

**SALINITY RESPONSIVE AQUAPORINS IN THE LARVAL MOSQUITO**

***AEDES AEGYPTI***

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## **ABSTRACT**

The anal papillae (AP) of the mosquito larva, *Aedes aegypti* are important sites for ionoregulation because they actively take up ions from a dilute external environment to help maintain appropriate ion levels in the hemolymph. An apparent paradox is that these structures are also permeable to water and because of the syncytial nature of the epithelium, aquaporins (AQPs) are likely to be involved. A previous study has revealed expression of AQP homologs in the anal papillae of freshwater reared larvae. In the present study, transcript expression levels of six AQP homologs in the AP were examined in larvae reared in ion poor water and 7.5 g L<sup>-1</sup> Instant Ocean® salts (~ 30% seawater). The mRNA expression of four of these AQP homologs (AaAQP2, AaAQP3a, AaAQP3b, AaAQP4) was salinity responsive suggesting the importance of these homologs in salinity acclimation. In addition, western blot analysis of AaAQP3a and AaAQP3b confirmed the salinity responsive nature of these two proteins. Immunohistochemistry also confirmed the presence of AaAQP3a and AaAQP3b in both the apical and basal membranes of the AP.

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## LIST OF ABBREVIATIONS

**Note:** only abbreviations used most frequently in this thesis have been listed here.

AMG	anterior midgut
PMG	posterior midgut
AP	Anal Papillae
AQP	Aquaporin
FW	Fresh water
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gastric caeca
IPW	Ion-poor water
HG	Hindgut
MG	Midgut
MT	Malpighian tubules
PMG	Posterior midgut
SW	Sea water
NKA	Na <sup>+</sup> / K <sup>+</sup> ATPase
VA	Vacuolar-type H <sup>+</sup> -ATPase
WB	Whole body

## **STATEMENT OF CONTRIBUTION**

This thesis was written by Hina Akhter with guidance from Dr. Andrew Donini. Training of RNA extraction was provided by Sima Jonusaite. Training for technical aspects of qPCR was provided by Sima Jonusaite and Phuong Bui. Training for qPCR data collection and analysis was provided by Phuong Bui. qPCR data was gathered and analysed in collaboration with Phuong Bui.

Training for western blot/densitometry, tissue embedding and sectioning was provided by Dr. Helen Chasiotis. Tissue dehydration and paraffin embedding in Figure 3.6 and Figure 3.7 was done by Lidiya Misyura and tissue sectioning and staining in Figure 3.6 and Figure 3.7 was done by Hina Akhter. Training for Olympus IX71 inverted fluorescent microscope and image acquisition was provided by Dr. Andrew Donini.

# **1 INTRODUCTION**

## **1.1 Osmoregulation**

Osmoregulation is a process whereby an animal maintains water and ion levels within a strict range. It allows animals to inhabit many ecological niches and survive under different physiological stressors. Aquatic animals can generally be divided into two types based on how they regulate their internal fluid composition: osmoconformers and osmoregulators. Osmoconformers are able to alter their internal osmolarity to match their external habitat (Bradley, 2008; Bradley, 2009). In contrast, osmoregulators maintain their internal osmolarity within a narrow physiological range as the external ion levels vary (Bradley, 2008). Some animals may osmoregulate within a certain range and osmoconform beyond that range, hence both osmoregulators and osmoconformers cannot be considered strict osmoregulators or strict osmoconformers (Bradley, 2009).

Ability to successfully osmoregulate is an important physiological mechanism for survival of aquatic animals including mosquito larvae and disrupting the osmoregulatory capacity of mosquito larvae may serve as an important strategy for population control. Therefore, it is necessary to examine the molecular and physiological mechanisms underlying osmoregulation that contribute to their survival in aquatic habitats.

### **1.1.1 Osmoregulation in Mosquito Larvae**

The larvae of mosquitoes (family: Culicidae) can be found in a variety of aquatic habitats such as streams, pools, marshes and any other synthetic/natural stagnant water reservoir (Clements et al., 1992). In Freshwater (FW) habitats, larvae risk gaining a lot of excess water and losing ions to their external habitat whereas in seawater (SW), larvae risk losing water and gaining a lot of excess ions from their external habitat (Bradley, 1987). The majority of the mosquito larvae are

freshwater species with only 5 % considered to be saline tolerant (Clements et al., 1992). Mosquito larvae can be divided into three categories depending on the range of salinities they can survive in. These include: obligate freshwater (FW) osmoregulators, euryhaline osmoregulators and euryhaline osmoconformers. Both freshwater species (obligate FW osmoregulators) and saline tolerant species (euryhaline osmoregulators) can osmoregulate the ionic composition of their hemolymph within a particular range, however saline-tolerant species can withstand a much larger range of external salinity (Bradley, 2008).

Larval species that show decrease survivorship once the external salinity is raised above the hemolymph ion concentration fall under the category of obligate FW osmoregulators (Bradley & Phillips, 1975). They have a limited salinity range under which they survive (Patrick & Bradley, 2000a). The hemolymph osmolarity of FW larvae (*Aedes aegypti*, *Culex pipiens*) is kept around ~250mOsm (Edwards, 1982c). *Aedes aegypti* larva can ionoregulate and survive in external media containing only 0-30 % sea water (Edwards, 1982b). Within this range, amino acids also increase in the hemolymph as the external salinity is raised to equalize osmotic gradient and prevent water loss from the larva (Edwards, 1982c). *Culex quinquefasciatus* are unable to synthesize amino acids to raise their hemolymph osmolarity, one of the many strategies allowing survival at higher salinities, which also restricts these to FW (Patrick & Bradley, 2000a).

Euryhaline osmoregulators are saline tolerant species that can survive in higher external salinity than FW obligates (Bradley, 2008). In dilute conditions, larvae osmoregulate by producing hypo-osmotic urine to their hemolymph (Ramsay 1950). In increased salinity levels, they produce hyperosmotic urine (Bradley, 2008). Due to their osmoregulatory capability, saline tolerant larvae of *Aedes sollicitans* and *Aedes campestris* can survive in 200 % SW (Bradley, 2008). *Anopheles nerus* can live in environmental salinity of about 50 % to 75 % and also survive

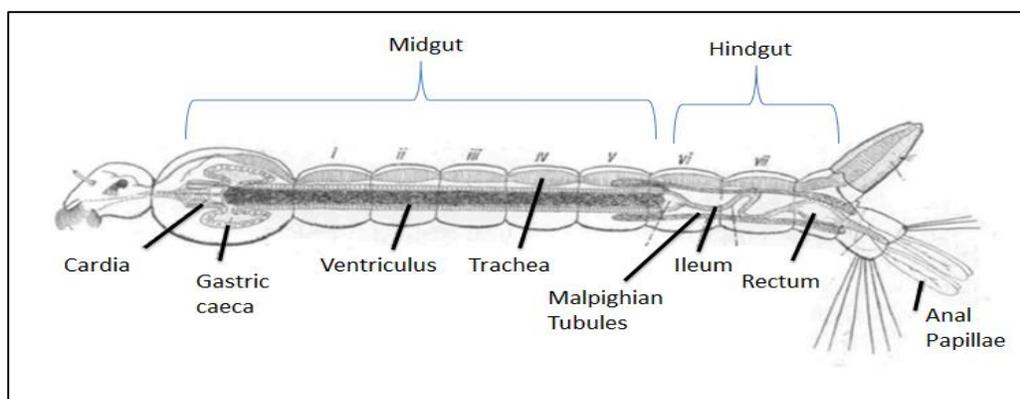
in 100 % SW (Bradley, 1987; Grueber & Bradley, 1994). *Aedes (Ochlerotatus) taeniorhynchus* can osmoregulate in salinities ranging from 20 % to 100 % SW (Grueber & Bradley, 1994). Saline tolerant species also increase their drinking rate to prevent dehydration except for *O. taeniorhynchus* which do not alter their drinking rate except for metabolic demand and/or in relation to their surface area (Bradley and Phillips, 1977; Bradley, 2008). Water absorption by the gut also increases in higher salinity (Wigglesworth, 1933b). Drinking excess water in SW also brings in added ions which are then actively secreted out by specialized rectal epithelia. Rectal morphology of euryhaline osmoregulators is unique and allows saline tolerant species to produce hyperosmotic urine in higher salinities. Saline tolerant mosquitoes (*Aedes detritus*, *Aedes campestris*, *Aedes taeniorhynchus*, *Aedes dorsalis*) have a two part rectum, in which the posterior part is involved in producing a hyperosmotic urine and actively secreting excess ions from the hemolymph for excretion (Grueber and Bradley, 1994; Bradley, 1987)

Euryhaline osmoconforming larvae maintain the osmotic concentration of the hemolymph similar to their external environment. In saline conditions, larvae prevent dehydration by synthesizing extra osmolytes such as amino acids (proline and serine) or trehalose that raise the internal osmotic pressure of their tissues and hemolymph (Patrick & Bradley, 2000a,b). Extra organic osmolytes act as a buffer against water loss and allow this group of species to survive in much higher salinities (Patrick & Bradley, 2000b). Most of the mosquito larvae of genera *Culex* and *Culiseta* can osmoconform in external media of greater than 30 % seawater (Bradley, 2008). *Culiseta inornata* can match their hemolymph ion concentration up to 1000mOsm of external salinity (Garrett & Bradley, 1987). *Culex tarsalis* increase hemolymph proline (50 x more than FW) and serine at 64 % SW to maintain osmotic pressure to prevent dehydration (Patrick & Bradley, 2000b). Unlike the morphological changes seen between the rectum of FW obligates and

euryhaline osmoregulators, there is generally no distinction in the morphology of FW obligates and euryhaline osmoconformers, showing that their osmoregulatory capacity solely depends on the accumulation of extra osmolytes (Patrick & Bradley, 2000a).

### **1.1.2 Osmoregulation in *Aedes aegypti***

*Aedes aegypti* larvae are FW obligates, which means that they must cope with the physiological stress of gaining excess water from their external habitat to prevent hemolymph dilution (Albers & Bradley, 2011). To maintain ionic homeostasis, the larvae are equipped with osmoregulatory tissues including the midgut (MG) which includes the gastric caeca (GC), Malpighian tubules (MTs), hindgut (HG) (ileum and rectum) and the anal papillae (AP) (Fig 1.1). These tissues all function together to maintain water and ion balance within the larvae (Clements, 1992). The water absorbed from the gut (through drinking) and the anal papillae moves into the MTs as a result of the osmotic gradient established by the active movement of ions ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ) from the hemolymph into the MTs to make the primary urine (Bradley, 1987; Beyenbach, 2003). This fluid then moves through the hindgut, which includes the ileum and rectum. The rectal epithelium reabsorbs the ions, producing dilute urine (Bradley, 1987). The morphology and physiological function of each of these organs is given below.



**Figure 1.1: Diagram of *A. aegypti* larva showing regions of the gut.** Midgut is divided into the cardia, gastric caeca and the ventriculus. The ventriculus is divided into anterior and posterior regions and is referred to in this thesis as the anterior midgut and posterior midgut. Malpighian tubules are attached at the region between midgut and ileum. Hindgut consists of the ileum and the rectum. The anal papillae are external tissues facing the external aquatic medium. The major tracheal branch that supplies oxygen to the tissues is also shown (Adapted from Wigglesworth, 1933a; JEB, vol 10, issue 1, p 17)

### 1.1.3 Midgut

The midgut (MG) of the larval mosquito functions in the digestion and absorption of nutrients, maintains ionic homeostasis, and regulates pH of MG lumen (Clark et al., 2005). MG content is generally isosmotic to the larval hemolymph (Ramsay, 1950). Water is passively transported across the MG epithelium by the gradient established by the uptake of nutrients and ions (Bradley, 1987). MG is divided into the cardia, gastric caeca (GC) and the ventriculus (tube-like structure or the “stomach”) (Fig 1.1). The cardia is involved in the secretion of the peritrophic membrane that extends into the midgut and partitions the gut into two distinct compartments: the endoperitrophic space and the ectoperitrophic space. The endoperitrophic space holds the ingested contents of the gut while the ectoperitrophic space is the region between the gut epithelium and the peritrophic membrane (Clements, 1992). The peritrophic membrane functions to protect the gut epithelium from physical abrasion, pathogens and the digestive enzymes (Hegedus et al, 2009). The GC are eight blind ended globular folds of the gut attached to the anterior portion of the MG. The GC are involved in absorption of nutrients and movement of ions and water (Clements, 1992;

Wigglesworth, 1933a). The two main types of cells found in the GC include the resorbing/secretory cells which form the anterior two thirds of each caecum and ion-transporting cells which form the posterior part of each caecum (Volkman & Peters, 1989a). GC also play a role in osmoregulation as GC of *Aedes argenteus* larva reared in seawater (SW) are able to absorb more fluid than in freshwater (FW) (Wigglesworth, 1933b). Furthermore, more chloride ions are present in GC of SW reared *Aedes togoi* larvae suggesting an ion transporting role of GC (Volkman & Peters, 1989b).

The ventriculus or the 'stomach' can be divided into two separate portions: the anterior ventriculus (AV) or the posterior ventriculus (PV) (Clements, 1992). These are also referred to as the anterior midgut (AMG) and the posterior midgut (PMG). The AMG and the PMG vary in both structure and function. Digestion of food mainly occurs in the anterior midgut (AMG). For this, the *A. aegypti* AMG has a very high pH (>10) compared to the PMG and is involved in dissociation of proteins from indigestible protein-tannin complexes (Linser et al., 2009). In addition, AMG with its high pH plays a role in protecting the larva from pathogens that may be ingested with food (Stiles & Paschke, 1980). In contrast, the PMG is involved in absorbing nutrients from the digested food (Clements, 1992). The PMG also represents an area of higher ion movement and metabolic activity because of the presence of a large number of mitochondria in the epithelial cells as compared to the AMG (Clark et al., 2005). The PMG also plays a role in lowering the pH of lumen and the exact processes involved in lowering the pH are currently unknown (Moffett et al., 2012).

#### **1.1.4 Malpighian Tubules (MTs)**

MTs in insects play a role in excretion of metabolic waste and maintain hemolymph ion and water balance (Beyenbach & Piermarini, 2011; Beyenbach et al., 2010). In the larval *A.*

*aegypti*, the 5 structurally and functionally similar MTs, are attached at the junction between the PMG and the ileum at its posterior end while the distal MTs are blind ended (Fig1.1) (Clements, 1992). The MTs are a single cell thick and are composed of two types of cells: the principal cells and stellate cells. Principal cells are the large cuboidal cells that make up the majority of the tubule cells (80%) while the star-shaped stellate cells are small and make up only 20 % of the tubule cells (Beyenbach et al., 2010). The stellate cells are found in between the principal cells where the extensions of the cells spread and make a large contact with the principal cell epithelium (Beyenbach et al., 2010). Water moves into the MT lumen as a result of the osmotic gradient produced by the active secretion of ions (NaCl and KCl) energized by vacuolar-type H<sup>+</sup> V-ATPase (VA) localized to the apical brush border of the principal cells (Spring et al., 2009; Beyenbach et al., 2010; Weng et al., 2003). Principal cells secrete K<sup>+</sup> and Na<sup>+</sup> ions while the Cl<sup>-</sup> moves into the lumen transcellularly by the stellate cells as well as via paracellular pathway (Beyenbach, 2003; Beyenbach et al., 2010; Lu et al., 2011). In high salinity, larval MTs tend to alter their ion transporting mechanism and secrete more Na<sup>+</sup> ions than K<sup>+</sup> ions (Donini et al., 2006). In larval MTs, the water coming in from the gut through drinking diffuses into the hemolymph and excess volume is removed by the passive transfer of water following active ion secretion in the lumen (Edwards, 1982b; Aly and Dadd, 1989). In adult female *A. aegypti*, MTs also play a role in removing excess salt and water load imbibed with the blood meal (Beyenbach, 2003). Fluid secretion at the MTs is under the control of the nervous system and many diuretic and antidiuretic factors such as corticotropin-releasing factor (CRF)-related peptides, calcitonin (CT)-like peptides and the insect kinins play a role in diuresis (Coast et al., 2002; Coast et al., 2009)

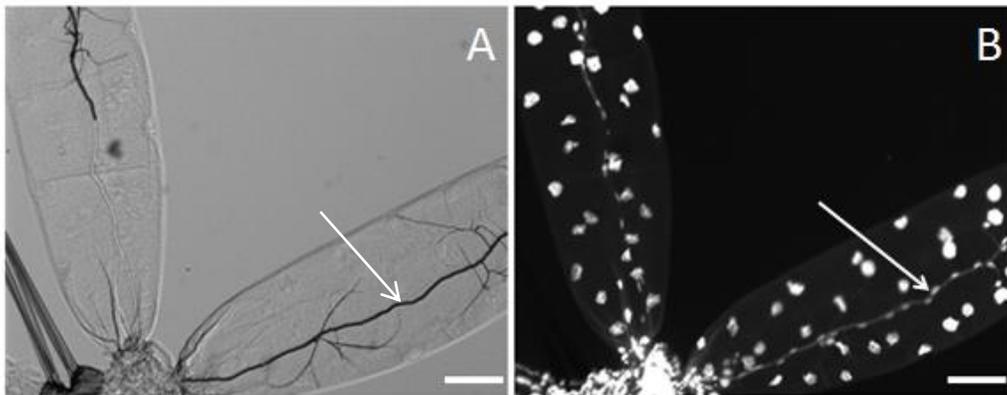
### **1.1.5 Hindgut**

The hindgut is divided into the ileum and the rectum (Fig 1.1). The ileum generally functions to transport the fluids from MG and the MTs to the rectum (Clements, 1992). The rectum is involved in removing excess water or secreting excess ions from the hemolymph to the lumen for excretion. The rectum of FW larval species differs from the saline-tolerant larva. The rectum of saline tolerant mosquitoes such as *Aedes campestris* and *Aedes taeniorhynchus* can be divided into two parts (anterior and posterior) while obligate FW species such as *A. aegypti* have a single rectal segment (Bradley, 2008; Grueber & Bradley, 1994; Meredith and Philips, 1973). In FW species, the rectum is the site of ion uptake ( $K^+$ ,  $Cl^-$ ) and in the saline tolerant forms, the posterior segment of the rectum functions as a salt gland (Bradley, 2008). Cells of the posterior rectum of *Aedes campestris* are much thicker than the anterior part and have highly folded apical membrane with a lot of mitochondria, which characterizes a typical ion-transporting epithelia (Meredith and Philips, 1973). These cells in the posterior rectal segment secrete excess ions from the hemolymph into the lumen to produce hyper-osmotic urine (Bradley, 2008)

### **1.1.6 Anal Papillae**

Anal Papillae (AP) are four sac-like structures that are in direct contact with the external habitat (Fig 1.1). The lumen of AP are filled with hemolymph which is continuous with the hemocoel (Edwards and Harrison, 1983). AP are composed of a single cell thick (simple) syncytial epithelium which is covered by a thin and permeable cuticle (Wigglesworth, 1933a). The AP lumen is also tracheated throughout for the delivery of oxygen (Fig 1.2) (Edwards and Harrison, 1983; Wigglesworth, 1983). Ultrastructure of AP shows highly folded apical and basal membranes with associated mitochondria which is a distinguishing feature of an ion transporting membrane (Clements, 1992; Edwards and Harrison, 1983). The AP can take up NaCl from their external habitat as well as secrete  $H^+$  and ammonia to their external habitat (Donini and O'Donnell,

2005; Stobbart, 1971). The current model suggests that sodium transport is linked to hyperpolarization of the cell by an apically located VA which pumps out  $H^+$  ions provided by the cytosolic carbonic anhydrase (CA) (Del Duca et al., 2011).  $Na^+$  is taken up by apically located sodium channels (currently unidentified) and then enters the hemolymph via basally localized  $Na^+/K^+$  ATPase (NKA) (Patrick et al., 2006; Del Duca et al., 2011).



**Figure 1.2: Trachea in the Anal Papillae.** Images of the Anal papillae (AP) showing a central tracheal branch that further divides into many smaller branches. A) shows the transmitted light image of the AP and B) shows the AP stained with DAPI. The arrow in A points to the central tracheolar branch running through the AP lumen. The arrow in B points to the Tracheolar cells (TC) associated with the central tracheal branch. Scale bar= 200  $\mu$ m.

