Chapter 3). Interestingly, AaAQP1 appears to be less important in DH_{31}-stimulated fluid secretion, as knockdown did not abolish overall secretion rates, while interestingly, there was a significant reduction in secretion rate due to AaAQP1 knockdown in DH_{31}-stimulated MTs treated with CAPA (Chapter 3). In previous studies on female *Ae. aegypti* MTs, DH_{31} and 5HT

caused significant increases in fluid secretion, which was then reduced to homeostatic levels by the anti-diuretic hormone, *Aedae*CAPA-1 (Sajadi et al., 2018). As a result of this research, the role of AaAQP1 has now been linked to the diuretic action of 5HT on fluid secretion in female *Ae. aegypti* MTs, participates in anti-diuretic hormone (CAPA) regulated MTs following DH_{31} stimulation as well as mediating basal, unstimulated rates of fluid secretion by the tubules.

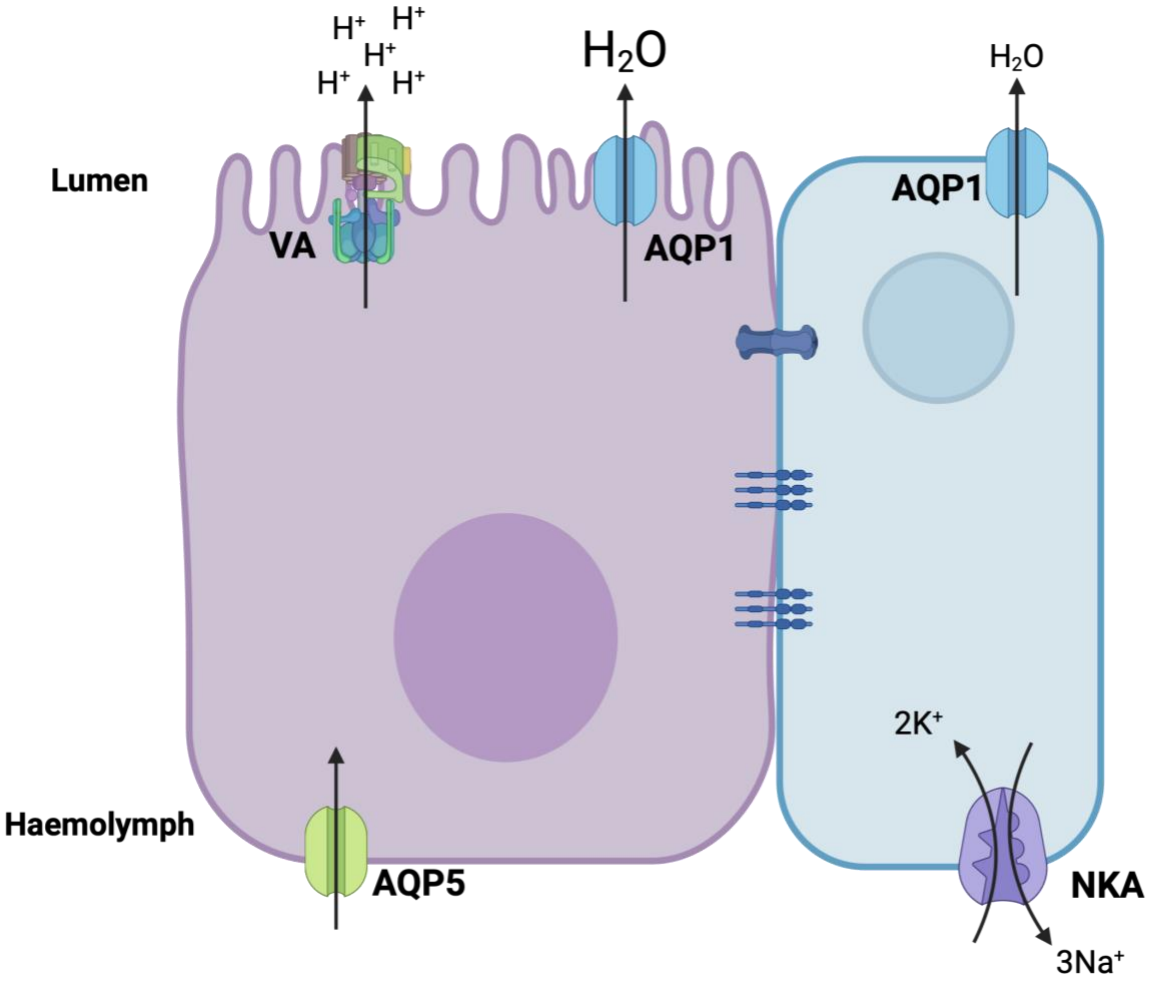

Figure 7-1. Proposed localization of AaAQP1 in female *Aedes aegypti* Malpighian tubule cells. Based on the findings in this thesis (Chapter 2 & 3), AaAQP1 is predominantly localized to the apical membrane of principal cells in adult female *Aedes aegypti* Malpighian tubules (MTs). Additionally, evidence suggests localization of AaAQP1 at the apical membrane of the stellate cells. Based on its localization pattern, AaAQP1 is likely involved in mediating water movement into the tubule lumen, which is believed to brought into the cell by the basolaterally-localized AaAQP5.

7.1.2 Localization of AQPs in Male *Ae. aegypti* and the Role of AaAQP1 in Water Transport Post Nectar Feeding

In adult male *Ae. aegypti*, it was previously suggested that their osmoregulatory response to nectar feeding is similar to that observed in female mosquitoes following a blood meal, nonetheless at a lesser magnitude (Plawner et al., 1991). In order to further investigate this idea, immunohistochemistry was used to localize AaAQP1, 2, 4, 5, and 6 in whole body tissue sections of sugar-fed adult male *Ae. aegypti* (Chapter 4). Interestingly, AaAQP1, 2 and 4 appeared apically localized in the male MTs (Chapter 4), which aligned perfectly with the observations on the same three AQPs in the MTs of females (Chapter 3). Additionally, AaAQP5 