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Salinity responsive aquaporins in the anal papillae of the larval mosquito, *Aedes aegypti*

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ABSTRACT

The larvae of the mosquito, *Aedes aegypti* normally inhabit freshwater (FW) where they face dilution of body fluids by osmotic influx of water. In response, the physiological actions of the anal papillae result in ion uptake while the Malpighian tubules and rectum work in concert to excrete excess water. In an apparent paradox, the anal papillae express aquaporins (AQPs) and are sites of water permeability which, if AQPs are expressed by the epithelium, apparently exaggerates the influx of water from their dilute environment. Recently, naturally breeding populations of *A. aegypti* were found in brackish water (BW), an environment which limits the osmotic gradient. Given that salinization of FW is an emerging environmental issue and that these larvae would presumably need to adjust to these changing conditions, this study investigates the expression of AQPs in the anal papillae and their response to rearing in hypo-osmotic and near isosmotic conditions. Transcripts of all six *Aedes* AQP homologs were detectable in the anal papillae and the transcript abundance of three AQP homologs in the papillae was different between rearing conditions. Using custom made antibodies, expression of two of these AQP homologs (AQP4 and AQP5) was localized to the syncytial epithelium of the anal papillae. Furthermore, the changes in transcript abundance of these two AQPs between the rearing conditions, were manifested at the protein level. Results suggest that AQP4 and AQP5 play an important physiological role in larval responses to changes in environmental salinity.

1. Introduction

In dilute freshwater conditions the internal body fluids of aquatic larval mosquitoes are hypertonic to their environment and hence they face osmotic influx of water. Under these conditions the anal papillae function to sequester ions through the active uptake of Na^+, K^+ and Cl^- from the habitat, across the epithelium and into the hemolymph that fills the papillae lumen (Donini and O'Donnell, 2005; Stobbart, 1971a). The anal papillae are also permeable to water and the uptake of water at the anal papillae accounts for roughly 33% of larval body weight per day (Stobbart, 1971b; Wigglesworth, 1932). The epithelium of the anal papillae is a syncytium with no paracellular pathway, and aquaporins have been implicated in mediating the observed water uptake at the anal papillae (Marusalin et al., 2012). Water uptake by the anal papillae seems counterproductive to the sequestering of ions in the dilute conditions and the physiological basis for expression of aquaporins in the anal papillae epithelium is not yet clear.

The mosquito *Aedes aegypti* is a vector of arboviral diseases such as dengue, chikungunya, yellow fever and Zika. The larvae of this mosquito are well known to inhabit freshwater and population control measures are routinely focussed on potential freshwater habitats (Surendran et al., 2012). More recently *A. aegypti* has been observed to complete development in brackish water (5 to 30% SW) in urban wells of Sri Lanka as well as artificial containers along coastlines (Ramasamy et al., 2011; Surendran et al., 2012). In the laboratory *A. aegypti* can tolerate up to 30% seawater (SW) which is roughly isosmotic to the larval hemolymph, with survival rates of about 50% when salinity reaches 40% SW (Clark et al., 2004). These observations are important for strategies of vector control because the salinization of freshwater from anthropogenic and natural sources is of global concern (Colombani et al., 2016; Kefford et al., 2016; Wallace and Biastoch, 2016).

Studies have examined how the anal papillae ultrastructure and physiology of ion uptake are affected by brackish water (Donini et al., 2007; Sohal and Copeland, 1966). When larvae are exposed to 30% SW for several hours, the Na^+ and Cl^- uptake kinetics are altered such that NaCl uptake is decreased (Donini et al., 2007). These observations are consistent with ultrastructural changes of the anal papillae epithelium where there is reduced membrane folding (surface area) and fewer mitochondria when larvae are exposed to isotonic and hypertonic solutions of NaCl (Sohal and Copeland, 1966). The effect of salinity on water permeability of the anal papillae has not been examined. Since the epithelium is a syncytium and aquaporins are implicated in water permeability of the anal papillae, a study on how salinity may affect aquaporin expression is a plausible starting point.