The role of AP in ionoregulation with fluctuating salinities has been supported by many studies. Removal of AP leads to the loss of ionic homeostasis in FW mosquito larva (Wigglesworth, 1933a). Previous experiments have shown that AP reduce  $NaCl$  uptake when transferred from FW to 30 % SW (Donini et al., 2007). The surface area of AP also enlarges in *A. aegypti* larvae exposed to ion- poor water in order to scavenge for ions in dilute external media (Wigglesworth, 1933a; Edwards and Harrison, 1983; Stobbart, 1971; Wigglesworth, 1938). AP of larval *Anopheles merus* is also observed to be twice as long in FW compared to SW reared larvae (Coetzee & Le Sueur, 1988). Furthermore, membrane infoldings and associated mitochondria decrease in AP of larvae acclimated to SW suggesting reduced ionic activity (Wigglesworth, 1933; Sohal and Copeland, 1996; Edwards and Harrison, 1983; Edwards, 1982a).

The syncytial epithelium of the AP is also permeable to water. In dilute conditions, water at the AP is absorbed and then secreted out by the MTs and this process is dependent on osmotic flow (Wigglesworth, 1933a). Water permeability in the AP suggests a conflicting functional role of the AP in absorbing ions from the environment to maintain hemolymph ionic concentration (Wigglesworth, 1983; Wigglesworth, 1933a). The evidence for water permeability at the AP was initially supported by the swelling of the posterior abdomen when larvae were ligated at the 5<sup>th</sup> and 6<sup>th</sup> abdominal segment, and if the AP were removed before ligation, this swelling disappeared (Wigglesworth, 1933a; Stobbart, 1971). The exact mechanism by which water flows into the AP lumen is not known however, transcellular transport by aquaporins (water-channels) has been suggested (Marusalin et al., 2012). Six aquaporin (AQP) homologs (AaAQP1a, AaAQP1b, AaAQP2, AaAQP3a, AaAQP3b, AaAQP4) have been found in the *A. aegypti* genome (Drake et al., 2010). Alternate names for these AQP homologs have been given in Table 1.

**Table 1:** Alternate names for *A. aegypti* AQP homologs. (Taken from Marusalin et al. 2012, J comp Physiol B, Vol 182, Issue 8)

<b>A. Aegypti AQPs</b>	<b>Alternate names</b>
AaAQP1a	AeaAQP (Pietrantonio et al., 2000) AeaDRIP (Campbell et al., 2008) Aedae1a (Kambara et al., 2009) AaAQP1 (Drake et al., 2010; Herraiz et al., 2011) AaAQP1a (Goto et al., 2011)
AaAQP1b	AeaPRIP (Campbell et al., 2008) Aedae1b (Kambara et al., 2009) AaAQP2 (Drake et al., 2010; Herraiz et al., 2011) AaAQP1b (Goto et al., 2011)
AaAQP2	AeaBIB (Campbell et al., 2008) Aedae2 (Kambara et al., 2009) AaAQP3 (Drake et al., 2010; Herraiz et al., 2011) AaAQP-transporter (Goto et al., 2011)
AaAQP3a	Aedae3a (Kambara et al., 2009) AaAQP4 (Drake et al., 2010; Herraiz et al., 2011) AaAQP3a (Goto et al., 2011)
AaAQP3b	Aedae3b (Kambara et al., 2009) AaAQP5 (Drake et al., 2010; Herraiz et al., 2011) AaAQP3b (Goto et al. 2011)
AaAQP4	Aedae4 (Kambara et al., 2009) AaAQP6 (Drake et al., 2010) AaAQP4 (Goto et al., 2011)

All six of these homologs are present in the AP of FW reared larva, however, transcript expression of AaAQP1b and AaAQP4 is greater in the AP relative to other homologs (Marusalin et al., 2012). How AQPs in the AP respond to changes in external salinity is the focus of this study.

## 1.2 Aquaporins (AQPs)

Aquaporins (AQPs) are members of a large family of major intrinsic proteins (MIP) and present ubiquitously in all organisms. They are small hydrophobic integral membrane proteins (~30 kDa, size of a monomer) that confer bidirectional water permeability in biological membranes

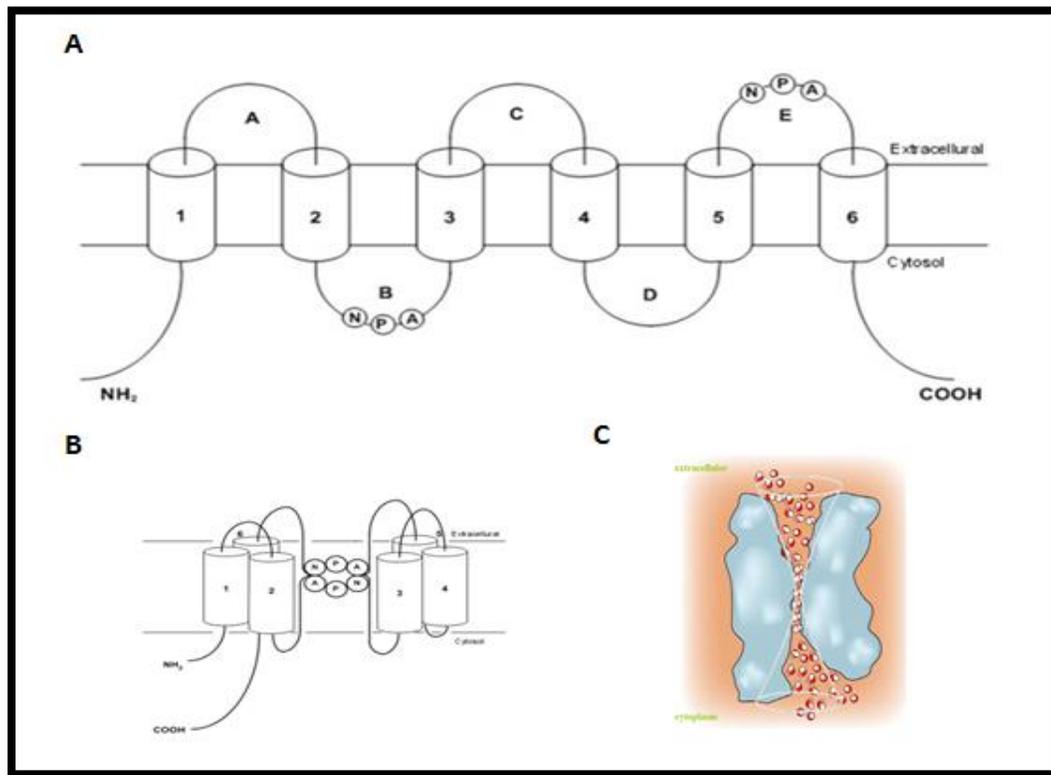
in response to changes in osmotic gradients (Murata et al., 2000, Campbell et al., 2008; Meinild et al., 1998). The water transport function of AQPs can be reversibly inhibited by mercurial compounds (Preston et al., 1992). AQPs do not allow the permeation of protons hence water is able to diffuse down its osmotic gradient without disrupting the electrochemical potentials of cells (Murata et al., 2000; Saparov et al., 2005, de Groot & Grubmuller, 2001; Van Hoek & Verkman, 1992). They play a role in a wide variety of physiological processes and often display a cell or tissue specific expression pattern (Magni et al., 2005; Yasui, 2004, Ishibashi et al., 2009). Currently, 13 different AQP homologs (named, AQP0-AQP12) have been identified in mammals (Ishibashi et al., 2009). They are distributed in almost all organs and tissues and play vital roles in human physiology (Verkman, 2008; Verkman, 2011; Krane & Kishore, 2003; Jeyaseelan et al., 2006; Gomes et al., 2009). AQPs were first discovered accidentally by Peter Agre in 1992 in human red blood cell (rbc) membranes when an unknown protein of 28 kDa was purified while trying to raise antibodies against a purified Rh peptide. It was originally named the channel-like integral membrane protein of 28 kDa (CHIP28) but after identifying its role in water transport, it was renamed aquaporin-1 (AQP1) (Preston & Agre, 1991; Preston et al., 1992). Before this discovery, water was thought to enter the cells across the lipid membranes; however, this could not explain the high rate of water flux in some cells such as the amphibian skin epithelia and the human rbc (Gomes et al., 2009). In addition, water permeability of human rbcs was known to be reversibly inhibited by mercurial compounds which suggested the water entry in the cell must be mediated by a then unknown protein present on the cell membrane which was later identified as an AQP (Macey, 1984; Benga, 2003). The newly found protein also showed high sequence similarity to the MIP protein of the bovine lens fibre and had a similar function, therefore, this protein was also re-named aquaporin-0 (AQP0) (Gorin et al., 1984; Verkman, 2011).

AQPs are generally grouped into two types: orthodox aquaporins (AQPs), which specifically only allow the permeation of water and aquaglyceroporins (GLP), which in addition to water, allow the permeation of glycerol and other small solutes (Zardoya et al., 2005; Campbell et al., 2008). Mammalian AQPs 1, 2, 4, 5 and 8 are water-specific whereas AQPs 3, 7, 9 and 10 are aquaglyceroporins (Verkman, 2008). Mammalian AQPs 11 and 12 are still unclassified and are only considered in the MIP family due to high sequence homology (Verkman, 2008). In some literature, AQPs have also been characterized into additional categories. A category of AQPs that can transport ammonia are referred to as aquaammoniaporins (Jahn et al., 2004; Litman et al., 2009).

### **1.2.1 Structure and Transport properties of AQPs**

In cell membranes, AQPs are often arranged in tetramers with each monomer acting as a distinct functional water channel (Tomkowiak & Pienkowska, 2010). Each monomer consists of six membrane spanning tilted alpha helical domains (labelled 1-6) connected by five loops (labelled A-E) (Fig 1.3a, 1.3b) (Tomkowiak & Pienkowska, 2010; Campbell et al., 2008; Wu & Beitz, 2007). Loops A, C, and, E are extracellular and loops B and D are intracellular (Fig 1.3a, 1.3b). The amino and carboxy termini are both cytoplasmic and share a 20 % sequence similarity (Preston et al., 1994; Verkman & Mitra, 2000; Pao et al., 1991; Wistow et al., 1991; Reizer et al., 1993). The symmetrical loops B and E both contain the highly conserved signature region of AQPs known as the asparagine-proline-alanine (NPA) motif. These two loops fold back into the cell membrane bringing the conserved NPA regions together to form a water transporting pore and give rise to what is known as the 'hour glass' model (Figure 1.3b, 1.3c). This hour glass model consists of wide external cytoplasmic and extracellular openings with a narrow central constriction formed by the interaction of the two NPA motifs (Tomkowiak & Pienkowska, 2010; Campbell et

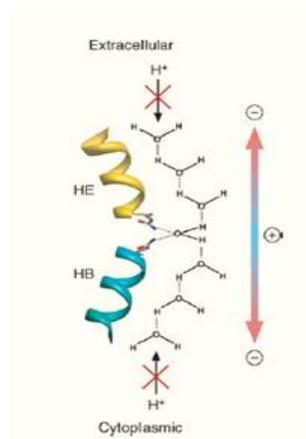
al., 2008; Wu & Beitz, 2007). The constricted pore acts as a filter that selectively allows the passage of water molecules while preventing the leakage of protons. Mutations in and around this region are shown to alter the function of AQPs (Bai et al., 1996; Jung et al., 1994; Kuwahara et al., 1997; Shi & Verkman, 1996).



**Figure 1.3:** Structure of an Aquaporin (AQP) monomer. A) Shows the six membrane spanning domains of the AQP water channel (labelled 1-6) with 5 connecting loops (A-E). The N-termini and C-termini are both cytoplasmic. B) The two highly conserved NPA motifs from loops B and E come together to form a central pore, and give rise to the 'hourglass model' configuration of AQP seen in C. (A and B, taken from Tomkowiak & Pienkowska., 2010, *Advances in cell biology*, vol 1, issue 1, p 2-3; C taken from Cohen (2012), *Entomol. Omithol. Herpetol.*, vol SI, issue 1, p 2162)

A second conserved site found in the extracellular region of the pore is known as the aromatic/arginine (ar/R) constriction which also functions as a selectivity filter (Sui et al., 2001; Beitz et al., 2006; Campbell et al., 2008). This site consists of conserved aromatic and arginine residues (Sui et al., 2001). This site is narrower than the NPA region and specific amino acid residues in this region determine the selectivity of the AQPs. In water specific channels, the

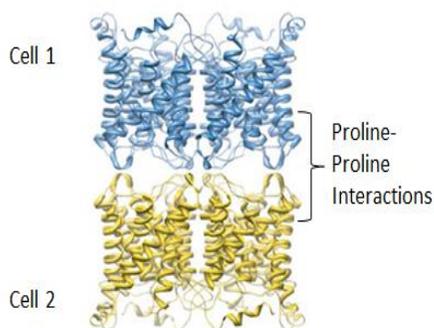
diameter of this constriction is 2.8Å while in GLPs, the diameter of this region is ~ 3.4 Å to permit the entry of more larger and hydrophobic molecules (deGroot & Grubmuller, 2001; Wang and Tajkhorshid, 2007; Hub and deGroot, 2008). In human AQP1, this constriction is formed by four residues: AQP1, Phe56, His180, Cys189, and Arg195 while in a GLP, GlpF from *E.coli*, the residues are Trp46, Gly191, Phe200 and Arg205 (Campbell et al., 2008; Sui et al., 2001; King et al., 2004). A histidine residue in this region is usually a characteristic of water specific AQPs. The cysteine residue provides a sulfhydryl group which extends into the pore and forms a binding site for mercurial compounds, which reversibly inhibit water transport function (Preston et al., 1993; Zhang et al., 1993). The lack of a histidine and cysteine in GLPs makes them hydrophobic and less efficient in transporting water and more suitable for the transport of glycerol, and other small solutes like urea and small polyols (Stroud et al., 2005). Arginine plays a role in proton exclusion as mutational studies have shown that replacing arginine with valine and hence removing the positive charge on arginine results in proton leakage across the channel (Beitz et al., 2006; Chen et al., 2003). Proton exclusion is very important for preserving electrochemical gradient and pH of the cell under homeostasis (Van Hoek & Verkman, 1992). It occurs due to steric hindrance, electrostatic interactions and hydrogen bonding. The arrangement of water molecules while permeating the AQP pore plays a role in proton exclusion (Fig 1.4) (Gonen & Walz, 2006). Water molecule entering the pore reorients itself so that its oxygen atom forms bonds with the hydrogen bonds of the two Asparagine (Asp) residues of the NPA motifs in the middle of the pore. In turn, the two hydrogen atoms are oriented perpendicular to the pore, breaking the continuous line of hydrogen bonds and preventing proton permeation (Gonen & Walz, 2006).



**Figure 1.4: Proton exclusion in the Aquaporin (AQP) pore.** The asparagine residues of the two NPA motifs from loop E and Loop B interact with the incoming water molecule so that two hydrogen atoms are oriented perpendicular to the pore. It allows other water molecules in the opposite sides of the pore to be oriented in opposite directions and the resultant electrostatic field prevents any protons from entering the pore (Taken from Gonen & Walz 2006, Quarterly reviews of biophysics, vol 39, issue 4, p 382).

AQP monomers are arranged in tetramer parallel to one another and form a fifth pore in the center of the tetramer (Gonen et al., 2006; Gomes et al., 2009). The exact role of this arrangement and the function of the central pore is often left unexplored. However, mammalian AQP1 is shown to allow the transport of ions when expressed in planar lipid bilayers and this ion permeability is suggested to occur via the central pore of the AQP tetramer and regulated by nucleotide (cGMP) gated pathway (Saparov et al., 2001; Yool et al., 2002; Kruse et al., 2006; Anthony et al., 2000). Mutation preventing the cGMP induction on the C- termini prevents ion transport in AQP1 without altering water permeation (Saparov et al., 2001). An AQP known as the big brain (BIB) protein found in *Drosophila* also functions as a voltage-insensitive cation channel through the central pore of AQP tetramer when expressed in *Xenopus* oocytes and it is suggested to play a role in membrane depolarization during neuronal signalling (Yanochko & Yool, 2002; Yool, 2007; Spring et al., 2009). Ammonia is also suggested to be transported across the central pore of Aq1aa tetramer in the gills of FW climbing perch, *Anabrus testudineus* (Ip et al., 2013).

In addition, AQPs can also be arranged in orthogonal arrays (OA)s, as in the human AQP4 and this arrangement is thought to support a rapid bulk flow of water in some tissues (Yang et al., 1996). Furthermore, lens AQP-0 forms junctions in the mammalian eye, in which the AQP tetramers interact with opposing tetramers via proline residues of extracellular loops (Fig 1.5) (Gonen & Walz., 2006). Junction formation functions to uphold the structure of the lens tissue and Junctions are only formed by the truncated form of the protein and enabled by the lack of glycosylation (Gonen & Walz., 2006).

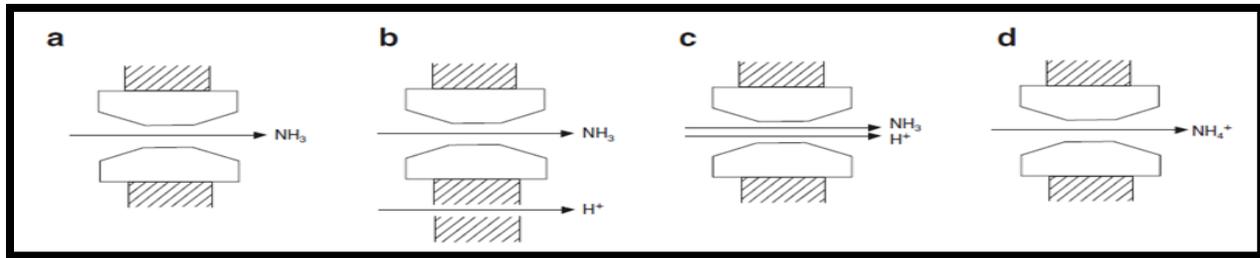


**Figure 1.5: Aquaporin (AQP) Junction formation in the lens.** Two AQP tetramers come together in AQP0 from lens and form junctions by interaction of proline residues on the extracellular side (Adapted from Gonen & Walz., 2006, Quarterly Reviews of biophysics, vol 39, issue 4, p 374)

AQPs monomers are often glycosylated (e.g., 50 % of mammalian AQP1 monomers), however, the significance of glycosylation in water-transport function has not been established (Van Hoek et al., 1995; Smith & Agre, 1991., Zhang et al., 1993; Shi et al., 1994). Human AQP1 form tetramers in the cell membrane and often one or two of its monomers are glycosylated (Verkman & Mitra, 2000). In addition to being present in cell membranes, AQPs have also been found in intracellular compartments such as the endoplasmic reticulum, mitochondria as well as in plant tonoplasts and vacuoles (Ishibashi et al., 2009; Ferri et al., 2003; Gena et al., 2008; Lee et al., 2006).

In addition to water, some AQPs are also able to transport other molecules such as urea, CO<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>, NO, NO<sub>2</sub>, and NH<sub>3</sub>, As(OH)<sub>3</sub>, Sb(OH)<sub>3</sub>, Si(OH)<sub>4</sub> however, the physiological significance of these is currently not known (Wu & Beitz, 2007; Nakhoul et al., 1998; Herrera et al., 2006; Campbell et al., 2008; Herraiz et al., 2011). Other chemicals such as boron have also been shown to permeate plant AQPs in boron-limiting conditions (Takano et al., 2006).

The gaseous form of Ammonia, NH<sub>3</sub>, has been known to be transported across some mammalian AQPs (AQP3, AQP7, AQP8 and AQP9) (Litman et al., 2009; Holm et al., 2005; Campbell et al., 2008). Ammonia is a toxic nitrogenous waste by-product of protein metabolism and animals must get rid of it. In general, a family of membrane proteins, ammonium transporter/methylamine permease/Rhesus (Amt/Mep/Rh) in microorganisms and Rhesus (Rh) proteins in mammals are involved in transporting charged ammonia, NH<sub>4</sub><sup>+</sup> (Wu & Beitz, 2007). In mammals, AQPs are thought to transport this nitrogenous waste in conjunction with the Rh proteins as the localization of ammonia transporting AQPs mirrors the location of Rh proteins in the human body (Litman et al., 2009). Litman et al (2009) have proposed four hypothetical models of ammonia transport across the AQPs (see Fig 1.6). In addition, NOD26, a plant AQP has been found in membranes surrounding nitrogen fixing bacteria and is suggested to be involved in nitrogen fixing by transport of NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup> (Krane & Kishore, 2003). Evidence of AQP involvement in ammonia transport was also seen in nodulin 26, a legume AQP, when ammonia permeability was inhibited by HgCl<sub>2</sub> (a known inhibitor of AQPs) (Tyerman & Niemietz et al., 2000). Wu & Beitz (2007) have suggested the role of ar/R constriction site in ammonia selectivity. Point mutation of this site (Phe56 and His180 with alanine and Arg195 was replaced with valine) allows the permeation of ammonia across mammalian AQP1 (Beitz et al., 2006; Wu & Beitz, 2007).