staining appeared basolateral in the male MTs, which was also shown in the female MTs (Chapter 3), and is supported by similar findings in larval *Ae. aegypti* MTs (Misyura et al., 2020). An interesting finding of this study was the localization of select AQPs to the epidermis of male *Ae. aegypti*, which has not been localized in any other mosquito to date. Therefore, it is possible that male mosquitoes utilize AQPs in their epidermis to retain moisture when the opportunity presents itself, to mitigate water loss to the dry environment (Beament, 1964), based on their surface area to volume ratio which is greater in comparison to their female counterparts. Additionally, the most prominent AQP in the FB tissue of sugar-fed adult males appeared to be the entomoglyceroporin AaAQP5, which is likely involved in sugar and glycerol transport occurring at the FB following a nectar meal (Arrese & Soulages, 2010; Drake et al., 2015). Further investigation was pursued for AaAQP1 and 4 as representatives for one water-specific AQP and for one entomoglyceroporin, in the MTs of adult male *Ae. aegypti* in response to nectar-feeding as well as starvation (Chapter 4). Localization patterns determined by immunohistochemistry revealed that AaAQP1 and 4 were found uniformly distributed at the apical membrane of the MTs, which implies that AaAQP1 and 4 are important in water and/or

solute secretion in male *Ae. aegypti* MTs (Figure 7-2). Furthermore, western blotting revealed a significant reduction in AaAQP1 and 4 protein abundance in male MTs following 48hr of starvation, relative to sugar-fed male MT protein (Chapter 4). As starvation leads to desiccation stress for the male mosquito, it is probable that a reduction in select AaAQPs in fluid-secreting organs such as the MTs is necessary to avoid excess removal of water. Although unknown in male *Ae. aegypti*, it has been shown in adult *Drosophila melanogaster* that a DRIP AQP channel is expressed in specific neuronal pathways that are linked to response to osmotic stress (Zandawala et al., 2021) and thus raises the possibility that starvation activates the reduction of AaAQP1 and 4 in male MTs as a physiological response to osmotic stress.