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Aquaporins (AQPs) are members of a large family of major intrinsic proteins (MIP) and are ubiquitous among organisms. They are integral membrane proteins that typically mediate bidirectional water permeability across membranes driven by osmotic gradients (Campbell et al., 2008). There are currently thirteen identified AQP homologs (AQP0 to AQP12) in mammals (Agre and Kozono, 2003; Ishibashi et al., 2011). They are divided into two main groups, orthodox AQPs which typically only allow permeation of water, and aquaglyceroporins which allow permeation of polyols (*e.g.* glycerol) and other small solutes (Campbell et al., 2008).

Six aquaporin genes have been identified in *Aedes aegypti* and four of these have recently been functionally characterised by expression in *Xenopus* oocytes (Drake et al., 2010, 2015; Goto et al., 2011; Kambara et al., 2009). There is a discrepancy in the nomenclature of these aquaporins and this has been previously presented (Marusalin et al., 2012). In the present study we will conform to the nomenclature presented in the recent study that functionally characterised the *Aedes* aquaporins (Drake et al., 2015). AQP1 and AQP2 were shown to be strong conductors of water without allowing permeation of polyols or other solutes (Drake et al., 2015). AQP4 is functionally an aquaglyceroporin showing strong conductance of glycerol and other solutes and very weak conductance of water (Drake et al., 2015). AQP5 is a strong water transporter but can also weakly allow permeation of other solutes such as urea and trehalose (Drake et al., 2015). A previous study examining the transcript expression of aquaporins in larval *A. aegypti* reared in freshwater (FW) (20 μmol l^{-1} Na^+ and 40 μmol l^{-1} Cl^-) revealed high transcript abundance of AQP2 and AQP6 in the anal papillae (Marusalin et al., 2012). In agreement with the functional expression, the observed water permeability of the anal papillae of FW reared larvae was attributed to AQP2 (Marusalin et al., 2012). Additional water permeability through AQP6 cannot be ruled out since the transport characteristics of AQP6 awaits further study (Drake et al., 2015; Marusalin et al., 2012).

Armed with the information of transcript expression of aquaporins in the anal papillae of FW reared larvae, this study aimed to examine if alterations to external salinity lead to changes in aquaporin expression in these important larval osmo and ion regulatory organs. We challenged larvae to two extremes, ion poor water (IPW), and 30% SW treatment representing brackish water (BW) which was near the upper salinity tolerance of *A. aegypti* larvae (Clark et al., 2004). The transcript abundance of the six aquaporins was examined and further studies at the protein level were conducted on the resulting salinity responsive aquaporins. Based on the changes in expression, the transport characteristics, and the localisation of these aquaporins we propose putative physiological functions for these transporters in the anal papillae of larval mosquitoes.

2. Materials and methods

2.1. Mosquito rearing and treatments

Eggs of *Aedes aegypti* from a colony maintained in the Department of Biology, York University (Toronto, Canada) were hatched in distilled water. Roughly 100 hatchlings were transferred into each of two containers, one with 600 ml water filtered by a Milli-Q water purification system (EMD Millipore, Etobicoke, Ontario, Canada) hereby referred to as ion poor water (IPW) and the other with 600 ml 10% seawater consisting of 2.5 g l^{-1} of Instant Ocean® sea salt (Spectrum Brands, Blacksburg, VA, USA) dissolved in Milli-Q purified water. After one day the larvae in 10% seawater were transferred to 600 ml of 20% seawater (5 g l^{-1} Instant Ocean®) and the larvae in IPW to fresh 600 ml of IPW. After another day the larvae in 20% seawater were transferred to 600 ml of 30% seawater (7.5 g l^{-1} Instant Ocean®), hereby referred to as brackish water (BW), and the larvae in IPW to fresh 600 ml of IPW. At this stage the larvae were reared until they reached the fourth larval stage (~1.5 weeks). Larvae were fed every two days with 2 ml of a 1:1 yeast:liver powder solution and the water was refreshed every 2 days prior to feeding.