**Figure 1.6: Four hypothetical models of ammonia transport across Aquaporins (AQP)s** a) Ammonia enters the pore as NH<sub>3</sub> b) NH<sub>3</sub> enters the pore and allows H<sup>+</sup> conduction through a separate pathway c) NH<sub>3</sub> and H<sup>+</sup> both enter through the pore d) Ammonia permeates in its ionic form, NH<sub>4</sub><sup>+</sup> (Taken from Litman et al (2009), Springer Berlin Heidelberg, p 332).

AQPs are known to be inhibited by many different compounds. Mercurial sulfhydryl compounds such as mercury chloride (HgCl<sub>2</sub>) inhibit water transport by interacting with the SH-groups of cysteine residues conserved in the extracellular loop E near the NPA motif and alanine residue in loop B (Pettersson et al., 2005; Savage et al., 2007; Murata et al., 2000). This inhibition can be removed by the reducing agent β-mercaptoethanol. However, not all AQPs are inhibited by HgCl<sub>2</sub> because of the lack of the cysteine residue such as in AQPz from the bacteria, *E.coli* (Calamita, 2000). AQPs can also be non-reversibly inhibited by gold and silver and reversibly inhibited by acetazolamide as shown in rat AQP4 (Niemi et al., 2002; Tanimura et al., 2009). Zinc and copper can also act as AQP inhibitors (Zelenina et al., 2004; Yukutake et al., 2009). Tetraethylammonium (TEA) chloride is also shown to inhibit AQP function in *Xenopus* oocytes but it is non-specific and blocks voltage gated potassium channels (Mackinnon et al., 1990; Yool et al., 2002).

### 1.2.2 Regulation of Aquaporins

AQP function can be regulated in short term or long term by different mechanisms such as intracellular vesicular trafficking and transcription which can both be hormonally regulated and undergo post-translational modifications such as phosphorylation. In times of water conservation,

both mammalian AQP2 and AQP3 are transcriptionally up-regulated in the collecting ducts of the mammalian kidney (Campbell et al., 2008). AQP2 is also trafficked to the cell membrane of collecting ducts from intracellular vesicles as a result of arginine-vasopressin (AV), an antidiuretic hormone (Terris et al., 1996; Neilsen & Agre, 1995; Gustafson et al., 1998; Campbell et al., 2008). AV binds to the V2 receptors of baso-lateral collecting duct membrane, activates a G-protein coupled adenylyl cyclase pathway that results in phosphorylation of AQP2 by protein kinase A at the conserved serine and threonine residues. Phosphorylation of AQP2 causes intracellular vesicles holding AQP2 protein to be inserted into the cell membrane (Campbell et al., 2008). AV also acts on the mammalian AQP4 but in an opposite way. It results in exocytosis and re-storage of AQP4 protein in glial cell vesicles (Moeller et al., 2009). AQP1 permeability also increases with AV and decreases with atrial natriuretic peptide (ANP) (Patil et al., 1997). Glucagon also regulates the intracellular trafficking of AQP8 in mammalian liver cells (Gradilone et al., 2003). In insects, Rp-MIT, an AQP in *Rhodnius prolixus* is up-regulated following a blood meal due to the release of 5-hydroxytryptamine (5-HT) in the hemolymph (Martini et al., 2004). Furthermore, role of cortisol in salinity adaptation of fish is suggested to involve AQPs (Martinez et al., 2005; Deane et al., 2011).

pH also plays a role in regulating permeability of the water channel such as in human AQP0, AQP3, AQP4, and AQP6 (Varadaraj et al., 2005; Yasui et al., 1999; Hara-Chikuma & Verkman., 2006; Zeuthen & Klaerke, 1999). Also, in the European eel, *Anguilla anguilla*, exposure to acidic pH inhibits the water transport activity of AQP3 by 91 % and its permeability also decreases at pH above 8.2 suggesting AQPs may have an optimum functional pH (MacIver et al., 2009). However not all AQPs are regulated by pH as lowering pH does not alter the water permeability of AQP1, AQP1dup and AQP10 in these eels (MacIver et al., 2009).

Gating also regulates the permeability of AQPs through conformational reorientation of the AQP structure (Cohen et al., 2012). A subfamily of plant AQP, PIP has been shown to block permeability of the AQP channel through gating initiated by phosphorylation or calcium binding which is related to changes in the cytoplasmic pH (Cohen et al., 2012). Gating in Plant AQP S0PIP2;1 occurs by the folding conformation of loop D which interacts with the C-terminus to block the pore (Nyblom et al., 2009).

### **1.2.3 Aquaporins in Insects**

Campbell et al (2008) subdivided invertebrate AQPs into three types based on sequence homology: *Drosophila* integral protein (DRIP), big brain (BIB) and *Pyrocoelia rufa* integral protein (PRIP). Amongst these, BIB homologs are unique in that they have very long C-termini and show no water transport activity (Campbell et al., 2008; Benoit et al., 2014b). AQPs in insects are involved in many physiological functions such as high volume liquid diets of blood and plant sap/nectar feeding insects, respiration, cyroprotection, and anhydrobiosis (Campbell et al., 2008; Spring et al., 2009; Cohen et al., 2012; Tomkowiak & Pienkowska., 2010; Benoit et al., 2014b).

Many terrestrial insects feed on vertebrate blood in order to gain essential nutrients (proteins) for vitellogenesis. AQPs in blood feeding insects are associated with the salivary gland (SG)s, MG, MTs and the ovaries (Benoit et al., 2014b). Blood feeding insects need to inject saliva produced by the salivary glands into the host to prevent an immune response that can initiate host blood coagulation. SG AQPs for saliva production have been found in many insects, however, the physiological role of these AQPs have only been studied in the ticks, where, *IrAQP1*, is important in water flux in the SGs (Campbell et al., 2010; Beniot et al., 2014b). Imbibed water after a blood meal is absorbed by the gut and excreted back into the host by the salivary glands in ticks instead of being excreted by the MTs as in other insects (Campbell et al., 2010). Ticks also absorb water

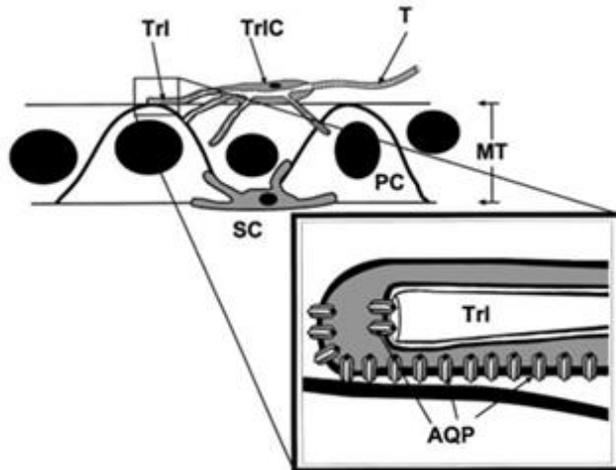
vapour from the air by the secretion of hygroscopic droplets that absorbs water onto the mouth of ticks and role of AQPs is suspected in this process but not experimentally verified (Campbell et al., 2010; Benoit et al., 2014b). In adult *A. aegypti*, AaAQP1a, AaAQP1b and AaAQP3b are transcriptionally expressed in the salivary glands (Ribeiro et al., 2007).

Blood consists of high water and salt content and poses an osmoregulatory challenge for these insects (Benoit & Denlinger, 2010; Beyenbach & Petzel, 1987). After a blood meal, these insects quickly excrete excess water and salt to maintain ion and water balance as well as to avoid predation as extra fluid in the abdomen prevents efficient flight (Beyenbach, 2003; Beyenbach, 2012). Fluid is uptaken up into the hemolymph by the midgut and AQPs may be responsible for this uptake (Beniot et al., 2014b). In adult *A. aegypti*, AaAQP1a and AaAQP1b are highly expressed in the midgut (Drake et al., 2010). In adult *Anopheles gambiae*, AgAQP1b is also expressed in the midgut (Tsujimoto et al., 2013).

In *A. aegypti* adult females, diuresis in the MTs begins before the completion of a blood meal and 40 % of excess water is excreted within the first hour post blood-meal (Clements, 1992; Clements, 2000; Bradley, 1987). AQPs play a role in regulating diuresis and avoid unnecessary physiological stress post blood meal. Four AQP homologs of *A. aegypti* are found in the MTs at the transcript level and knockdown of three of these reduces diuresis (Drake et al., 2010). In *A. gambiae*, AgAQP1 is highly expressed in the proximal principal cells of MTs and distal stellate cells (Tsujimoto et al., 2013). AQPs have also been found in the MTs of blood feeding *Glossina moritans morsitans* (Benoit et al., 2014a) and *R. prolixus* (Staniscuaski et al., 2013). AQP associated with MTs are not limited to blood feeding arthropods but can also be found in other insects such as the *Drosophila*, in which DRIP is localized to the stellate cells (Dow et al., 1995; Kaufmann et al., 2005) and the house cricket, *Acheta domesticus*, where it localizes to the middle

and distal MTs (Spring et al., 2007). AQPs are also expressed in the ovaries of blood feeding insects such as ticks and mosquitoes where they are thought to play a role in water uptake into oocytes, however, this has not yet been experimentally shown (Benoit et al., 2014b).

AaAQP1a, a water-specific, DRIP homolog in *A. aegypti* is implicated in insect respiration (Fig 1.7) (Pietrantonio et al., 2000). AaAQP1a is highly similar to the mammalian AQP4 that forms orthogonal arrays (OAs) in the lung tissue and also forms OAs when expressed in *Xenopus* oocytes. It is localized to the tracheolar cells associated with the MTs where it is thought to regulate fluid in response to metabolic needs (Pietrantonio et al., 2000). Insect respiratory system consists of a branching network of tracheal tubes, known as the tracheal system (Wigglesworth, 1983). Spiracles on exoskeletons of insects allows the entry of oxygen from the external environment which spreads through the network of tracheal tubes that further divide into smaller tubes, called the tracheoles. These tracheoles are filled with fluid in metabolically inactive cells. During metabolic demand (such as flight), the fluid is withdrawn from the tracheoles and oxygen is supplied to the surrounding tissue (Wigglesworth, 1983). Immunological staining of AaAQP1a showed that tracheolar cells of newly emerged *A. aegypti* females are more frequently and intensely stained than older females (Duchesne et al., 2003). This supports an indirect role of AaAQP1 in MTs during diuresis.



**Figure 1.7: Localization of AaAQP1 at the ends of tracheoles associated with the MTs.** Trl: Tracheole; TrIC, tracheolar cell; T, trachea; PC, principal cell; SC, stellate cell; MT, Malpighian Tubule. (Taken from: Pietrantonio et al (2000), *Insect Mol Biol*. Vol 9, issue 4, p 9)

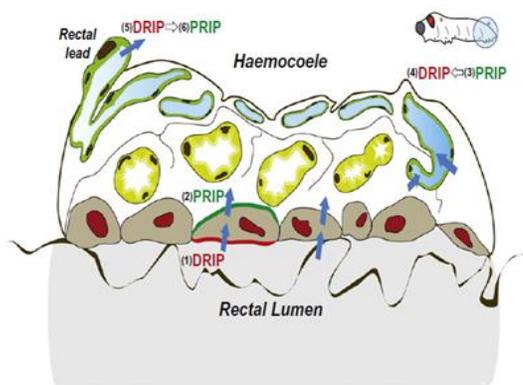
Homopteran insects feed on the xylem (e.g. green leafhoppers) and phloem (e.g. aphids) from plants. Xylem feeders ingest large volumes of low nutrient sap and must transfer excess water out of the gut quickly to concentrate nutrients that can then be absorbed by the MG (Le Caherec et al., 1997). Xylem feeding insects are equipped with a water shunting complex called the filter chamber. Filter chamber is formed by the gut that loops upon itself to bring the anterior midgut (AMG) and hindgut (HG) (along with MTs) adjacent (juxtaposition) to each other (Hubert et al., 1989). The close contact between the AMG and MTs allows a quick shunting of excess water directly to the excretory system to concentrate the ingested nutrients before absorption by the MG (Hubert et al., 1989). The first AQP associated with the filter chamber was cloned from the green leafhoppers, *Cicadella viridis*, known as AQP<sub>cic</sub> (Le Caherec et al., 1996). It was found to be a water-specific channel, functionally similar to mammalian AQP1 (Le Caherec et al., 1996). This AQP also forms OAs when expressed in *Xenopus* oocytes showing the importance of this arrangement in tissues requiring large water influxes (Le Caherec et al., 1996).

Phloem feeding insects are at a risk of losing a lot of water from their hemolymph as they feed on highly concentrated fluid. The osmotic pressure of the phloem is higher (4-5 x) than the hemolymph as it has high sucrose (Douglas, 2006; Shakesby et al., 2009). The distal end of the intestine loops to be in direct contact with the proximal end of the intestine or the 'stomach' for quick water shuttle. The water from distal intestine is absorbed and directly transferred to the proximal intestine without compromising hemolymph water balance (Shakesby et al., 2009; Mathew et al., 2011). In the phloem feeding insect, pea aphid, *Acyrtosiphon pisum*, the high water flux in this modified gut is the result of ApAQP1 (Shakesby et al., 2009). It is a highly water permeable AQP and induces an 18 fold increase in water permeability when expressed in *Xenopus* oocytes. ApAQP1 is also found in the gut and salivary glands of the mature embryo in preparation for phloem feeding soon after birth (Shakesby et al., 2009). BtAQP1, water specific channel, is also localized to the similarly modified gut of the adult whitefly, *Bemisia tabaci* (Mathew et al., 2011).

In lepidopteran and coleopteran insects, such as the silkworm larva, *Bombyx mori*, there is a high demand to conserve water (Kataoka et al., 2009). In most of these terrestrial insects, AQPs are associated with a modified excretory system known as the cryptonephric complex (Azuma et al., 2012). The cryptonephric complex is a modified arrangement of the excretory system in which the distal ends of the MTs (called cryptonephric Malpighian tubules (cMTs) are in close association with the rectum (Fig 1.8). This close association allows constant and rapid reabsorption of fluid from the rectum to produce very dry faeces (Azuma et al., 2012). In the silkworm larva, *Bombyx mori*, three APQs (AQP-Bom1, AQP-Bom2 and AQP-Bom3) have been identified, two of which are associated with the cryptonephric complex (Kakaota et al., 2009; Azuma et al., 2012). AQP-Bom1 and AQP-Bom2 belong to the DRIP and PRIP families,

respectively (Azuma et al., 2012). AQP-Bom1 is expressed on the apical membranes of colonic and rectal cells while AQP-Bom 3 is expressed on the basal membrane of the cells. Water flows out from the lumen of the rectum through the rectal (AQP-Bom1) DRIP, then (AQP-Bom2) PRIP, the outer cMT (AQP-Bom2) (PRIP) and the outer cMT (AQP-Bom1) DRIP. Water then enters the cMT lumen and moves toward the rectal lead MT, where its moves through the apical (AQP-Bom1) DRIP and basal (AQP-Bom3) PRIP to reach the haemocoel (Fig 1.8) (Azuma et al., 2012).

On the other hand, AQP-Bom2 isoform is expressed in the PMG and MTs and it has been suggested to function as an aquaglyceroporin because of its permeability to water, glycerol and urea (Kakaota et al., 2009; Azuma et al., 2012).



**Figure 1.8: Proposed water movement in the cryptonephric complex of silkworm larva, *Bombyx mori*.** Water from the rectal lumen is transported into the haemocoel through AQP-Bom1 (DRIP) and AQP-Bom3 (PRIP) in series. The number (1-6) denote putative recycling of water to the haemocoel (Taken from Azuma et al (2012), J. Insect Physiol. Vol 58, issue 4, P 531)

In the honeybee, *Apis mellifera*, an AQP has been implicated in the concentration of the nectar in the honeybee crop, an organ involved in temporarily storing the nectar (Serrão et al., 2014). It is mostly localized to apical epithelium of the crop and some in the cytoplasmic vesicles. Nectar ingested by the honeybee consists of 90 % water and is converted into honey in a process that involves digestion and dehydration of the nectar. This AQP plays a role in removing excess water to be transported out of the crop minutes after the ingestion of nectar. It is important in

preventing dilution of digestive enzymes required to convert nectar to honey (Serrão et al., 2014). The current model suggest that this water is then transported from the hemolymph to MTs where it is excreted by the hindgut (Serrão et al., 2014)

In freeze tolerant insects, water is withdrawn from cells as ice is formed in the hemolymph to prevent cell death during winter. Freeze tolerant insects use AQPs to remove water from their cells during ice formation while accumulating cryoprotectants such as glycerol (Philip et al., 2008; Izumi et al., 2007). AQPs play a role in the survival of stem borer, *Chilo suppressalis* larvae over winter which can tolerate temperatures of about  $-25^{\circ}\text{C}$  (Izumi et al., 2007). AQP involvement in water and glycerol permeation in the fat body of larval *C. suppressalis* was inhibited using an AQP inhibitor (Izumi et al., 2007; Izumi et al., 2006).

EsAQP1, a water specific channel is also implicated in the freeze-tolerance of larval gall fly, *Eurosta solidaginis*, where it is highly expressed in the brain and suggested to cryoprotect the brain over winter (Philip and Lee., 2010; Philip et al., 2011). *E. solidaginis* larvae can survive temperatures of  $-80^{\circ}\text{C}$  over winter and experiments have shown that they are unable to survive in the presence of AQP inhibitors (Bennett and Lee., 1997; Philip and Lee., 2010; Philip et al., 2008). Even though EsAQP1 exhibited low water permeability in *Xenopus* oocytes, it has been suggested that in vivo permeability of this AQP may be different as activity can be regulated by post-translational modifications (Philip et al., 2011).

AQPs also play a role in anhydrobiosis (complete dehydration) which also involves removing water from the cells. In the sleeping chironomid, *Polypedilum vanderpanki*, two water-specific, mercury sensitive AQPs (pvAqp1 and pvAQP2) play opposing roles in anhydrobiosis (Kikawada et al., 2008). pvAqp1 is ubiquitously expressed and involved in removing water from the larval body during dehydration and pvAqp2 is mainly found in the fat body and its expression

prevents dehydration (Kikawada et al., 2008). Role of AQPs have also been implicated in survival of the Antarctic midge, *Belgica antarctica*, larva in both desiccation and freezing conditions (Yi et al., 2011). Previous work has also shown that an external salinity increases tolerance to freezing and desiccation in these midges suggesting AQP involvement (Elnitsky et al., 2009).

AQPs are also found in other physiological tissues of insects. For example, an AQP (cfAQP) transcript was found in the water sacs (organs specific to termites), epidermis and the antenna of the termite, *Ooptotermes formosanus* (Kambara et al., 2009). In the blowfly, *Phormia regina*, an AQP (PregAQP1), from the DRIP subfamily, has been associated with maintaining water balance in the olfactory organs (Ishida., 2012). Nagae et al (2013) also identified a first known AQP from rectal epithelium of the coleopteran (soil feeding) beetle, *Tribolium castaneum*. Similar to mammals, insect AQPs seem to be ubiquitously present in all tissues.

#### **1.2.4 Aquaporins in aquatic/semi-aquatic animals**

AQPs have also been found in many aquatic and semi-aquatic animals such as amphibians (Suzuki et al., 2007; Suzuki & Tanaka, 2009), crustaceans (Chung et al., 2012), molluscs (Pienkowska et al., 2014) and fish (Cerdà & Finn, 2010).

In amphibians such as anurans, AQPs are generally associated with the tight junction epithelium of the kidney, urinary bladder and the ventral pelvic skin where they play a role in water absorption in terrestrial habitats when animals are at risk of evaporative water loss across their skin (Ogushi et al., 2010; Suzuki & Tanaka, 2009). Water absorption across these tissues is under the control of an antidiuretic hormone, arginine vasotocin (AVT) (Suzuki & Tanaka, 2009). In the kidney, AVT induces a decrease in glomerular filtration and increases water uptake across the collecting ducts of kidney tubules through its action on AQPs. In the anuran, *Hyla japonico*, AQP-h2k is translocated from the cytoplasm to the apical membranes of the principal cells of

collecting ducts to increase water reabsorption after AVT stimulation (Sasaki & Noda, 2007). In contrast, AQP-h3BL is localized to the basolateral membrane of these cells and is unaffected by AVT and its role is restricted to constant transportation of water between the principal cells and tissue fluids (Akabane et al., 2007).