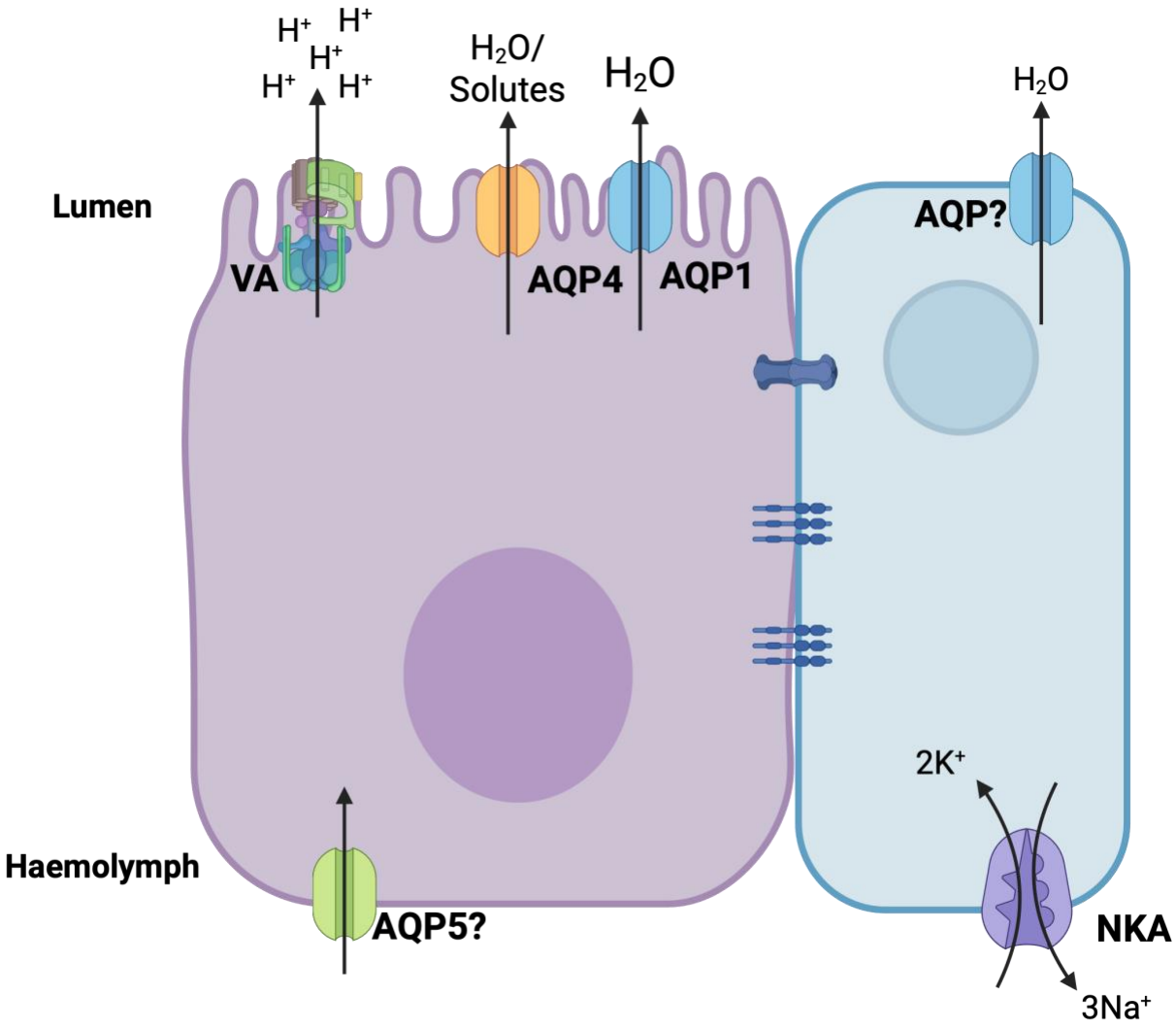

Figure 7-2. Proposed localization of AaAQP1 and 4 in male *Aedes aegypti* Malpighian tubule cells. Based on the findings of this thesis (Chapter 4), the water selective AaAQP1 and the entomoglyceroporin AaAQP4 are likely found at the apical brush border in the principal cells of male *Aedes aegypti* Malpighian tubules (MTs). It is probable that AaAQP1 mediates water movement into the tubule lumen while AaAQP4 may mediate solute movement into the tubule lumen, in response to sugar-feeding in the male mosquito.