2.2. RNA extraction and cDNA synthesis

One biological sample consisted of anal papillae (AP) collected from a group of 60–90 larvae after rearing to the fourth larval stage as described above. In total there were eight biological samples for IPW reared larvae and eight biological samples for BW reared larvae. The anal papillae were removed from larvae and immediately transferred to 200 μl of RNAlater® (Qiagen, Toronto, ON, Canada). Once all AP for a single biological sample were collected the RNAlater® was carefully removed and 1 ml of TRIzol® (Invitrogen, Burlington, ON, Canada) was added. The samples were sonicated for 5 s at 5 W using an XL 2000 Ultrasonic Processor (QSonica, Newtown, CT, USA) and RNA was extracted according to the TRIzol® instructions. Samples were treated with the TURBO DNA free™ kit (Ambion, Burlington, ON, Canada) to remove genomic DNA. The quality and quantity of RNA was determined using a Multiskan spectrum spectrophotometer (Thermo Scientific, Mississauga, ON, Canada). All sixteen samples had an optical density absorption ratio (260/280) of >1.8. cDNA for each sample was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad, Mississuaga, ON, Canada) using 1 μg/μl of total RNA.

2.3. Quantitative real-time PCR (qRT-PCR)

To determine the relative mRNA abundance of *AQP4* and *AQP5* in anal papillae of larval *A. aegypti* reared in IPW and BW, quantitative PCR (qPCR) was performed using primers designed and described by Marusalin et al. (2012) and listed in Table 1 (Marusalin et al., 2012). Each reaction consisted of 10 μl of SsoFast™ Evagreen® Supermix (Bio-Rad), 2 μl of cDNA template, 0.5 μl of 10 μmol l^{-1} forward primer, 0.5 μl of 10 μmol l^{-1} reverse primer and 7 μl of PCR water (DNAse free, RNAse free, Sterile, Invitrogen). Reactions were performed in two technical replicates with a no cDNA template control included for each gene in the CFX96™ real-time PCR detection system (Bio-Rad). Reactions ran with the following settings, 2 min of enzyme activation at 95 °C, followed by 39 cycles of 5 s denaturation at 95 °C and 5 s of annealing/extension at 58 °C. In order to confirm the presence of a single product, a melting curve analysis was performed after each reaction and consisted of holding the product at 95 °C for 10 s followed by holding the product at 65–95 °C with 0.5 °C increments held for 5 s each. For each gene, a standard curve was generated to optimize reaction efficiency. Quantification of transcripts was determined using the Pfaffl method (Pfaffl, 2004). The *18S rRNA* gene was used as an internal control because it showed consistent levels of transcript expression under experimental treatments. Primers for *18S rRNA* were designed and described by Jonusaite et al. (2016) and included in Table 1.

2.4. Antibodies for AQP4 and AQP5

Custom designed antibodies against AQP4 and AQP5 were produced by GenScript (Piscataway, NJ, USA). *In silico* analysis of specific antigenic epitopes within the AQP4 and AQP5 protein sequences was conducted by Genscript using the GenScript OptimumAntigen™ design tool. Sequence specificity of epitopes was confirmed by a BLAST search (NCBI database; http://www.ncbi.nlm.nih.gov). For AQP4 a rabbit polyclonal antibody was raised against the unique epitope PAEQAPSDVGKSNQ in the N terminal cytoplasmic region of the protein. For AQP5 a rabbit polyclonal antibody was raised against the unique epitope FRREVPEPEYNRELT in the C-terminal cytoplasmic region. A cysteine was added to the C terminal glutamine and N terminal phenylalanine, respectively, to facilitate conjugation with keyhole limpet hemocyanin (*KLH*) which was used as a carrier protein (GenScript).

2.5. Western blotting for AQP4 and AQP5

Anal papillae from 80 larvae were collected in ice-cold saline for each biological replicate (Clark and Bradley, 1996). The saline was removed and samples were stored at −80 °C until further processing. Samples