In the pelvic skin of *H. japonica*, AVT induces AQP-h2 and AQP-h3 to translocate from the cytoplasm to the apical membrane of the principal cells of first reacting cell layer, the outermost layer of the stratum granulosum (Hasegawa et al., 2003; Suzuki & Takana, 2009). AQP-h3BL is localized to the basal membrane of these cells and is not affected by AVT (Akabane et al., 2007). In *Hyla*, AQP-h3BL is suggested to play a role in water and glycerol transport across both the ventral and dorsal skin (Akabane et al., 2007). However, in *Xenopus laevis*, AQP-h3BL is mainly found in the basal membrane of ventral skin only and its very low expression in dorsal skin is suggested to contribute to comparatively low survival of this anuran in terrestrial habitat (Mochida et al., 2008; Suzuki & Takana, 2009). In the urinary bladder of *H. japonica*, AQP-h2 and AQP-h3BL are found in the granular cells and also translocate to the apical membrane upon AVT stimulation while AQP-h3BL localizes basolaterally and is not sensitive to AVT (Hasegawa et al., 2003; Akabane et al., 2007).

In Anurans, AQPs are also associated with the skin glands where they are suggested to be involved in keeping the skin moist and regulating body temperature by excreting water (Kubota et al., 2006). In *X. laevis*, AQP-x5 localizes to the apical membrane of secretory cells of small granular and mucous glands (Kubota et al., 2006). In the *H. japonica*, *Rana japonica* and *Bufo marinus*, AQP-x5 localizes to the apical membrane of acinar cells of mucous and seromucous glands (Kubota et al., 2006). In contrast, AQP-x3BL localizes to the basal membranes of small

granular glands and mucous glands of *X. laevis* and mucous glands of other *Hyla* (Mochida et al., 2008; Akabane et al., 2007).

In the blue crab, *Callinectes sapidus*, CasAQP-1 is suggested to play a role in adaptation of early stage larvae to salinity during development (Chung et al., 2012). Females of *C. sapidus* migrate to higher salinity waters for spawning and all 8 larval stages (Z1-Z8) develop in those waters and migrate back to lower salinity once they reach the megalopa stage after which they metamorphose into the first crab stage. CasAQP-1 is ubiquitously present in crab tissues at the mRNA level including the osmoregulatory tissues, the hepatopancreas and gills where its expression is greater in the hepatopancreas than the gills. Under constant conditions, expression of CasAQP-1 of the whole body larvae is highest during Z1-2 and Z7-8 larval stage, however, upon being exposed to hyposalinity, Z3-Z4 larvae increase expression of CasAQP-1 whereas the other larval stages are unaffected (Chung et al., 2012). An AQP transcript has also been found in the gills of the green shore crab, *Carcinus maenas*, where its expression does not significantly change in lower salinity (Towle et al., 2011).

Recently, for the first time, Pienkowska et al (2014) identified three AQPs LsAQP1, CoAQP1, and SpAQP1 belonging to three freshwater snail species *Lymnaea stagnalis*, *Stagnicola palustris* & *Catascopia occulta (terebra)*, respectively. Structural analyses revealed that these AQPs are water specific and highly similar to human AQP4. Transcript of LsAQP1 were present in the digestive tract, cerebral ganglia, kidney, reproductive system and foot of *L. stagnalis* where it is suggested to be involved in water transport however, its exact physiological role within these tissues is currently unknown (Pienkowska et al., 2014).

In fish, role of AQPs during changes in salinity has mainly been studied in euryhaline teleosts (Aoki et al., 2003; Martinez et al., 2005). Euryhaline teleosts can move between FW and SW during different phases of life such as spawning/mating. AQPs are found in many fish tissues/organs but the main focus here will be the three osmoregulatory organs: the gills, intestine and kidney (Marshall and Grosell, 2006; Cerdà & Finn, 2010).

FW teleosts are at a risk of gaining excess water and losing ions to their habitat (Marshall and Grosell, 2006). In these animals, kidneys produce a large volume of dilute urine and ions are uptaken across the gills to maintain ion and water balance. In SW, fish risk losing water to their external habitat and drink large volumes of SW in compensation. Ingested ions are absorbed by the intestine and then secreted out by the kidneys in the form of an isotonic urine. Gills also actively secrete ions to maintain ionic balance by ion transporting chloride cells (Marshall and Grosell, 2006).

Along with ion regulation, fish gills are also involved in water regulation, acid-base regulation, and excretion of nitrogenous wastes such as ammonia (Evans et al., 2005). Many AQP homologs are found in the gills but much of the work has focussed on AQP3 isoforms. AQP3 (AQP3a and AQP3b) is mainly associated with fish gills appearing as two isoforms in some fish, such as in the Zebrafish (*Danio rerio*) (Cutler et al., 2007). In the European sea bass (*Dicentrarchus labrax*) and the European eel (*A.anguilla*), AQP3 is suggested to function as a non-specific water channel, permeable to both water and urea (Giffard-Mena et al., 2007; MacIver et al., 2009). In the Killifish, it is also suggested to play a role in ammonia permeability (Jung et al., 2012). AQP3 expression generally tends to decrease in gills of SW acclimated fish (Cutler et al., 2007). Transcript of AQP3 decrease in gills of sea bass (*Dicentrarchus labrax*) 2 days after FW to SW transfer (Giffard-Mena et al., 2007). AQP3 transcript also downregulates in gills of

European eel up to 94 % after FW to SW transfer with a 3-fold decrease in AQP3 protein (Cutler & Cramb., 2002; Lignot et al., 2002). AQP3 transcript and/or protein expression also decreases in gills of silver sea bream (*Sparus sarba*), and the Japanese eel (*Anguilla japonica*) after FW to SW transfer (Deane and Woo, 2006; Tse et al., 2006). In the gills of Killifish (*Fundulus heteroclitus*), kfAQP3 mRNA decreases with FW to SW transfer with no change in protein abundance and this inconsistency is suggested to be the result of differential expression of proteins in different gill regions (Jung et al., 2012). It is suggested that in pillar cells of gills, kfAQP3 decreases in SW and plays a role in cell volume regulation and gas exchange whereas in the mitochondrial rich cells of gills, it increases to excrete ammonia and regulate acid base balance (Jung et al., 2012). In addition, other AQP homologs present in the gills respond differently to external salinity. For example, in the Japanese medaka (*Oryzias latipes*) gills, AQP3a expression decreases in gills of SW with a concurrent increase in AQP10 (Kim et al., 2014). In the river pufferfish (*Takifugu obscurus*), AQP3 expression in gills also decreases with salinity while AQP1 which is generally expressed at low levels remains unaffected (Jeong et al., 2014). Due to its localization to the basolateral membrane of ion transporting chloride cells of gills, AQP3a in tilapia (*Oreochromis mossambicus*) is suggested to play an osmosensory role by regulating cell volume (Watanabe et al., 2005).

In the fish intestine, AQP homologs are suggested to play a role in increasing water uptake during SW acclimation (Cerdà & Finn, 2010). Expression of AQP1 isoforms in the intestine generally increases in SW (Whittamore, 2012). In the European eel, eel AQP1 is shown to be water specific (MacIver et al., 2009; Hill et al., 2007) while in zebrafish, AQP1 is permeable to water, glycerol and urea (Tingaud-Sequeira et al., 2010). In SW, AQP1 transcript is up-regulated in the Atlantic salmon (*Salmo salar*), European eel, Japanese eel, and the gilt-head Sea bass to

increase water absorption across the intestine (Tipsmark et al., 2010; Martinez et al., 2005; Aoki et al., 2003; Giffard-Mena et al., 2008). AQP1 transcript, along with AQP3 is also up-regulated in the SW acclimated puffer fish, *Takifugu obscurus*, intestine (Jeong et al., 2014). In contrast, recent work has shown a decrease in mRNA and/or protein of AQP homologs (Aqp1aa, Aqp8ab and Aqp10a) in SW reared Japanese medaka (*Oryzias latipes*) challenging the current model that suggests that transepithelial water absorption across the intestine generally increases in SW (Madsen et al., 2014). Madsen et al (2014) have suggested that paracellular water permeability across the intestine may be more important in these fish in SW adaptation.

AQP3 (AQP3a and/or AQP3b) transcripts have also been found in the intestines of both FW and SW acclimated teleosts localized to the macrophage-like cells of anterior intestine and mucous/goblet cells in the rectum (Lignot et al., 2002). In comparison to FW, SW acclimated European eels show greater staining of AQP3 in rectal goblet/mucus secreting cells implying its function in mucus fluid secretion (Lignot et al., 2002). AQP8 is also up-regulated in the intestine of SW acclimated Japanese eel, *A. japonica*, and sockeye salmon, *Onocorhynchus nerka* (Kim et al., 2010; Choi et al., 2013). Along with four other AQP homologs, AQP8b is also transcriptionally up-regulated during smoltification in the intestine of Atlantic salmon (Tipsmark et al., 2010; Choi et al., 2013).

In FW, fish kidney produces large hypotonic urine and in SW, the kidney conserves water by increasing water uptake and producing isotonic urine and this process involves AQPs (Engelund & Madsen, 2011). In the sea-bass, AQP1 transcript is up-regulated in SW acclimated kidney (Giffard-Mena et al., 2007). AQP1aa is also up-regulated in kidney of SW acclimated Atlantic salmon, while AQP1b is down-regulated, concurrently (Tipsmark et al., 2010). In SW acclimated rainbow trout (*Oncorhynchus mykiss*), AQP1b is found in apical sides of both kidney tubules

whereas in FW, it localizes sub-apically; suggesting that AQP activity at the kidney is not solely dependent on transcriptional control (Enguland & Madsen, 2011).

AQP3 isoforms have also been found in the kidney of some teleosts where it is upregulated following a transfer to SW (Watanabe et al., 2005; Tipsmark et al., 2010). In the stenohaline fish, *Cyprinus carpio*, staining of distal kidney tubules for AQP3 increases in SW (Salati et al., 2014). Most of the literature on fish AQPs is focussed on transcriptional changes with salinity. More functional studies of individual AQP isoforms will determine the exact physiological roles these AQPs play in fish during salinity acclimation.

### 1.3 Significance of this study

*Aedes aegypti* prefer to reside near human habitats and lay eggs on surface of stagnant water. The eggs hatch in water and the aquatic larvae go through four instar stages before pupating and emerging as adults. The whole life cycle takes ~18-20 days (Clements, 1992). *Aedes aegypti* females require vertebrate blood for reproduction. The protein rich blood plasma is used for the production of eggs (Clements, 1992). Due to the vertebrate blood requirements for their life cycle to continue, *Aedes aegypti* have become a vector for many human arboviral diseases such as the dengue, yellow fever and chikungunya virus (Cavrini et al., 2009; Weaver & Reisen, 2010). These viruses have been difficult to treat due to lack of proper vaccination/medication. Chikungunya virus has been on the rise in the Caribbean islands since Dec 2013 with 8000 suspected cases reported in March 2014 (WHO, 2014a). With no vaccine available, cases are expected to rise. Cases of dengue have also increased in recent years and with no specified treatment or vaccine against it, it is the leading cause of illness and death amongst children in many countries (Halstead, 2003; Chun et al., 2007; Monath et al., 2007). Prevention of dengue is mainly depending on controlling the mosquito vector population (WHO, 2014b). Current vaccination for yellow fever is also preventative and once an infection has occurred, there is no treatment (WHO, 2014c).

An effective means to controlling disease is to control the vector (*A. aegypti*) population. Water homeostasis is critical for *A. aegypti* larval survival in aquatic habitats and disrupting the osmoregulatory capacity of *A. aegypti* larvae by targeting AQP water channels can have implication for the effective control of these diseases. A fundamental understanding of how AQPs are involved in larval mosquito osmoregulation could provide new avenues to affect population level control measures.

## 1.4 Hypothesis and Experimental Objectives

Water permeability across the Anal Papillae (AP) has been supported by many studies (Wigglesworth, 1933a; Stobbart, 1971). This permeability is suggested to involve AQPs because the Anal Papillae (AP) have a syncytial epithelium. Previous work by Marusalin et al (2012) identified the presence of all six AQP homologs (AaAQP1a, AaAQP1b, AaAQP2, AaAQP3a, AaAQP3b, AaAQP4) in the anal papillae (AP) of *A. aegypti* larvae at the transcript level. This work also showed that HgCl<sub>2</sub>, an AQP inhibitor, decreases water uptake across the AP epithelium (Marusalin et al., 2012). Role of these AQP homologs in osmoregulation across the AP epithelium of *A. aegypti* larvae is currently unknown. Previous studies on aquatic animals have shown that AQP abundance in osmoregulatory tissues changes as the external salinity is modified, and it is suggested that AQPs play an important role in maintaining osmotic balance (Tipsmark et al., 2010; Cutler & Cramb, 2002; Lignot et al., 2002; Chung et al., 2012). Therefore, we hypothesize that AQPs expressed in the AP play a role in larval osmoregulation. To examine the role of these AQPs in osmoregulation, AP are exposed to either ion-poor water (IPW) or 30 % sea water (SW). Hence, the first objective of this study is to determine the mRNA abundance of six AQP homologs in the AP of larvae reared in IPW and 30 % SW using real-time polymerase chain reaction (qPCR). The second objective is to determine protein level abundance of AQP homologs in AP of larvae reared in IPW and 30 % SW using western blot. The third objective is to localize AQPs in the AP using immunohistochemistry. The fourth objective is to determine the presence of these AQP homologs in other osmoregulatory tissues which may form a basis for future studies examining osmoregulatory role of AQPs in these tissues. By examining expression and localisation of AQPs in response to changes in salinity one can begin to understand if and how AQPs participate in osmoregulation.

## 2 METHODS/MATERIAL

### 2.1 Experimental Animals

Eight biological samples of larvae were acclimated to ion-poor water (IPW) and another eight samples of larvae were acclimated to ~ 30 % seawater (SW) (7.5 g L<sup>-1</sup> Instant Ocean® salts). For this, the eggs of *Aedes aegypti*, obtained from the colony kept at York University, were initially hatched in Milli-Q water. Upon hatching, the larvae were transferred to either Milli-Q water (IPW control) or to 10% SW (SW treatment; 2.5 g salt/L). After one day of exposure to 10 % SW, the SW larvae were transferred to a higher concentration of ~ 20% SW (5g salt/L), while the IPW larvae were transferred to new IPW. Following a day of exposure to ~ 20% SW, the SW larvae were transferred to ~ 30% SW (7.5 g salt/L) while the IPW larvae were also transferred to new IPW. The larvae were then kept in either IPW or 30% SW for two weeks until they reached third to fourth larval instar stage. The larvae were fed ~ 2 ml of a 1:1 (yeast and liver powder) almost daily until dissection. The larvae were dissected in *Aedes* saline [5mM L-proline, 9.1 mM L-glutamine, 8.74 mM L-histidine, 14.4 mM L-leucine, 3.37 mM L-arginine-HCL, 10mM Glucose, 5 mM Succinic acid, 5 mM Malic acid, 10mM Citric Acid (tri sodium salt), 30 mM NaCl, 3 mM KCL, 5mM NaHCO<sub>3</sub>, 0.6mM MgSO<sub>4</sub>, 5mM CaCl<sub>2</sub>, 25 HEPES; pH ~ 7.0] (Clark & Bradley, 1997).

### 2.2 RNA extraction and cDNA synthesis

The Anal papillae (AP) collected from 60 -90 larvae were isolated in *Aedes* saline and transferred to a tube containing 200 µl of RNeasy® Stabilization reagent (Qiagen, Toronto, ON, Canada) or RNA protect® Reagent (Qiagen, Toronto, ON, Canada). After collecting the Anal Papillae, RNA later Stabilization reagent or RNA protect reagent was removed and 1000 µl of

TRIzol<sup>®</sup> RNA isolation reagent was added (Invitrogen, Burlington, ON, Canada). The collected tissues were then sonicated for 5 sec at 5 watts using an XL 2000 Ultrasonic Processor (Mandel, Guelph, ON, Canada). Thereafter, total RNA was extracted according to the manufacturer's instruction (Invitrogen, Burlington, ON, Canada). Subsequently, isolated RNA was treated with TURBO DNA free<sup>™</sup> kit in order to remove any genomic DNA (Ambion, Burlington, ON, Canada). The quality and quantity of RNA was determined using a Multiskan spectrum spectrophotometer (Thermo Electro Corporation, USA). All 16 samples had an optical density (OD) absorption ratio (OD<sub>260/280</sub>) of >1.8. An iScript<sup>™</sup> cDNA synthesis kit was used to synthesize first strand cDNA using 1 µg/µl of total RNA for each sample (Bio-rad, ON, Canada).

### **2.3 Quantitative real-time PCR (qPCR)**

For 6 AQP homologs, the primers designed by Marusalin et al (2012) were used. Quantitative real-time PCR (qPCR) was performed using these primers in order to find the relative abundance of these genes in IPW and SW treatments. The annealing temperature used for each primer set was 58°C. Each reaction was performed in two technical replicates and no cDNA template control for each gene was also included. Each reaction well consisted of 10µl of SsoFast<sup>™</sup> Evagreen<sup>®</sup> supermix (Bio-Rad, Mississauga, ON, Canada), 2 µl of cDNA template, 0.5 µl of 10µM forward primer, 0.5 µl of 10µM reverse primer and 7 µl of PCR water. All reactions were performed in the CFX96 Touch<sup>™</sup> PCR detection system (Bio-Rad, Mississauga, ON, Canada). The qPCR reactions were run using the following settings: 2 minutes of enzyme activation at 95°C, followed by 39 cycles of 5s denaturation at 95°C and 5s of annealing/extension at 58°C. Each reaction was followed by a melting curve analysis in order to confirm the presence of a single amplicon. The setting for the melting curve analysis consisted of holding the product at 95°C for 10 sec; followed by holding the product at 65-95°C with a 0.5°C increment held for 5

sec. The results of the qPCR were analyzed using the CFX Manager Software V2.1 (Bio-Rad, Mississauga, ON, Canada). Results of the mRNA quantification were normalized to 18S ribosomal subunit (18s) gene (annealing temperature: 58°C). Quantification of transcripts was calculated using the Livak method (deltadeltaCT) (Pfaffl 2004).