7.1.3 Road De-Icers Impact Osmoregulatory Physiology in Select Freshwater Arthropods

Prior to the research presented in this dissertation, there was a limited understanding on the effects of road de-icers, and particularly, the organic alternative brine beet juice de-icer (BBJD) on the success and osmoregulatory physiology of freshwater (FW) fauna. It had been previously demonstrated that BBJD, which is derived from sugar beets and is naturally high in K^+ (Fay and Shi, 2012; Wruss et al., 2015), caused internal K^+ levels to drastically increase in a FW mussel species (Gillis et al., 2021). Relatedly, using an aquatic larval insect *Chironomus dilutus*, the effects of NaCl and BBJD on survival were similar and thus, BBJD should not be considered as an "eco-friendly" alternative to traditional road de-icers without a further understanding of its effects on FW osmoregulation (Nutile and Solan, 2019b). Prior to this research, there was no understanding on how BBJD affects water transport in important FW invertebrate models. The evidence provided in this dissertation highlights a putative water-selective AQP (CrAQP2) in the osmoregulatory organs of the larval midge *Chironomus riparius* (Chapter 5). Interestingly, CrAQP2 appeared to play an important role in water transport from a whole body perspective, in the typical FW environment of the midge, as well as under osmotic stress caused by NaCl-based de-icer and BBJD (Chapter 5). This was determined through CrAQP2 knockdown in midge larvae prior to exposure to FW, NaCl, and BBJD. Following acute exposures to each treatment, a significant reduction in total body water content was seen in each group with knockdown and additionally, CrAQP2 knockdown reduced the ability of midges to survive long term in de-icer contaminated water, relative to FW (Chapter 5). This study is the first of its kind to observe the effects of BBJD on the osmoregulatory physiology of an important FW aquatic insect in combination with the role of an AQP in response to BBJD and, based on the findings of this research, it is evident that organic de-icers such as BBJD have a noticeable impact on the omsoregulatory physiology of FW invertebrates.

Additionally, the effects of BBJD as well as NaCl were observed in a FW amphipod species known as *Hyalella azteca* (Chapter 6; Picinic et al., 2022). Previously, significant decreases in *H. azteca* populations were measured in FW lakes and were attributed to a few factors, including worsening water quality by anthropogenic activities (Anteau et al., 2021). Therefore it is likely that the use of road de-icers is a source of amphipod declines in FW habitats. This research spatially localized the important enzymatic ion transporters including the V-type H^+-ATPase (VA) and the Na^+/K^+-ATPase (NKA) in the ion-transporting gills of *H. azteca* (Chapter 6; Picinic et al., 2022). With de-icer treatment, both NKA and VA activity in whole body samples was reduced in response to NaCl and BBJD relative to FW (Chapter 6; Picinic et al., 2022). It was also found that NaCl exposure resulted in elevated internal Na^+ levels, whereas BBJD exposure resulted in elevated internal K^+ levels (Chapter 6; Picinic et al., 2022). Therefore, corroborating results on *C. riparius* (Chapter 5), the findings of this research also confirm that BBJD is not necessarily a less harmful de-icing agent, as it appears to affect the osmoregulatory physiology of FW arthropods like *H. azteca*, similarly to the effects of NaCl.

7.2 Future Directions

The research presented in this dissertation provides important insights on the effects of osmotic stress caused by diet and or changes in salinity on the osmoregulatory physiology of select terrestrial and freshwater aquatic arthropods. More specifically, this research has localized all of the true AaAQPs in the adult female and male disease vector mosquito *Aedes aegypti*, as well as providing a comprehensive understanding of an important water-selective AQP known as AaAQP1 enriched in the MTs of both sexes (Chapter 2; Chapter 3; Chapter 4). The understanding of how water transport occurs and the role of AQPs in response to feeding in adult *Ae. aegypti* mosquitoes

serves as an important piece to a larger puzzle that is the research behind the prevention of mosquito-borne disease transmission. Furthermore, this research also emphasized the importance of studying the osmoregulatory physiology of FW invertebrates in response to environmental stress posed by anthropogenic activites. The salinization of freshwater has been deemed as a major threat to FW ecosystems, as it has been proven to cause signficant impacts on invertebrate survival and diversity (Meter et al., 2011; Shuler & Relyea, 2018; Nowghani et al., 2019; Hintz et al., 2021; Dugan & Arnott, 2022). As shown in this research, alternative road de-icers (like BBJD) are not necessarily an ecofriendly alternative to NaCl, given the detrimental effects observed on the osmoregulatory physiology and survival of important FW bioindicator species , *H. azteca* and *C. riparius*.

7.2.1 Future Directions for Research with AQPs in *Ae. aegypti*

To gain a better understanding of how AQPs are involved in the post-prandial diuresis of female *Ae. aegypti* mosquitoes, as well as post nectar feeding in the male mosquitoes, research must be conducted on the post-translational regulation of insect AQPs. It has been predicted that insect AQPs are phosphorylated at their C-terminus which typically occurs as a post-translational method of regulation (Campbell et al., 2008). Furthermore, this has been modelled in the mammalian kidney with a water-specific AQP (AQP2) which is phosphorylated and translocated from storage in vesicles and inserted in the membrane of the kidney collecting ducts when further reabsorption of water is required (Campbell et al., 2008). With that being said, it is possible based on the rapid secretion of fluid and ions occurring after the onset of a blood meal (Yang & Piermarini, 2017), that AQPs are likely being shuttled to and from the membrane, as a readily available response to the osmotic stress of a blood meal. Furthermore, in male *Ae. aegypti*, feeding