## 2.4 Production of antibodies specific to AaAQP3a and AaAQP3b

For AaAQP3a, a rabbit polyclonal antibody was raised against the unique amino acid sequence PAEQAPSDVGKSNQS in the N-terminal (cytoplasmic) region (Genscript, NJ, USA). For AaAQP3b, a rabbit polyclonal antibody was raised against the unique amino acid sequence FRREVPEPEYNRELT in the C-terminal (cytoplasmic) region (Genscript, NJ, USA). Unique sequences used for AaAQP3a and AaAQP3b are shown in Fig 2.1.

```

AaAQP1a -----MTESAGVKQLVGVADITE--NRNIWRML 26
AaAQP1b -----MGTSYKLGMDELSGRSSNSIWKAL 24
AaAQP3a -----MSTNHQNGKDSLHPMPAEQAPSDVGKSNQSFLTGTGHNARDVMSIF 45
AaAQP3b -----MKKS-----TLDTISIL 12
AaAQP2 MAEESLHVPDSNIDFHIVQLFERLENLRREPGGAKLSSSIQGRSTSMHNELRSLEFWRSI 60
AaAQP4 -----MAIEQLGYSVFFIALTSAIAAVARRVNDKLS 31

AaAQP1a NPARSFGPAVVMG---NNTDQWVYVWGPVIVGGILAGAVYRLFFKVRK--GDEESYDF--- 249
AaAQP1b NPARSFGTAFIGD---NWNANHWIYWAGPILGGICASLLYCQVFKAPQPEGESASERYRVA 248
AaAQP3a NPARTLPPAVWNG---SYKSIWIYFIAPPLAGMVMPLIYKYVFRRELQDEQTAMVIKTP 279
AaAQP3b NPARSLGPVLWNG---DWTAHWVYVWGPLTAAFSIAFLYKTVFRREVPEPE-----YN 239
AaAQP2 NPARSLGPSFVLN---KWDNHVYVWGPLIGGMVSGLLHEFIFSTKKTSKKSKDDVDSSV 286
AaAQP4 NPVLATALKWGCAGNSALEHIIVYVWIGSCAGAVLSVPLFKTASFRNMFLADKAKAE---- 261

AaAQP1a -----
AaAQP1b ADEKELKRLDGKRDMA----- 264
AaAQP3a EEMKAHIVEQNR----- 292
AaAQP3b RELTALNTEK----- 249
AaAQP2 NSDEDINVDMDMDKPPQSGKYHTYRPSSTGLVVSQQRFQSIYTAEPTSKTDRVESIYGGT 346
AaAQP4 -----

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**Figure 2.1:** Sequences used for the production of AaAQP3a (underlined in Red) and AaAQP3b antibody (underlined in Blue). Protein sequences were aligned using Clustal 2.1. Aquaporin UnitProt accession numbers are as follows: AaAQP1a (Accession #: Q9NHW7), AaAQP1b (Accession #: Q1HQY7), AaAQP2 (Accession #: Q17BX1), AaAQP3a (Accession #: Q17BC8), AaAQP3b (Accession #: Q1HQ54), AaAQP4 (Accession #: Q16GU8).

## 2.5 Western Blot

For western blot analysis of whole body (WB), 10-11 larvae were collected in microtubes while other tissues (GC, AMG, PMG, MTs, HG and the AP) were collected in *Aedes* saline. All tissues were homogenized in RIPA buffer (48.7 mM Tris, 0.15 NaCl, 0.03 M deoxycholic acid, 0.01 % Triton-X-100 and 10 % SDS, 1 M DTT, 100 mM PMSF, 100 mM EDTA) containing 0.005 % of protease inhibitor cocktail (Sigma-Aldrich, Oakville, ON, Canada). Each biological sample of GC, AMG, PMG, MTs and HG were collected from 30 larvae. Each biological sample for the AP was collected from 80 larvae. Tissue homogenates were centrifuged at 13K RPM for 20 minutes at 4° C using Sorvall™ Legend™ Micro 21 Centrifuge (Thermo Scientific, USA). After centrifugation, the supernatant was collected. Protein concentration was measured with a Multiskan spectrum spectrophotometer (Thermo Electro Corporation, USA) at 595 nm using the Quick Start™ Bradford assay (Bio-rad, Mississauga, ON, Canada). For the Bradford assay, bovine serum albumin (BSA) was used as a standard. Prior to gel electrophoresis, the samples were boiled at 100° C for 5 minutes with 6x loading buffer (0.36 M Tris/HCL, 30 % Glycerol, 12 % SDS, 9.3 % DTT, 0.03 % bromophenol blue) and cooled on ice. Each well was loaded with 10 µg of protein from the Anal Papillae for IPW and 30 % SW treatments. 15 µg of protein homogenates were loaded in each well for WB, GC, AMG, PMG and the HG (Fig 3.5). The samples were then electrophoresed on a 4% stacking/12 % acrylamide gel at 60 V until the protein reached the 12 % acrylamide gel when the voltage was increased to 110 V. After electrophoresis, the proteins were transferred onto a polyvinyl difluoride (PVDF) membrane using a semi-dry transfer unit, Electrophoresis power supply – EPS 301 (Amersham, Biosciences, NJ, USA). After the transfer, the membrane was washed in Tris-buffered saline (TBS) [ 9.9 mM Tris, 0.15 M NaCl, PH 7.4] with Tween-20 (TBS-T) (3 x) for 10 mins. The PVDF membrane was then blocked for 1

hour in 5 % skim milk powder in TBS-T. The membrane was then incubated overnight at 4° C with the primary (1°) antibodies (1:500 in 5 % skim milk in TBS-T). Following incubation with the 1° antibody, the membrane was washed again with TBS-T (3 x) for 10 minutes and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:5000 in 5 % skim milk in TBS-T) (Bio-rad, ON, Canada). The membrane was washed again in TBS-T (3x) for ½ hour. Immunoreactivity was visualized using Clarity™ Western ECL substrate (Bio-Rad, ON, Canada). A rabbit monoclonal antibody for Glyceraldehyde 3-phosphate dehydrogenase (GADPH) (1:1000 in 5 % skim milk in TBS-T) (Cell signalling, Danvers, MA, USA) was used as the loading control. Western blot of GAPDH was carried out as outlined above by subsequently stripping the membrane with stripping buffer [20 mM Magnesium acetate, 20 mM potassium chloride, 0.1 M Glycine; pH 2.2] and using HRP-conjugated goat anti-rabbit antibody (1:5000 in 5 % skim milk in TBS-T) (Bio-rad, ON, Canada). For the peptide control experiment, two tubes were prepared. One tube contained the antibody mixed in TBS and the other had antibody plus 2 x molar excess of antigen/peptide in TBS. Tubes were pre-incubated overnight at 4°C before using them in the western blot protocol.

## **2.6 Densitometry**

Image J 1.47 v software (USA) was used to quantify the protein abundance. The background density of the blot was subtracted from the density of the protein signal. All treatment group densitometry ratios were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (GADPH (14C10) Rabbit mAb #2118, Cell signalling, Danvers, MA, USA) protein, which was the loading control in all samples.

## 2.7 Immunohistochemistry

For whole mount, whole guts were dissected in *Aedes* Saline and fixed in 2 % paraformaldehyde for 2 hrs at RT. The tissues were then washed 3 x for 30 mins in phosphate buffered saline (PBS) [1.37 M NaCl, 0.03 M KCl, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.02 M KH<sub>2</sub>PO<sub>4</sub> ; PH 7.4] at RT. After washing, the tissues were blocked with 10 % antibody dilution buffer (ADB) (containing 10 % goat serum, 3 % BSA, 0.05 % Triton-X and 1 x PBS) at RT. Tissues were then incubated for ~ 46 hrs at 4<sup>0</sup>C with the primary antibody (1:500 diluted in ADB). The control tissues (omitting primary antibody) were also incubated in ADB alone for ~ 46 hrs at 4<sup>0</sup>C. After incubation, the tissues were washed again in 1 x PBS 2 times for 2 hrs at RT and probed with secondary antibody diluted in ADB (Alexa Fluor 594-conjugated goat anti-rabbit, dilution 1:500). The control tissues were incubated in ADB alone. Tissues were then washed again in 1x PBS 2x for 2hrs, mounted on slides with Prolong<sup>®</sup> gold antifade reagent with DAPI (Invitrogen, Burlington, ON, Canada).

For paraffin embedding, tissues were dehydrated in succession by placing them in a series of increasing ethanol solutions (70 % ethanol, 90 % ethanol, 100 % ethanol). The tissues were then placed in 100 % xylene and then 1:1 xylene/paraffin. Tissues were then transferred to 100 % paraffin and embedded in embedding rings. The tissues were sectioned (4-5 µm thick) using a Leica RM2125 RTS Microtome (Leica Microsystems, Concord, ON, Canada). The sections were floated on warm water and then collected on glass slides. The slides were left to dry overnight at ~ 48<sup>0</sup>C. Sections were then de-paraffinized by transferring slides to xylene for 2 x 5 mins. The tissues were then hydrated by sequentially placing slides in decreasing concentration of ethanol solutions (100 % ethanol for 2 x 2 min, 95 % ethanol 1x 2 min, 70 % ethanol 1 x 2 min, 50 % ethanol 1 x 2 mins) and then letting them sit in distilled water for 25 minutes. Heat-Induced Epitope

Retrieval (HIER) was done by placing the slides in 10-slide Wheaton staining jar filled with 10mM sodium citrate buffer. The jar with slides was heated for 6 minutes in the microwave oven. The sodium citrate buffer was allowed to cool with the lid on and then reheated for another 4 minutes. After the solution was allowed to cool for ~ 10 minutes, the slides were washed three times for 10 minutes in 1 x PBS. To quench endogenous peroxidase activity, 3 % hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was pipetted onto the slides for 30 minutes. The slides were then washed in distilled water. After quenching, the slides were washed successively for 10 minutes each in 0.4 % Kodak Photo-Flo 200 (PF/PBS) in 1 x PBS, 3 drops of Triton X-100 in 150 ml 1xPBS (TX/PBS) and ~ 10 % ADB in 1 x PBS (ADB/PBS) solutions. The slides were double stained with either polyclonal AaAQP3a rabbit antibody (1:20 in ADB) and monoclonal NKA mouse antibody, a5 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) (1:10 in ADB) or polyclonal AaAQP3b rabbit antibody (1:50 in ADB) and the NKA mouse antibody, a5, (1:10 in ADB) and left in the dark at RT. Negative control slides omitting the primary antibody were also incubated overnight with 10 % ADB alone. For the peptide negative control, antibody was pre-incubated with 10x molar excess of antigen/peptide overnight at 4°C in ADB. The following day, the tissues were again washed successively with PF/PBS, TX/PBS and ADB/PBS solutions for 10 minutes each. After washing, all tissues, including controls were incubated with Alexa 594 anti-rabbit antibody (1:500 in ADB; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and cy2 goat anti-mouse antibody (1:500 in ADB; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hr at room temperature. The slides were rinsed in distilled water and then successively washed in PF/PBS, TX/PBS and PF/PBS solutions (10 min each). The slides were then rinsed 3 x in 0.4 % PF in distilled water (PF/dH<sub>2</sub>O) for 1 min and then in distilled water for 1 min. After air drying, slides

were mounted with prolong<sup>®</sup> gold Anti-fade reagent with DAPI (Invitrogen, Burlington, ON, Canada).

Preparations were viewed with an Olympus IX71 inverted fluorescent microscope (Olympus Canada, Richmond Hill, ON, Canada) and images of preparations taken using CellSens<sup>®</sup> 1.12 Digital Imaging Software (Olympus Canada, Richmond Hill, ON, Canada).

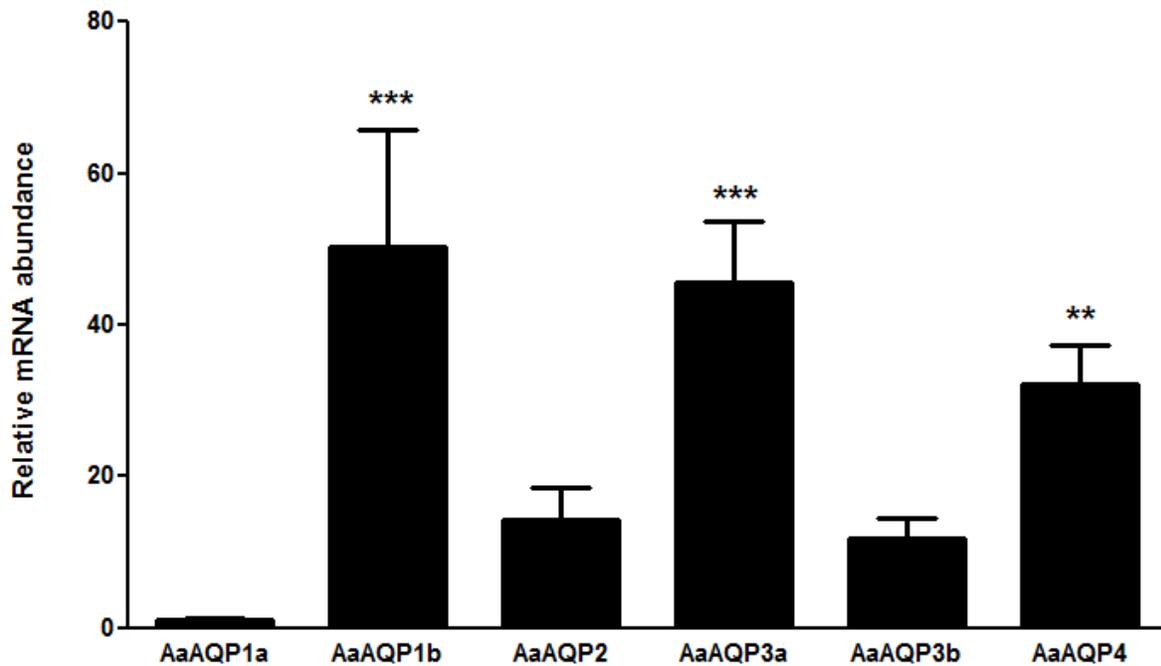
## **2.7 Statistical Analysis**

Data was analyzed using Microsoft Excel, SigmaPlot V 11.0.0.77 (USA), and GraphPad Prism 5 V 5.03 software (La Jolla, CA, USA). For the relative transcript abundance of AQP homologs in relation to the lowest expressed AQP, Anova was followed by the Dunnett's Multiple Comparison test (Fig 3.1). A t-test was used on the qPCR data to examine differences between IPW and 30% SW treatments (Figure 3.2). A t-test was similarly used for protein quantification data (Fig 3.3 and 3.4). A p value of less than 0.05 was considered to be an indicator of statistical significance. Data are expressed as mean  $\pm$  standard error of the mean (SEM) unless stated otherwise.

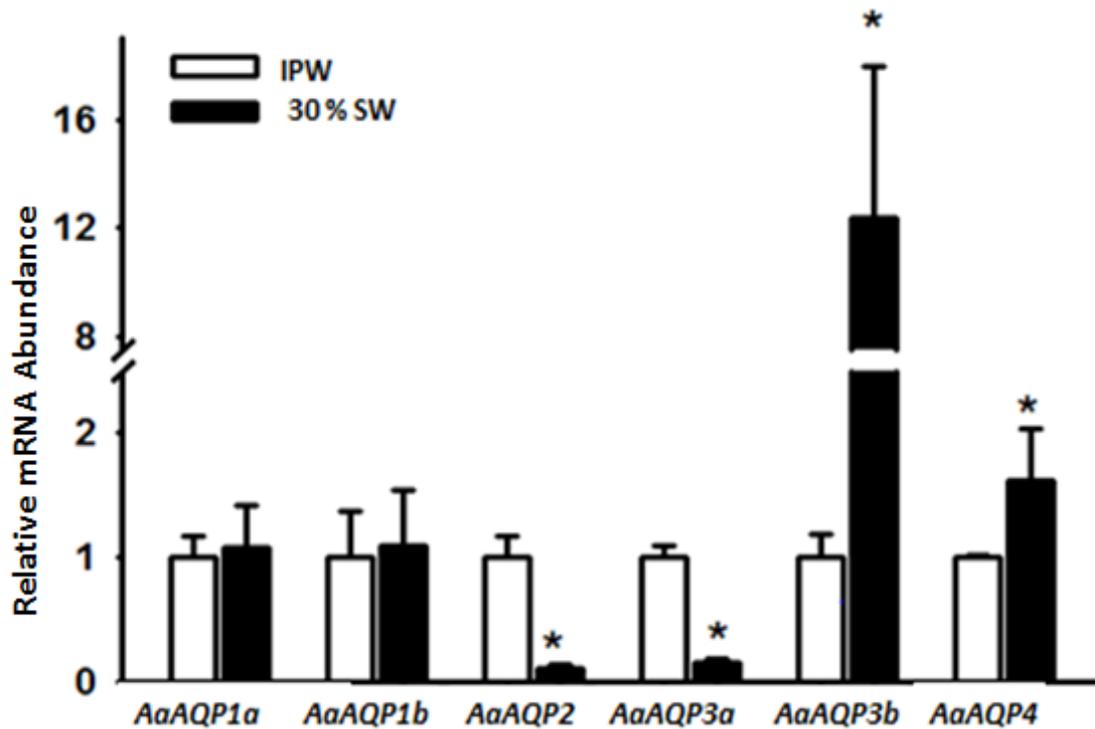
### 3 RESULTS

#### 3.1 Relative mRNA abundance of AQP homologs in the Anal Papillae of larval *Aedes aegypti*

All six AQP transcripts were found in the anal papillae of larval *A. aegypti* (Fig 3.1). In the IPW, AaAQP2a, AaAQP3a and AaAQP4 were present at a higher level compared to AaAQP1a (Fig 3.1). AQPs were found to vary in their response to salinity. AaAQP1a and AaAQP1b mRNA levels did not change with salinity (Fig 3.2). There was a 9-fold decrease in mRNA abundance of AaAQP2 and a 6.7-fold decrease in mRNA abundance of AaAQP3a upon salinity acclimation (Fig 3.2). There was a 12-fold increase in the mRNA abundance of AaAQP3b and a 1.6-fold increase in the mRNA abundance of AaAQP4 upon salinity acclimation (Fig 3.2).



**Figure 3.1: Relative mRNA abundance of AQPs in Ion-poor water.** Relative mRNA abundance of 6 Aquaporin (AQP) homologs (AaAQP1a, AaAQP1b, AaAQP2, AaAQP3a, AaAQP3b and AaAQP4) in the anal papillae (AP) of larval mosquito, *Aedes aegypti*, reared in ion-poor water. Gene expression was normalized to 18S (18S ribosomal RNA) gene. Each AQP gene is expressed relative to AaAQP1a, lowest expressed aquaporin gene and AaAQP1b, AaAQP3a and AaAQP4 are expressed significantly higher than AaAQP1a in IPW larvae. The data are expressed as mean values  $\pm$  SEM (n=4-7, \*\*\*P < 0.0001, \*\*P < 0.005, Anova followed by Dunnett's Multiple Comparison test).



**Figure 3.2:** Relative mRNA abundance of Aquaporin homologs with changes in salinity. Relative mRNA abundance of 6 Aquaporin (AQP) homologs (AaAQP1a, AaAQP1b, AaAQP2, AaAQP3a, AaAQP3b and AaAQP4) in the anal papillae (AP) of larval mosquito, *Aedes aegypti*, in 30 % SW relative to IPW. Larvae were chronically exposed to either 30% SW or IPW. All genes have been normalized to 18S (18S ribosomal RNA) gene. The data are expressed as mean values  $\pm$  SEM; n=4-7. (\*) denotes difference from IPW ( $P < 0.05$ ) (two-tailed student's t-test).

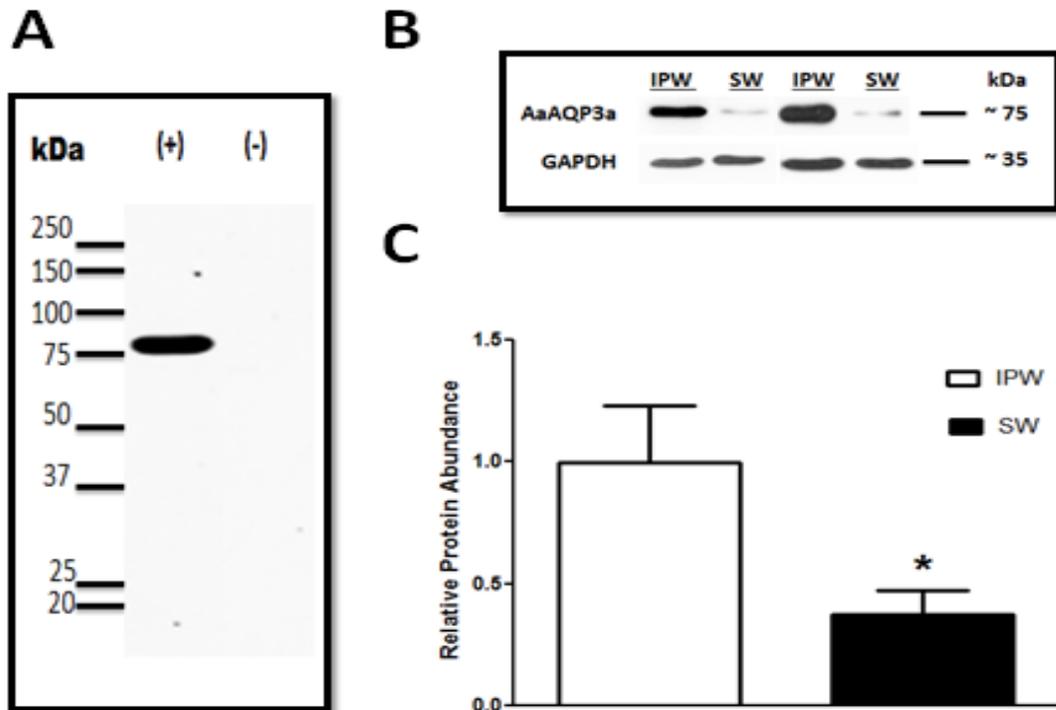
### **3.2 Protein quantification of AaAQP3a and AaAQP3b in anal papillae of larvae reared in ion-poor water and 30 % sea water**

Western blot analysis using a polyclonal antibody against a specific, unique region of AaAQP3a showed a band of approximately 75 kDa (Fig 3.3a). The specificity of the band was validated by western blot of anal papillae (AP) tissue homogenate using antisera that was pre-incubated with twice the amount of immunization peptide, resulting in the disappearance of the 75 kDa band (Fig 3.3a). Although the predicted size of an AaAQP3a monomer is ~31 kDa, the band at 75 kDa may represent a glycosylated form. Glycosylated forms of AQPs have been frequently observed in western blots detecting AQP proteins in other experiments (Cabral & Herrera, 2012; Beitz et al., 2006; Butler et al., 2006; Kim et al., 2000; Rutkovskiy et al., 2013). AaAQP3a protein levels decreased in salinity acclimated larvae as seen by a decrease in band intensity of AaAQP3a at ~75 kDa (Fig 3.3b, 3.3c) consistent with the observed decrease in mRNA levels of AaAQP3a (Fig 3.2).

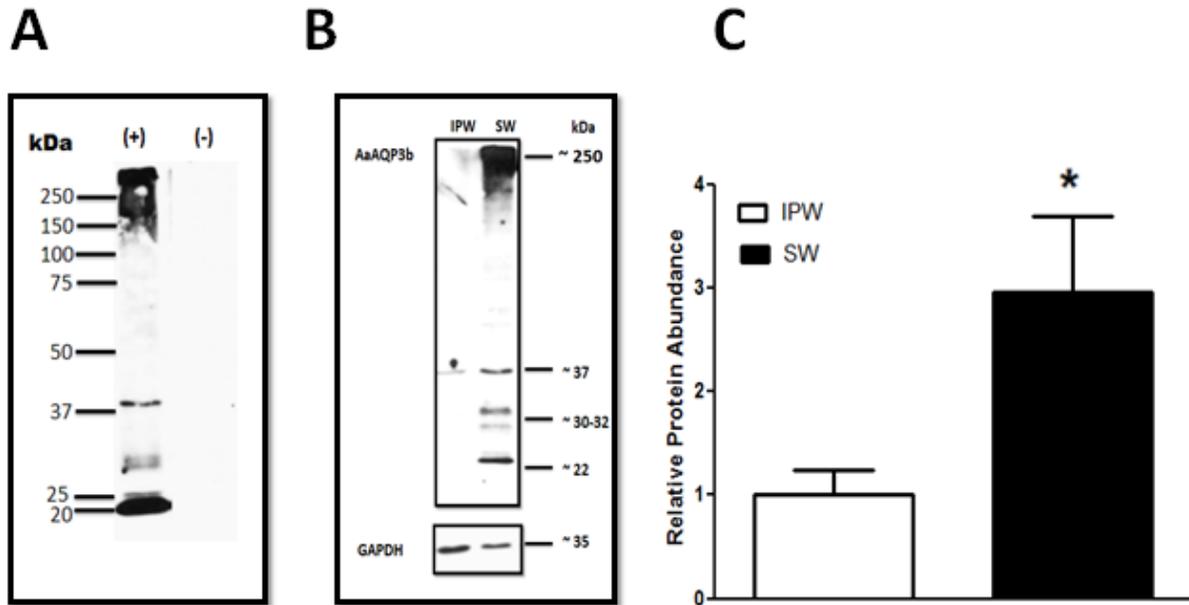
Western blot analysis using a polyclonal antibody against a specific, unique region of AaAQP3b showed several bands of various sizes (approximate sizes: ~ 250 kDa, 37 kDa, 30 kDa, 32 kDa, and 22 kDa) (Fig 3.4a, 3.4b). All of these bands disappeared when blots were probed with antisera pre-incubated with twice the amount of immunization peptide suggesting that all of the bands represent a form of AaAQP3b (Fig 3.4a). These different size proteins may represent glycosylated forms and/or oligomerized forms. AQP oligomers have also been previously observed in other conventional western blots (Le Caherec et al., 1997; Goodyear et al., 2009; Pietrantonio et al., 2000; Azuma et al., 2012). AaAQP3b protein levels increase in salinity acclimated larvae as seen by an increase in the amount of protein in SW (Fig 3.4b, 3.4c), corresponding to the observed increase in mRNA levels of AaAQP3b in the AP (Fig 3.2).

A preliminary western blot analysis of all relevant osmoregulatory tissues (WB, GC, AMG, PMG, MTs and the HG) showed bands of different sizes (Fig 3.5a). In WB, AaAQP3a appeared at around ~ 75 kDa, similar to the band seen in the AP (Fig 3.5a). No immunoreactivity for AaAQP3a was seen in the GC. Immunoreactive bands for AaAQP3a were seen at ~ 100 kDa for AMG and HG. In the PMG band of ~250 kDa was seen (Fig 3.5a). MTs showed many bands of various sizes indicating a high amount of this protein in the MTs (Fig 3.5a). These different sizes may also represent glycosylated forms and/or oligomerized forms of the protein.

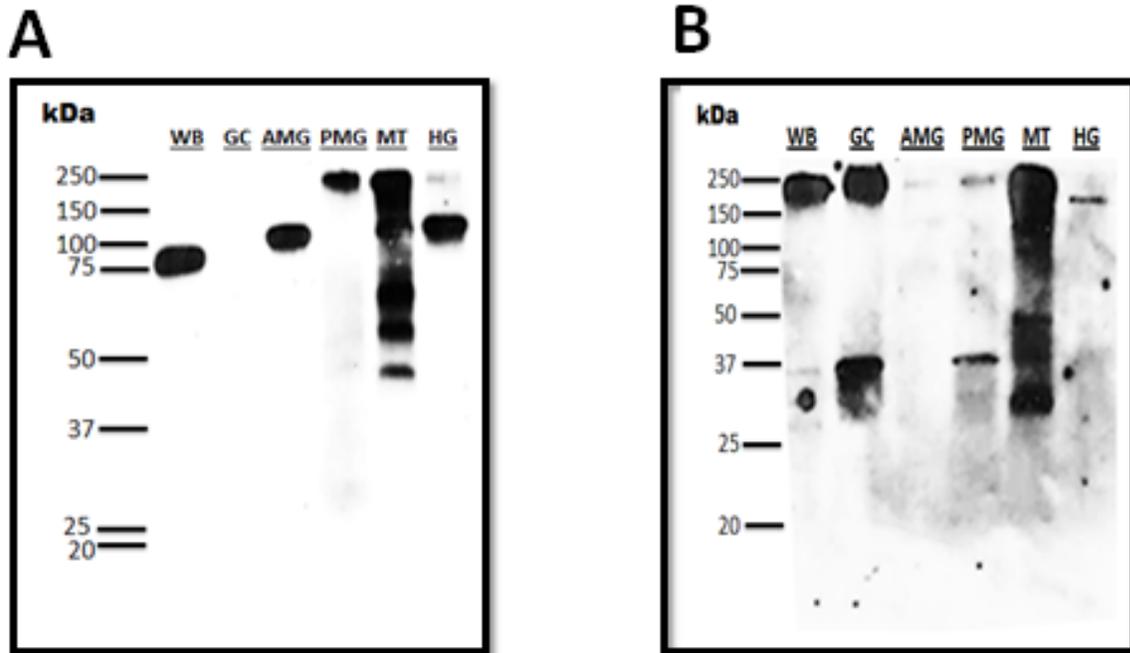
In the whole body (WB), a band immunoreactive to AaAQP3b antibody was seen at ~250 kDa (Fig 3.5b). This is similar to the band present in the AP at ~250 kDa indicating that this form of AQP is the major form present in the larvae. Based on the intensity of the band, immunoreactivity for AaAQP3b was seen in all osmoregulatory organs with low expression in the AMG, PMG and the HG (Fig 3.5b).



**Figure 3.3:** Protein expression of AaAQP3a in Anal Papillae (AP) of larval *Aedes aegypti* using AaAQP3a specific antibody. A) Western blot showing peptide blocking treatment. A band of ~ 75 kDa corresponds to a glycosylated protein in the first lane (+). This band disappears when the antibody is pre-incubated with peptide in the second lane (-); n=4. B) Western blot of AaAQP3a at ~ 75 kDa showing less protein in larvae reared in SW relative to IPW. The bands for control protein (GAPDH) at ~ 35 kDa are also shown. C) Relative protein abundance of AaAQP3a in larvae reared in ion-poor water (IPW) and 30 % seawater (SW). Protein abundance is relative to IPW and has been normalized to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). GAPDH did not vary with treatment (salinity) in these larvae ( $P < 0.05$ ) (t-test). The data showing relative expression are expressed as mean values  $\pm$  SEM; n=7. There is a significant decrease in AaAQP3a protein levels as a response to salinity exposure ( $P < 0.05$ ) (t-test). (\*) denotes significance.



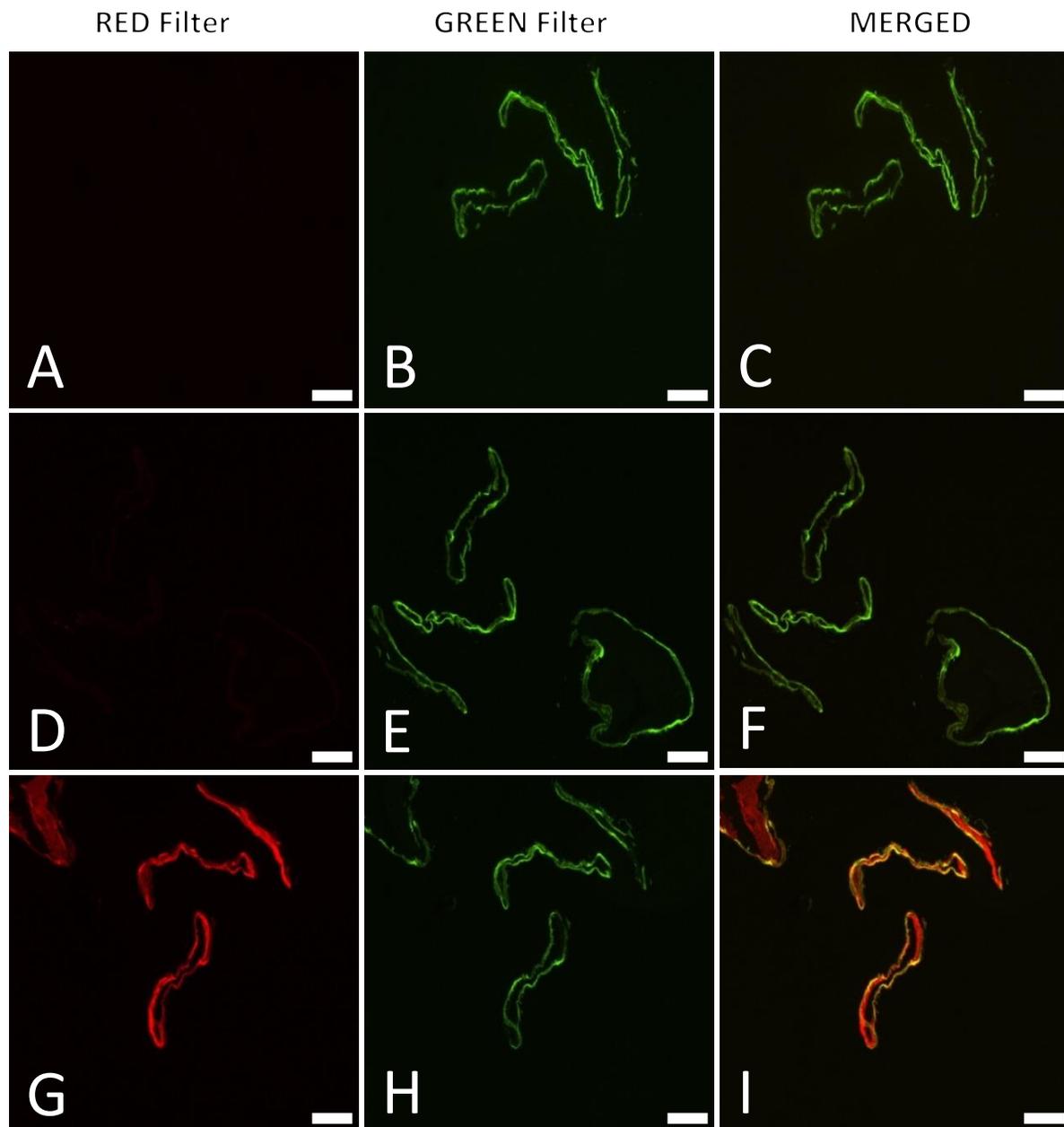
**Figure 3.4:** Protein expression of AaAQP3b in Anal Papillae (AP) of larval *Aedes aegypti* using AaAQP3b specific antibody. A) Western blot showing peptide blocking treatment. All five bands (approximate sizes; ~ 250 kDa, 37 kDa, 30 kDa, 32 kDa and 22 kDa) disappear when the antibody is pre-incubated with peptide in the second lane (-); n=4. B) Western blot of AaAQP3b showing a relative increase in protein in animals reared in SW. The bands for control protein (GAPDH) at ~ 35 kDa are also shown. C) Relative protein abundance of AaAQP3b in larvae reared in ion-poor water (IPW) and 30 % seawater (SW). Protein abundance is relative to IPW and has been normalized to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). GAPDH did not vary with treatment (salinity) in these larvae ( $P < 0.05$ ) (t-test). The data showing relative expression are expressed as mean values  $\pm$  SEM; n=6. There is a significant increase in AaAQP3b protein levels as a response to salinity exposure ( $P < 0.05$ ) (t-test). All five bands were added to quantify the protein. (\*) denotes significance.



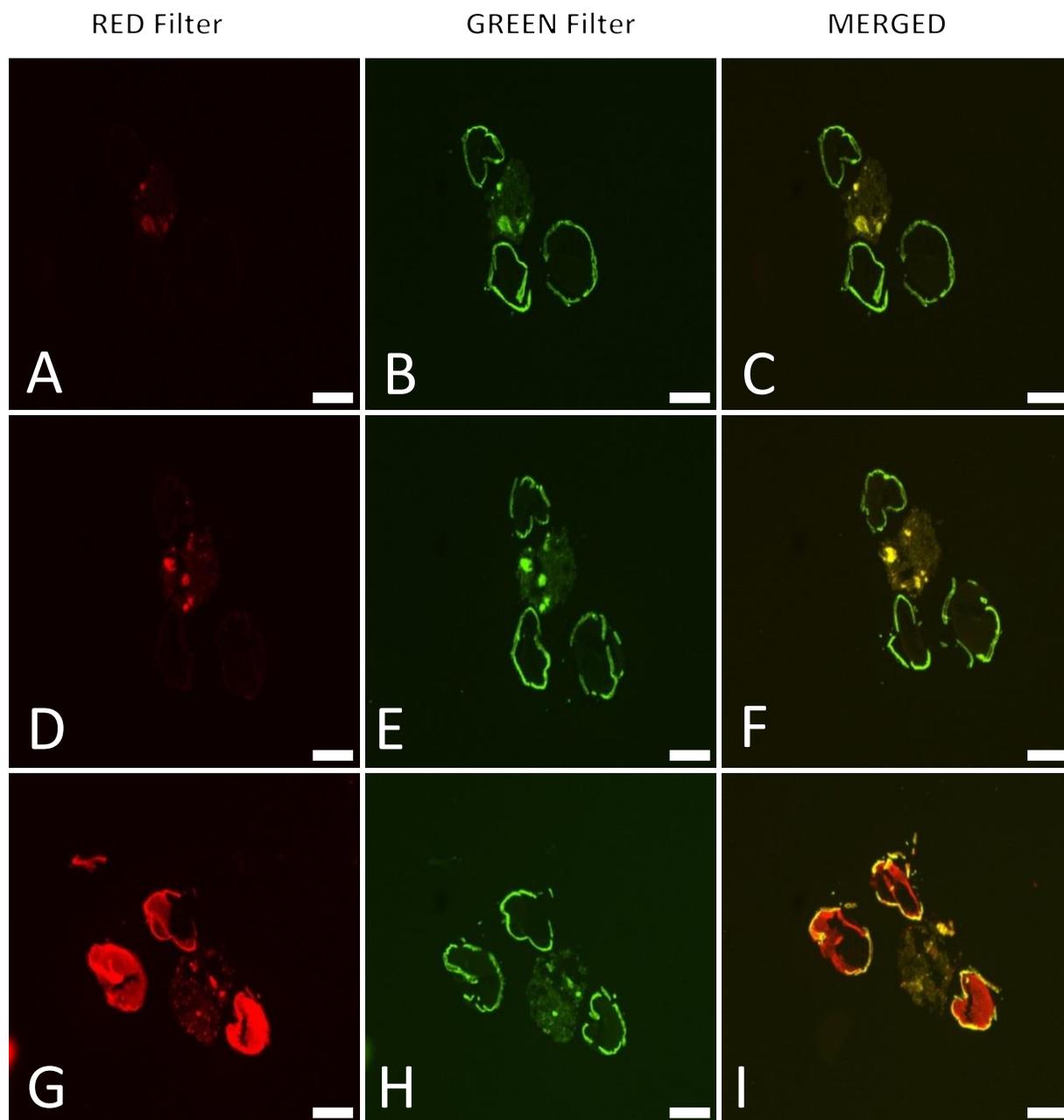
**Figure 3.5:** Preliminary western blots of AaAQP3a and AaAQP3b in whole body and other osmoregulatory tissues of larval *A. aegypti*: western blot of AaAQP3a (A) and AaAQP3b (B) in whole body (WB), gastric caeca (GC), anterior midgut (AMG), posterior midgut (PMG) and hindgut (HG) of larval mosquito, *A. aegypti*. Different band sizes may correspond to different states of AQP oligomerization and/or they may represent the glycosylated forms of the corresponding protein.

### **3.3 Immunolocalization of AaAQP3a and AaAQP3b in the anal papillae and other osmoregulatory tissues of larval *Aedes aegypti***

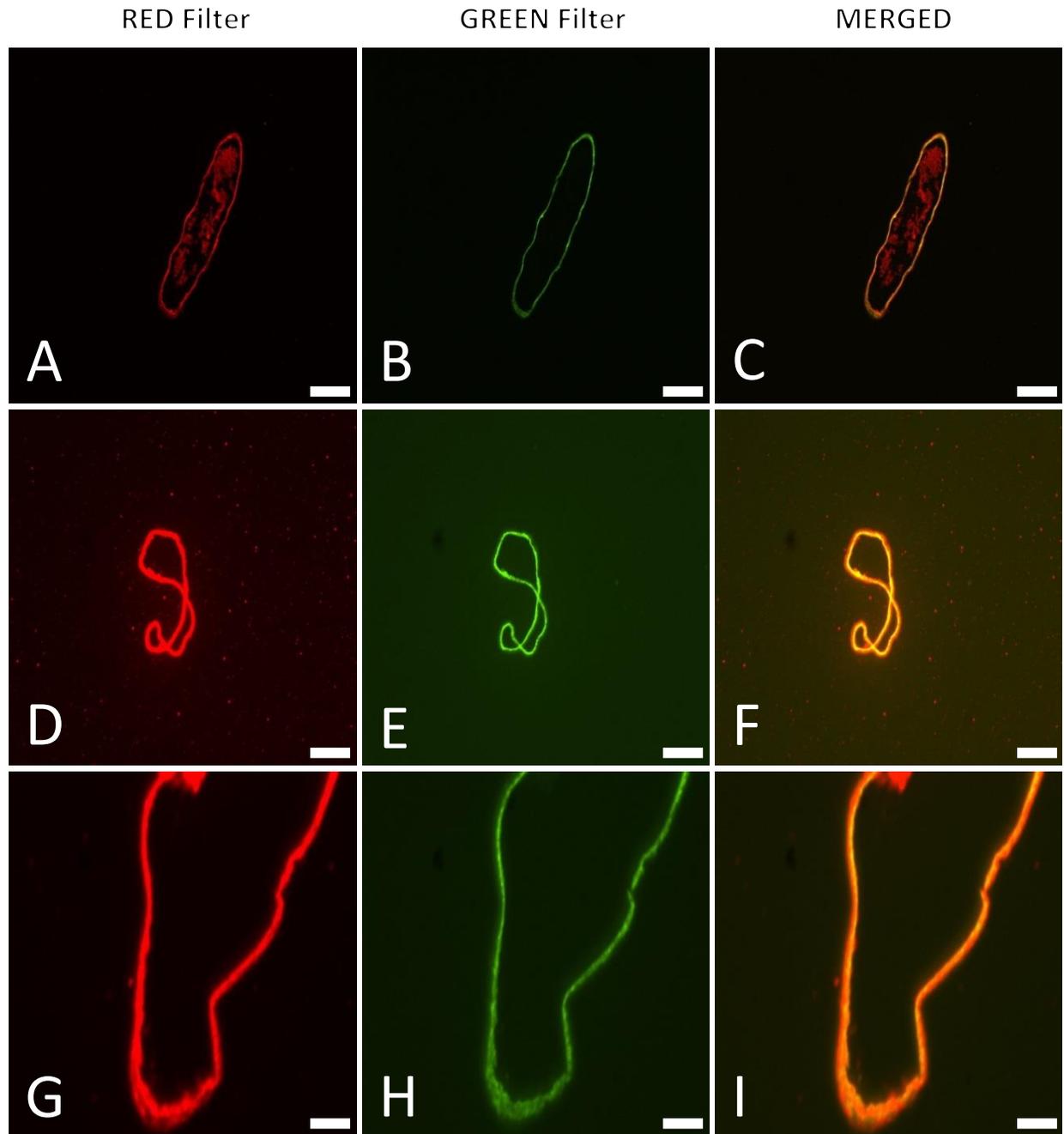
AaAQP3a and AaAQP3b were both immunolocalized to the AP epithelium. Both AQPs appeared in the apical and basal sides of the epithelium (Fig 3.6, 3.7). AaAQP3a and AaAQP3b appeared on the apical side and co-localized with NKA on the basal side. NKA has previously been localized to the basal epithelium of the AP (Patrick et al., 20 $\mu$ ). The specificity of the staining was confirmed using an antigen/peptide blocking control (Fig 3.6, 3.7). No staining was seen on the AP without the AaAQP3a antibody as well as with antisera pre-incubated with peptide control (Fig 3.6, 3.7). Figures 3.8 and 3.9 also show the staining of AaAQP3a and AaAQP3b at different magnifications. AaAQP3a and AaAQP3b were both localized to the MTs of the *A. aegypti* larvae of principal cells throughout the entire length of the MTs (Fig 3.10). AaAQP3b was also localized to the GC cells (Fig 3.11, 3.12). The point of attachment of the GC to the AMG shows a much intense staining at the apical cell membrane facing the MG lumen with NKA localized to the basal side (Fig 3.12).



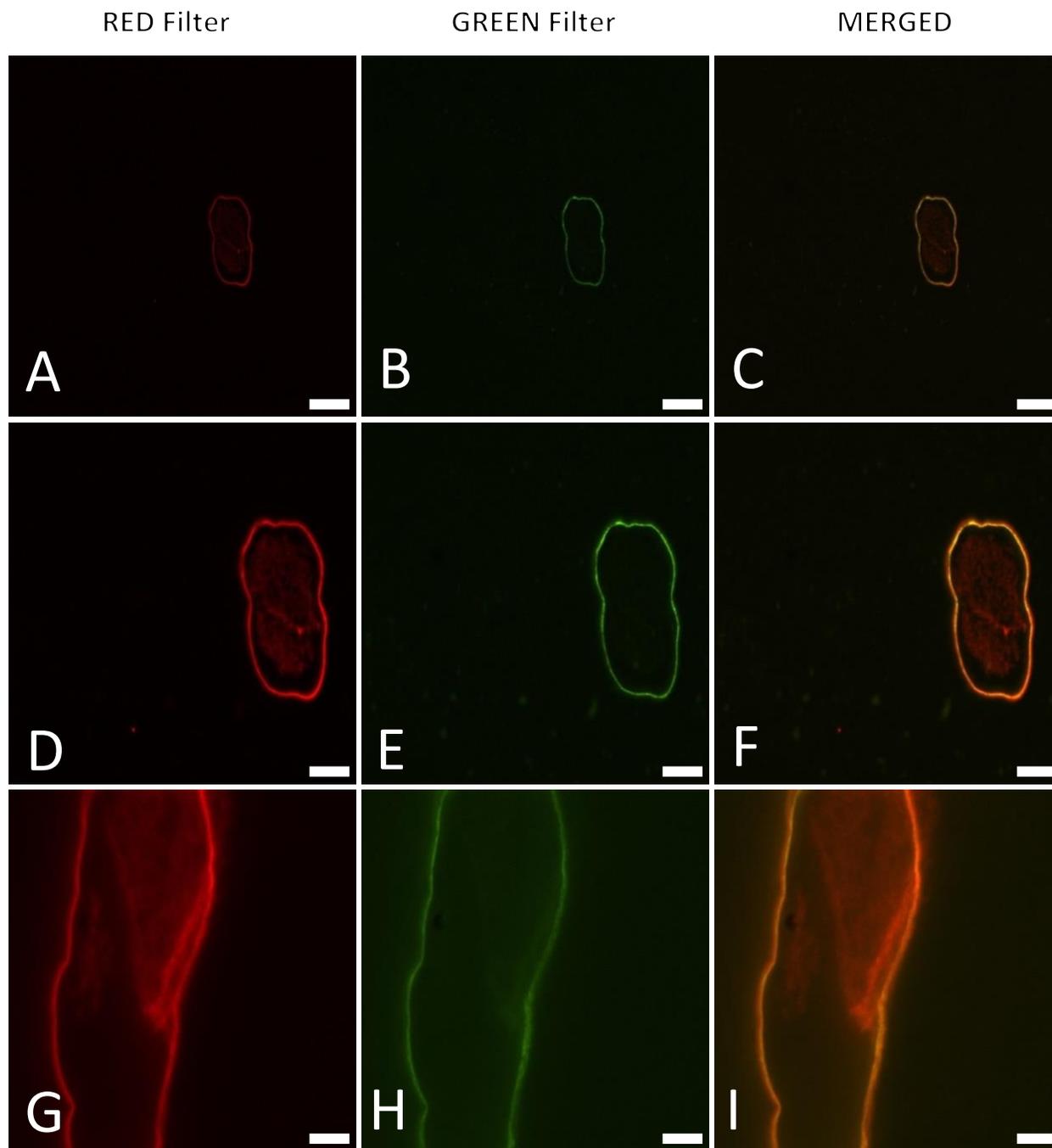
**Figure 3.6:** Immunostaining of the Anal Papillae (AP) with AaAQP3a (Red) and NKA (green) antibodies. A, B and C show a series where primary antibody for AaAQP3a was omitted (negative control). Image in A shows preparation with red filter only (AaAQP3a), B shows staining for green filter only (NKA) and C shows merged red and green filters (AaAQP3a and NKA merge). D, E and F show a series where the antibody for AaAQP3a was pre-incubated with the antigen (10x the antibody concentration). Image in D shows staining for red filter only (AaAQP3a), E shows staining for green filter only (NKA) alone and F shows merged red and green filters (AaAQP3a and NKA merge). Images in G, H and I show a series of AaAQP3a, NKA and AaAQP3a and NKA merged, respectively. Scale bar= 100  $\mu$ m.



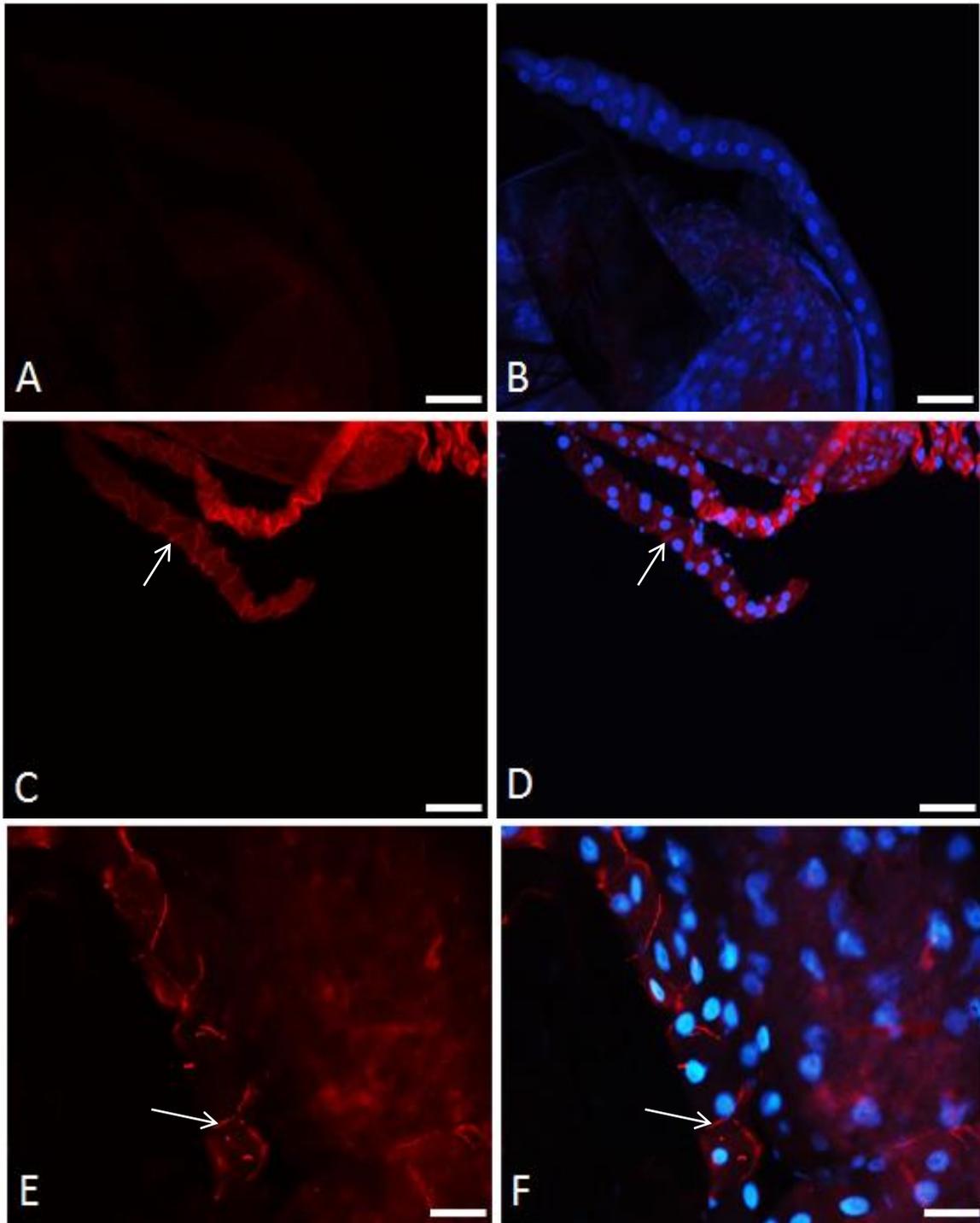
**Figure 3.7:** Immunostaining of the Anal Papillae (AP) with AaAQP3b (Red) and NKA (green) antibodies. A, B and C show a series where primary antibody for AaAQP3b was omitted (negative control). Image in A shows preparation with red filter only (AaAQP3b), B shows staining for green filter only (NKA) and C shows merged red and green filters (AaAQP3b and NKA merged). D, E and F show a series where the antibody for AaAQP3b was pre-incubated with the antigen (10x the antibody concentration). Image in D shows staining for red filter only (AaAQP3b), E shows staining for green filter only (NKA) alone and F shows merged red and green filters (AaAQP3b and NKA merge). Images in G, H and I show a series of AaAQP3b, NKA and AaAQP3b and NKA merged, respectively. Scale bar= 100  $\mu$ m.



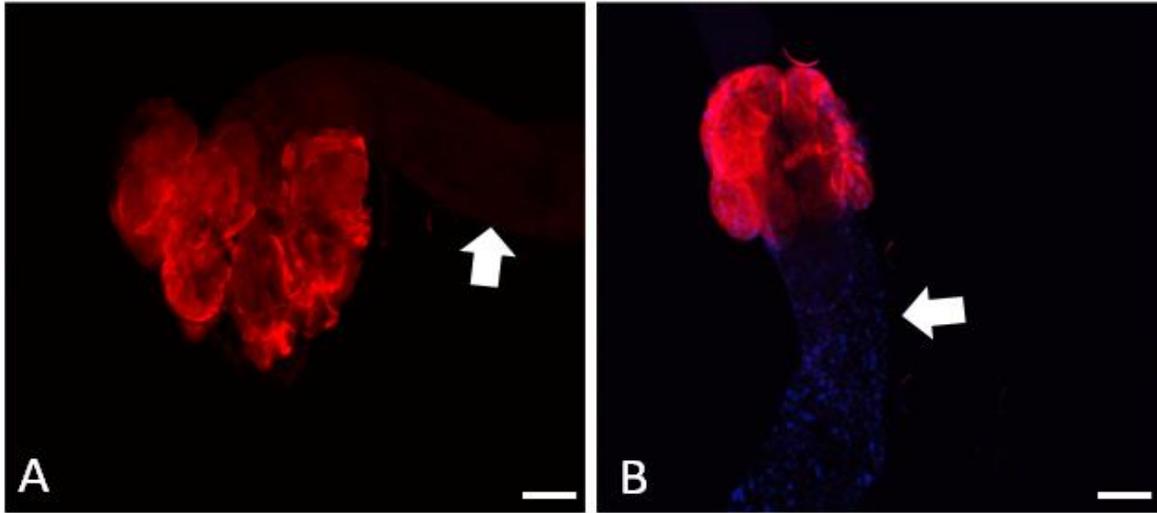
**Figure 3.8:** Immunostaining of Anal Papillae (AP) for AaAQP3a (Red) and NKA (green) at different magnifications. Images A, D and G show AaAQP3a staining of AP epithelium at 10 x, 20 x, and 40 x magnification, respectively. B, E and H show the basal membrane of AP stained with NKA at 10 x, 20x, and 40x magnification, respectively. C, F and I show localization of AaAQP3a on the apical membrane and co-localization of AaAQP3a with NKA on the basal membrane at 10 x, 20x and 40 x magnification, respectively. Scale bar (A-C)= 200  $\mu$ m; Scale bar (D-E)= 100  $\mu$ m; Scale bar (G-I)= 50  $\mu$ m.



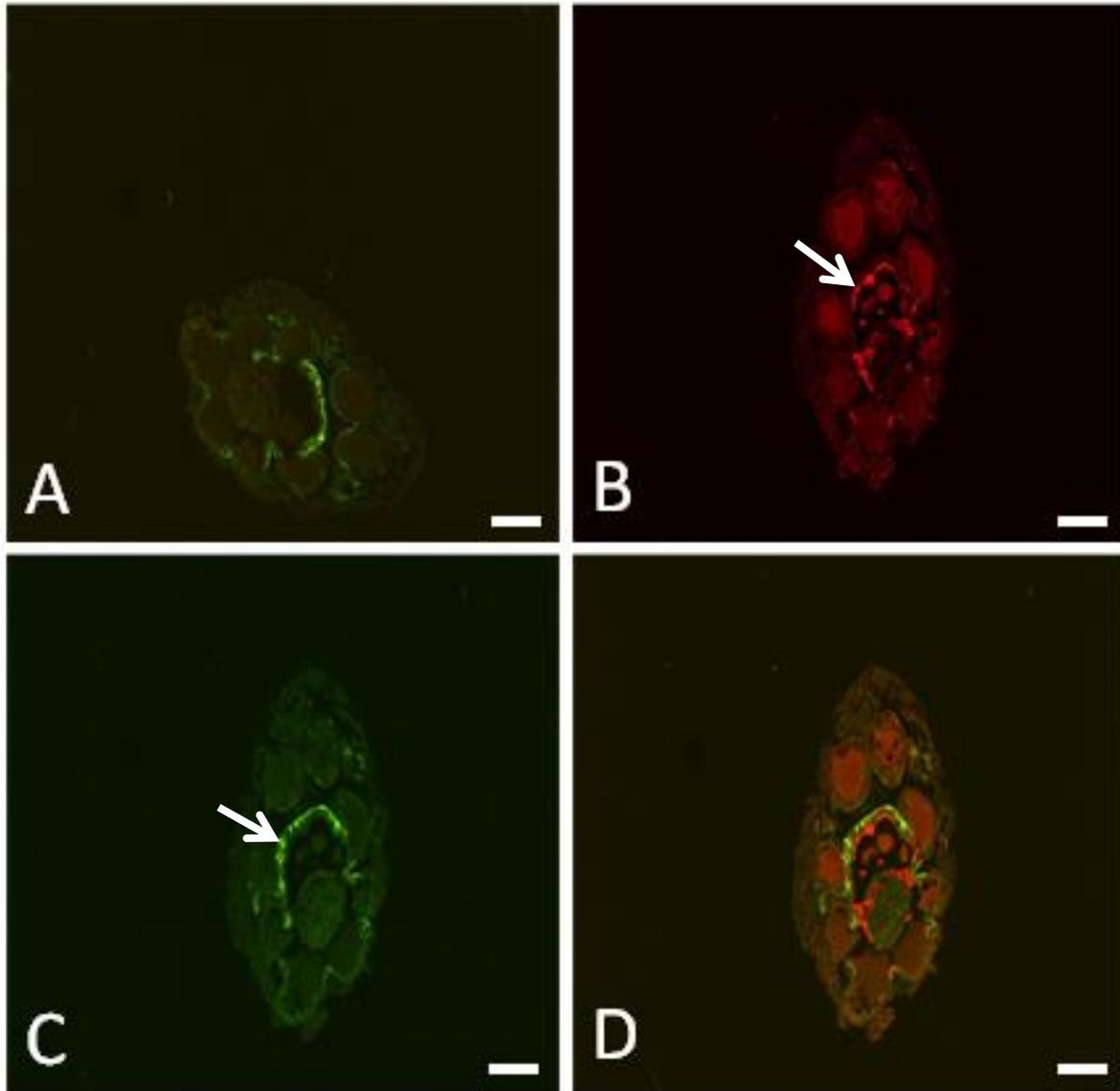
**Figure 3.9:** Immunostaining of Anal Papillae (AP) for AaAQP3b (Red) and NKA (green). Images A, D and G show AaAQP3a staining of AP epithelium at 10 x, 20 x, and 40 x magnification, respectively. B, E and H show basal membrane of AP stained with NKA at 10 x, 20x, and 40x magnification, respectively. C, F and I show localization of AaAQP3b on the apical membrane and co-localization of AaAQP3b with NKA on the basal membrane at 10 x, 20x and 40 x magnification, respectively. Scale bar (A-C) = 200  $\mu$ m; Scale bar (D-E) = 100  $\mu$ m; Scale bar (G-I) = 50  $\mu$ m.



**Figure 3.10:** Immunofluorescent staining of AaAQP3a and AaAQP3b in whole mount Malpighian tubules (MTs). A shows the negative control (no primary antibody) for MTs and B is the negative control MT stained with DAPI (shows nuclei). C shows the staining of the principal cells (arrow) of MTs for AaAQP3a (Red) and D shows the same staining with nuclei. E shows the staining of the principal cells (arrow) of MTs for AaAQP3b (Red) and F shows the same staining with nuclei. Scale bars = 100  $\mu$ m.



**Figure 3.11:** Immunostaining of AaAQP3b (Red) in whole mount gastric caeca (GC) of larval *A. aegypti*. The arrow points to the anterior midgut (AMG). Nuclei are stained with DAPI (blue) in (B). Scale bars = 200  $\mu\text{m}$ . No immunoreactivity was seen in the negative control that omitted AaAQP3b antibody (not shown here).



**Figure 3.12:** Immunostaining of the gastric caeca (GC) cross-section with AaAQP3b (Red) and NKA (green). A shows the negative control (for antigen/peptide blocking treatment). D shows the staining for AaAQP3b, C shows the staining for NKA and D shows the combined staining for AaAQP3b and NKA. Arrow in B points to the apical membrane of the anterior midgut (AMG) showing AaAQP3b staining and Arrow in C points to the basal membrane of AMG showing NKA staining. Scale bar = 200  $\mu$ m.

## 4 DISCUSSION

### 4.1 Aquaporins in the Anal Papillae of larval *A. aegypti* and the effects of salinity

In addition to being a site of ion uptake/secretion, AP is also implicated in the permeability of water across its syncytial epithelium. Experiments have shown that when the larvae is ligated at the 5<sup>th</sup> and 6<sup>th</sup> abdominal segments, the posterior segment of the larva swells due to excess water coming in from the AP and when the ligation is removed, this swelling disappears (Wigglesworth, 1933a; Stobbart, 1971). Furthermore, water gain across the AP is estimated to be up to 33% of larval body weight per day (Wigglesworth 1933a; Stobbart, 1971).