on a nectar meal results in increased water and sugar content in the body and thus would likely require a similar mechanism for removal of fluids by AQPs by the osmoregulatory organs. Therefore, further research should investigate the translocation of AQPs in membrane-bound vesicles in response to feeding in both sexes in order to gain a comprehensive understanding of the mechanism behind rapid urine excretion in this disease vector mosquito. In addition, the hormone signaling mechanisms mediating diuretic and anti-diuretic action in adult female *Ae. aegypti* has made significant progress in recent years (Sajadi et al., 2018; Sajadi et al., 2023); however, further investigation into the regulation of AQPs by hormone signalling must be conducted. The role of other known diuretic factors such as CRF and kinin, which have been shown to illicit a diuretic response in adult female *Ae. aegypti* MTs (Sajadi et al., 2018), should be explored in relation to AaAQP1 in the tubules to gain a thorough understanding of its role in water secretion post hormonal stimulation. As there are four additional AaAQP genes expressed in adult female MTs (Drake et al., 2010), the influence of diuretic and anti-diuretic neurohormones used in this study should also be examined on these remaining AQPs, as they are likely working together to aid in passive water transport. Furthermore, it would be important to study the functional role of AQPs in other organs such as the fat body (FB), which is important in nutrient storage and in the production of yolk proteins necessary for egg maturation (Chung et al., 2017). Another important organ where AQPs likely play a functional role is the ovaries (OV), which are the main reproductive organ of the female mosquito where proteins and fats are taken from the fat body after a blood meal and used to produce mature oocytes (Pinch et al., 2021). In previous studies, the FB and OV have shown changes in AaAQP gene expression 24-72hr post blood meal, which correlates with the timeline in which vitellogenesis occurs (Price et al., 2011; Drake et al., 2010; Drake et al., 2015). Therefore, it would be interesting to understand the specific role of both water-

specific AQPs and entomoglyceroporins in these reproductive-related organs of female *Ae. aegypti* to gain insight on the transport of water and/or other solutes that occurs after blood feeding in pursuit of producing mature oocytes.

7.2.2 Future Directions for Research on the Effects of Road De-Icer Contamination on Aquatic Invertebrate Osmoregulatory Physiology

Although the research conducted and presented in this dissertation provided a thorough understanding on the effects of road de-icer contamination on larval *C. riparius* and the amphipod *H. azteca*, there is an abundance of other important macroinvertebrates that are also likely being affected by the salinization of freshwater. For example, the effects of salinization by NaCl have been studied on the osmoregulatory function of mayfly nymph gills (Nowghani et al., 2019); however it would be important to also understand the effects of BBJD on mayfly nymph gill function, as they are generally considered to only tolerate small changes in salinity (Chadwick & Feminella, 2001) and thus are a great example of a freshwater invertebrate that may be severely impacted by habitat contamination with BBJD. Furthermore, an important aspect to consider in future research is the additive effects of temperature and salinization on the osmoregulatory physiology of freshwater invertebrates. It has been previously shown that increasing temperature of freshwater habitats with increased Cl^- levels caused by NaCl-contamination causes reduced tolerance and survivability of aquatic invertebrates (Kaushal et al., 2021). Therefore, it would be important to understand the combined effects of BBJD and increasing temperatures, as ambient global temperatures continue to rise and, with that, add another pressure on freshwater aquatic organisms that may result in difficulties in adapting to changes in salinity. The effects of temperature and salinity could be studied on the activity of NKA and VA in ion-transporting

epithelia, as well as the presence and function of AQPs during both temperature and osmotic stress. Additionally, to add to this research and develop a comprehensive understanding of the environmental impact road de-icer contamination has on freshwater fauna, it is important to study the effects of salinization by NaCl and BBJD on reproductive capacity of freshwater aquatic invertebrates such as *C. riparius*. Previously, it has been shown that midges belonging to the *Chironomus* genus are least tolerant to changes in salinity during their pupal life stage, where there are no organs present to regulate the osmotic change and thus result in pupal death prior to adult molting (Berezina, 2003). Therefore, it would be interesting to observe the effects of BBJD on the reproductive capacity as well as egg viability in *C. riparius* larvae, and as a comparative model, in a freshwater crustacean like *H. azteca*.

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