The role of AQPs in the transport of water at the AP has been supported by previous studies. Transcripts of all six AQP homologs identified by Drake et al (2010) in the *Aedes* genome were previously shown to be present in the AP of fresh water (FW) reared larvae (Marusalin et al., 2012). In addition, water transport across the AP epithelium was also decreased in the presence of HgCl<sub>2</sub>, an AQP inhibitor (Marusalin et al., 2012). In this experiment, we show that salinity response of six AQP homologs at two different extremes (ion-poor water vs. 30 % seawater) in the AP of larval mosquito varies at the transcript level. This change in transcript levels suggests that some AQP homologs may play a role in osmoregulation after the animals have acclimated to salinity.

AaAQP1a and AaAQP1b transcript levels did not change with salinity suggesting that these two homologs do not play a role in osmoregulation in the AP (Fig 3.2). Expression of AaAQP1a and AaAQP1b in *Xenopus* oocytes confirmed that they are both water specific channels (Drake et al., 2015). AaAQP1a homolog has previously been localized to the tracheoles associated with MTs in adult *A. aegypti* (Pietrantonio et al., 2000). It has been suggested that AaAQP1a plays a role in insect respiration. Tracheoles are filled with fluid under normal conditions, however, as

the metabolic demands for oxygen to the tissues increase; fluid leaves the tracheoles via AQPs and allows oxygen to reach the cytoplasm of the cells (Pietrantonio et al., 2000). Pietrantonio et al (2000) suggested that AaAQP1a is under transcriptional control in the tracheolar cells associated with MTs based on its localization to only some tracheolar cells. It is suggested to be regulated by cAMP due to the presence of *Aedes* 5-HT<sub>7</sub>-like receptors in the tracheoles associated with the MTs (Lee and Pietrantonio, 2003). Furthermore, AaAQP1a stained intensely in newly emerged females and it is suggested that this homolog may be involved in removing fluid from the tracheoles post eclosion (Duchesne et al., 2003). Hence, it is plausible that AaAQP1a is also localized to the tracheoles present in the AP lumen and supplies oxygen to AP cells (Edwards and Harrison, 1983). Edwards (1982a) showed the oxygen consumption of *A. aegypti* whole body larvae did not change in salinities ranging from 0 to 80 %. Even in the isolated AP with intact trachea, no significant change in oxygen consumption was eminent between 0, 2 and 20 % SW although the amount of mitochondria is reduced in SW (Edwards, 1982a). Hence, it may not be necessary to transcriptionally up-regulate AaAQP1a in salinity when there is no increase in demand for oxygen consumption.

AaAQP1b also did not change with salinity. In adult females *A. aegypti*, AaAQP1b is highly expressed in MTs, MG and ovaries and mRNA abundance increases 3 hours post blood feeding (Drake et al., 2010). Any role of AaAQP1b in water regulation seems to be limited to general low water permeability as knockdown of this AQP in adult MTs does not decrease diuresis after the blood meal (Drake et al., 2010). Two scenarios may be considered for AaAQP1b regulation in the AP. First, it can be concluded that AaAQP1b does not play a role in osmoregulation at all and it is possible that it is co-localized to the tracheolar cells along with AaAQP1a. On the other hand, it can be suggested to play a role in osmoregulation without being transcriptionally

regulated. AQPs are known to be present in intracellular trafficking vesicles and are transported to the cell membrane when required, often stimulated by hormones (Campbell et al., 2008). Localization of AaAQP1b would be essential to make any further conclusions. In addition, water permeability of AQPs can be altered by other mechanisms such as gating and the exact role can only be found through structural studies and/or mutational studies (Cohen et al., 2012; Törnroth-Horsefield et al., 2010; Nyblom et al., 2009).

AaAQP2 mRNA levels decrease in 30 % SW and hence it likely plays a role in osmoregulation. *A. aegypti* AaAQP2 is a homolog of the *Drosophila* BIB which does not function as a water channel in *Drosophila* but rather is involved in cell adhesion (Drake et al., 2010; Tatsumi et al., 2009). It is up-regulated in the ovaries and fat body of adult females *A. aegypti* after a blood meal and may play a role in vitellogenesis (Drake et al., 2010). In *Drosophila*, it functions as a voltage-insensitive cation channel through the central pore of an AQP tetramer (Yanochko & Yool, 2002; Spring et al., 2009). Perhaps, this homolog is involved in ion transport through its central pore in the AP in dilute water and in salinity, it decreases to prevent further ion uptake.

AaAQP3a decreased in SW at both the mRNA and protein level (Fig 2; Fig 3b, c). The lower change in protein levels compared to the mRNA levels might result from the time lag between protein translation and degradation. Protein changes in gill epithelia of European and Japanese eel have been quantitatively less intense than the changes observed at the mRNA level in response to salinity (Lignot et al., 2002; Tse et al., 2006). Drake et al (2015) have characterized AaAQP3a as an aquaglyceroporin because its expression in *Xenopus* oocytes allows the permeation of many different solutes including glycerol, urea, erythritol, adonitol, mannitol and trehalose. AaAQP3a is also capable of transporting water and its knockdown in adult MTs along with three other AQP homologs reduces post-blood meal diuresis (Drake et al., 2010; Drake et al.,

2015). Furthermore, AaAQP3a is unusual as it has an NPS motif in loop B instead of a typical NPA motif generally found in water specific channels. Hence, it is no surprise that this homolog is capable of transporting other solutes instead of just water. Further studies looking at specific additional solute transport capacity of AaAQP3a will allow for a more conclusive role of this homolog in osmoregulation in the AP. However, two possible roles of this AaAQP3a in the AP may be suggested.

Localization of AaAQP3a to the apical and basal epithelium of the AP suggests that it can allow water entry from the external habitat and its reduced role in SW suggests that it may prevent water loss across the AP epithelium in SW reared larvae. In high salinity, water would move out of the AP as a result of the high osmotic gradient and the papillae would collapse. AaAQP3a may allow regulated water uptake in ion-poor conditions and prevent water efflux in salinity altogether. In previous studies, AQP3 in gills have been down regulated in fish transferred from FW to SW to maintain osmotic balance. This decrease in AQP3 was shown in the sea bass (Giffard-Mena et al., 2007), European eel (Cutler and Cramb, 2002; Lignot et al., 2002), the Japanese eel (Tse et al., 2006) and the river pufferfish (Jeong et al., 2014). In the European eel, transcript levels of Eel AQP3 reduced by 97 % upon FW to SW and this decrease was due to the increasing levels of the hormone cortisol in plasma of SW eels (Cutler et al., 2007; MacIver et al., 2009).

Furthermore, AaAQP3a may be involved in regulating other solutes in the AP as salinity levels are altered such as organic osmolytes which can include amino acids as well as sugars. Organic osmolytes are important in maintaining osmotic pressure similar to the external environment and allows animals to osmoregulate without significantly changing their internal ion composition. Previous work showed that *A. aegypti* larvae raised in 2 % SW had lower levels of total amino acids in the hemolymph (40mM) compared to those raised in 30 % SW (125mM)

(Edwards, 1982c). Amino acids play an important role in osmoregulation by increasing the osmotic pressure of the cells as well as the hemolymph. In increased salinity, mosquito larvae can increase the levels of organic osmolytes such as proline, serine and trehalose to prevent water loss (Patrick & Bradley, 2000b). AaAQP3a may decrease in salinity to prevent loss of an intracellular osmolyte and maintain osmotic pressure of AP cells similar to that of the external habitat. It has been shown that an increase in external salinity increases the accumulation of proline in the hemolymph, which is both an intracellular and extracellular osmolyte, in *Culex tarsalis* to conform to their external environment (Patrick & Bradley, 2000b). In addition, increasing external osmolarity also increases trehalose levels in the *C. tarsalis* hemolymph which is an extracellular osmolyte (Patrick & Bradley, 2000b). Perhaps in AaAQP3a decreases to prevent trehalose from entering the cells or proline from leaving the cell and hence plays a role in maintaining cellular and/or hemolymph osmolarity.

In addition AQPs that transport glycerol are generally permeable to ammonia but not vice versa (Litman et al., 2009). It is possible that AaAQP3a is involved in decreasing ammonia efflux across the AP epithelium. AP are involved in excreting ammonia, a breakdown product of protein metabolism (Donini & O'Donnell, 2006). This decrease in ammonia efflux may allow for ammonia to be assimilated into amino acids such as proline which may be essential in maintaining osmotic pressure within the larvae during salinity exposure (Scaraffia et al., 2005).

In contrast to changes in AaAQP3a, AaAQP3b increases at both the mRNA and protein level in the AP of SW reared larvae (Fig 2; Fig 4b, 4c). Although, the predicted size of an AaAQP3b monomer is ~26 kDa, bands of higher molecular weights ~ 32-37 kDa may represent various glycosylated forms of the protein. Glycosylated forms are commonly observed in AQP western blots (Cabral & Herrera, 2012; Beitz et al., 2006; Tanya et al., 2006; Kim et al., 2000;

Rutkovskiy et al., 2013). The band greater than ~250 kDa may represent the oligomerized form of the protein similar to what has been seen in other conventional western blots detecting AQPs (Le Caherec et al., 1997; Goodyear et al., 2009; Pietrantonio et al., 2000; Azuma et al., 2012). Some AQPs exist as orthogonal arrays (OAs) in the membrane such as the mammalian AQP4 and AQPcic in the filter chamber of green leafhopper and this AQP may also form OAs and may not dissociate (Yang et al., 1996; Le Caherec et al., 1997). AQPs often oligomerize even after heating and SDS conditions of western blot (Pietrantonio et al., 2000). The size of the monomer at 22 kDa is slightly smaller than the predicated 26 kDa (Fig 4 a, b). This discrepancy may be due to the expression of a truncated form of the protein.

In SW, AaAQP3b increases both at the mRNA level and protein level and this seems to contradict the osmoregulatory role of the AP as an increase in AaAQP3b would allow osmotic efflux of water resulting in loss of ionic and water homeostasis. Expression of AaAQP3b in *Xenopus* oocytes suggested that it is highly permeable to water as well as trehalose (Drake et al., 2015). Even though AaAQP3b was localized to the AP epithelium, this was only done in untreated animals reared in freshwater and its localization in SW is necessary to determine its exact physiological role.

Furthermore, it is possible that AaAQP3b plays an osmosensory role in the AP in salinity by localizing to the basal membrane only. AQPs are able to act as osmosensors by regulating cell volume (Watanabe et al., 2005). It is suggested that in FW acclimated tilapia, cell volume changes regulated by AQP3 induces the release of prolactin from the prolactin releasing cells of the pituitary (Watanabe et al., 2005). Previous work has also shown that anal papillae cells swell when FW larva *A. argenteus* are exposed to 1-1.5 % NaCl (Wigglesworth, 1933b). This swelling would only be possible with water entry from the basal membrane. The high permeability of AaAQP3b

as shown in *Xenopus* oocytes would allow this homolog to effectively regulate cell volume (Drake et al., 2015). Perhaps a similar phenomenon is occurring in the cells of *A. aegypti* larvae and AQPs are involved.

In addition, it can be speculated that AaAQP3b plays a role in transport of additional and an increased expression in salinity allows regulation of other solutes. Anal papillae are involved in removal of nitrogenous waste by ammonia excretion (Donini & O'Donnell, 2005). AQPs have been previously shown to allow permeation of other solutes such as urea and ammonia (Campbell et al., 2008). Likewise, AaAQP3b may be involved in the excretion of ammonia in SW rather than in regulation of water balance. In Killifish kfAQP3 expression increases in the mitochondrial rich cells of the gills as a response to increase in external salinity and this increase is suggested to play a part in ammonia/ammonium transport (Jung et al., 2012). In the euryhaline teleost, *Anabas testudineus*, Aq1aa is not involved in osmoregulation in the gills as exposing these fish to 1 or 6 day salinity does not alter transcript abundance (Ip et al., 2013). Instead, it plays a role in ammonia excretion as its abundance increases in gills and skin when fish are exposed to terrestrial environments. During locomotion, these fish increase protein metabolism and produce more ammonia which is suggested to be excreted by the central pore of Aq1aa tetramer (Ip et al., 2013). Furthermore, increase in NH<sub>4</sub>Cl in the aquatic habitats of *A. testudineus* results in reduced expression of Aq1aa to prevent influx of ammonia (Ip et al., 2013). It is also suggested that Aq1aa may be involved in ammonia excretion during long term salinity acclimation since initially these fish reduce ammonia excretion and accumulate amino acids with gradual increases in salinity to regulate cell volume, however, in long term SW acclimation, ammonia excretion increases again (Ip et al., 2013).

AQPs permeable to ammonia differ in specific residues in the ar/R region (Wu & Beitz., 2006; Litman et al., 2009). The amino acid residue at position 180 has been considered the most important in determining aquaporin permeability to ammonia (Litman et al., 2009). Water-specific mammalian AQP1 becomes permeable to ammonia when histidine at position 180 is replaced with alanine (Beitz et al., 2006). Histidine at position 180 is also replaced by isoleucine of a known ammonia permeable mammalian AQP8 (Beitz et al., 2006; Campbell et al., 2008). *Aedes* AaAQP3b also has alanine at position 180, hence, it is possible that it is permeable to ammonia and an increase in SW corresponds to the excretion of ammonia. In addition, ammonia permeable AQPs have generally been co-localized with ammonia transporting Rhesus (Rh) proteins (Litman et al., 2009). AP also expresses two Rh proteins and perhaps one or more of the AQP homologs co-localizes with Rh proteins (P. Bui and A. Ionescu, personal communication).

AaAQP4 also increases in salinity. Even though it is was not localized in the AP, it can be speculated to be co-localized with AaAQP3b and play a similar role. AaAQP4 is more related to the mammalian unclassified AQPs (AQP11 and AQP12) and *Drosophila* CG12251 and highly expressed in *A. aegypti* adult foregut (Drake et al., 2010). It contains a NPVLA instead of the usual NPA region in its second loop E and it is likely to serve as a non-specific water channel. Future structural studies would determine the significance of this AQP homolog.

#### **4.2 Aquaporins in other osmoregulatory tissues of larval *A. aegypti***

Western blot analysis of AaAQP3a showed the homolog to be present in all osmoregulatory tissues albeit in different quantities (Fig 3.5). AaAQP3b was also present in all osmoregulatory tissues except for the GC (Fig 3.5). Bands appearing at a higher MW than the predicted sizes are likely to represent undissociated AQP oligomers or the glycosylated form of the proteins (Verkman & Mitra, 2000). AQPs often exist as tetramers in cell membranes and some exist as orthogonal

arrays such as the mammalian AQP4 (Yang et al., 1996; Le Caherec et al., 1997). In a previous study, a western blot analysis of AaAQP1a in the MTs of female adult *A. aegypti* also showed higher MW bands than the predicted size (~26.2 kDa) and was concluded to be AQP oligomers (Pietrantonio et al., 2000). Furthermore, western blot of colon and rectum of *Bombyx mori* for AQP-Bom1 also showed a monomer (27 kDa) and a higher band of tetrameric state (118 kDa) and AQP- Bom 3 also showed a band of ~ 21 kDa and a higher band of tetrameric state (107 kDa) (Azuma et al., 2012). Higher MW bands may also be due to glycosylation. Glycosylation is important in cell surface expression of AQPs as well as protein stabilization in the plasma membranes although water transport function does not depend on glycosylation (Hendriks et al., 2004; Buck et al., 2004). *A. aegypti*, AaAQP1a, is reported to have a potential glycosylation site in loop E (Asn211-Trp212-Thr213) (Pietrantonio et al., 2000).

Immunostaining and a preliminary western blot both confirmed the presence of AaAQP3a and AaAQP3b in the MTs of *A. aegypti* larvae. Staining of AaAQP3a and AaAQP3b in larval *A. aegypti* MTs is consistent with the functional role of these tissues in fluid excretion (Fig 3.10). Different molecular weights present in the western blot may correspond to different forms of AQPs (glycosylated and/or tetramerization) and gives an indication of high protein levels of these AQP homologs in the MTs. High protein concentration of AQPs in MTs is in support of the functional role that MTs play in fluid excretion. Previously, high transcript of both AaAQP3a and AaAQP3b were found in the larval MTs along with AaAQP1a and AaAQP2 (Marusalin et al., 2012). In addition, previous work by Drake et al (2010) showed that both AaAQP3a and AaAQP3b, along with AaAQP1a are the main AQPs associated with the adult female *A. aegypti* and play a role in diuresis after a blood meal. AaAQP1a was previously localized to the tracheolar cells associated with the MTs in adult *A. aegypti* where it is recognized for transporting fluid into and out of the

tracheoles with altering metabolic demands (Pietrantonio et al., 2000; Duchesne et al., 2003). Our work now shows that AaAQP3a and AaAQP3b are also present in the MTs of larval *A. aegypti*. In this study, AaAQP3a and AaAQP3b both localized to the principal cells (Fig 3.10). AQP homologs have been associated with MTs of many blood feeding and non-blood feeding insects. Presence of AQPs in MTs have also been identified in MTs of other insects such as the *Rhodnius prolixus* (Echevarria et al., 2001; Martini et al., 2004; Staniscuaski et al., 2013), *Bombyx mori* (Azuma et al., 2012), *Drosophila* (Kaufmann et al., 2005) and *Anopheles gambiae* (Liu et al., 2011).

Presence of AaAQP3b in the GC but not AaAQP3a is consistent with previous work showing that AaAQP3b is the only abundant AQP homolog present in the GC at the transcript level (Marusalin et al., 2012). GC cells of *A. argenteus* are involved in fluid absorption and in SW reared larvae, fluid absorption becomes more rapid (Wigglesworth, 1933b). It can be suggested that AaAQP3b is also involved in fluid absorption in the GC of *A. aegypti* larvae whose role may be more significant in higher salinity.

A more intense band for AaAQP3a compared to AaAQP3b in the MG is also in agreement with a relatively higher expression of AaAQP3a at the transcript level (Marusalin et al., 2012) (fig 3.5a, 3.5b). In the hindgut, AaAQP3a appears to be expressed at higher levels than AaAQP3b which is also consistent with the previously reported relative transcript expression (Marusalin et al., 2012).

## 5 CONCLUSION AND FUTURE DIRECTIONS

The freshwater larvae of *A. aegypti* must be able to maintain ion and water balance for survival in their dilute habitat. Regulation of water across the Anal Papillae (AP) epithelium is important for larval survival. This study determined the relative abundance of six AQP homologs in the AP of larvae acclimated to ion-poor water and 30 % SW. Results indicated that four out of six AQP homologs are salinity responsive. In addition, two of these homologs (AaAQP3a and AaAQP3b) are also salinity responsive at the protein level and localized to the AP epithelium. Future work looking at the functional role of AaAQP3a and AaAQP3b in the AP will determine the exact mechanism by which these two homologs are involved in osmoregulation. Specific transport properties of these AQPs in the Anal Papillae can be determined using gene knockout and subsequently measuring changes in specific ion transport properties of AQPs using Scanning ion-selective electrode technique (SIET) including Ammonia. SIET has been described in detail by Donini & O'Donnell (2005). However, gene knockdown studies are limited as multiple AQPs are usually associated with each tissue/cell and knockdown of one may result in increased function of another. For example, a knockdown of an AQP in pea aphid did not affect survival or body weight gain, possibly due to some other AQP homologs taking over the water transport function (Shakesby et al., 2009).

In addition, additional solute transport capacities of both AaAQP3a and AaAQP3b can be assessed using African clawed frog, *X. laevis*, oocyte functional assays as done by Drake et al (2015) for six different solutes. *Xenopus* oocytes are water impermeable and have conventionally been used to understand transport properties of AQPs (more examples- Kikawada et al., 2008; Goto et al., 2011). AQP properties can also be identified using yeast expression systems (as done by Staniscuaski et al., 2013). Some AQPs also alter their permeability as a result of pH and

hemolymph pH is inversely altered in relation to external salinity as shown for *A. aegypti* larvae (Donini et al., 2007). Future experiments can also consider changes in AQP permeability induced by changes in hemolymph pH. Experiments can also look at the hormonal regulation of AQPs in the AP with changes in salinity. It has previously been suggested external salinity increases the ion concentration of hemolymph (Donini et al., 2007). External salinity causes the release of 5-HT which increases fluid secretion in the larval MTs (Donini et al., 2006; Clark & Bradley, 1997)

In addition, preliminary western blot showed the presence of AaAQP3a protein in all osmoregulatory tissue except for the gastric caeca and presence of AaAQP3b protein in all the osmoregulatory tissues. Furthermore, AaAQP3a and AaAQP3b were both immunolocalized in the Malpighian of larval *A. aegypti* and AaAQP3b was immunolocalized to the gastric caeca. Future work can examine the changes in abundance of these proteins in relation to changes in salinity to determine their significance in osmoregulation.

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