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Larval anopheline mosquito recta exhibit a dramatic change in localization patterns of ion transport proteins in response to shifting salinity: a comparison between anopheline and culicine larvae

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SUMMARY

Mosquito larvae live in dynamic aqueous environments, which can fluctuate drastically in salinity due to environmental events such as rainfall and evaporation. Larval survival depends upon the ability to regulate hemolymph osmolarity by absorbing and excreting ions. A major organ involved in ion regulation is the rectum, the last region for modification of the primary urine before excretion. The ultrastructure and function of culicine larval recta have been studied extensively; however, very little published data exist on the recta of anopheline larvae. To gain insight into the structure and functions of this organ in anopheline species, we used immunohistochemistry to compare the localization of three proteins [carbonic anhydrase (CA9), Na^+/K^+ P-ATPase and H^+ V-ATPase] in the recta of anopheline larvae reared in freshwater and saline water with the localization of the same proteins in culicine larvae reared under similar conditions. Based on the following key points, we concluded that anophelines differ from culicines in larval rectal structure and in regulation of protein expression: (1) despite the fact that obligate freshwater and saline-tolerant culicines have structurally distinct recta, all anophelines examined (regardless of saline-tolerance) have a structurally similar rectum consisting of distinct DAR (dorsal anterior rectal) cells and non-DAR cells; (2) anopheline larvae undergo a dramatic shift in rectal Na^+/K^+-ATPase localization when reared in freshwater *vs* saline water. This shift is not seen in any culicine larvae examined. Additionally, we use these immunohistochemical analyses to suggest possible functions for the DAR and non-DAR cells of anopheline larvae in freshwater and saline conditions.

Key words: carbonic anhydrase, V-ATPase, Na^+/K^+-ATPase, DAR cells, anopheline, culicine.

INTRODUCTION

Larval mosquitoes are aquatic organisms that live in habitats of varying salinity ranging from rain pools and streams to salt marshes and hypersaline lakes. Although the majority of mosquito species live in freshwater, having variable tolerance for low levels of salinity, approximately 5% live in either brackish or saline water (O'Meara, 1976; Clements, 1992). A major challenge faced by all mosquito larvae is the tendency for these habitats to fluctuate widely in salinity due to occurrences such as rainfall or evaporation (Clements, 1992). For most larvae to survive in waters that are at times greatly hyper- or hypo-osmotic to their hemolymph, they must possess highly developed systems for regulating the absorption and excretion of ions.

To survive in dynamic aquatic environments, larvae must regulate their hemolymph osmolarity (~300 osmol l^{-1}) accordingly. In freshwater, larvae tend to gain water by osmosis and lose salts by diffusion. In saline water, larvae tend to lose water to their environment and gain salts (Clements, 1992). In fresh or dilute water, all larvae hyper-regulate their hemolymph osmolarity by resorbing ions and nutrients to produce a dilute urine. By contrast, depending on the species, larvae in saline water respond in one of three ways: obligate freshwater species are restricted to waters that are iso- or hypo-osmotic to their hemolymph and cannot survive in higher osmolarities; saline-tolerant osmoregulators regulate their osmotic and ion concentrations by secreting a hyper-osmotic urine to maintain a hemolymph osmolarity of ~350 osmol l^{-1} over a species-specific range of external osmolarities (Bradley, 1994); and saline-tolerant osmoconformers increase hemolymph osmolarity with increasing media osmolarity (in water exceeding 300 osmol l^{-1}) by producing organic compounds, such as proline and trehalose, and accumulating them as non-toxic osmolytes in the hemolymph (Bradley, 1994). As saline-tolerant osmoconforming larvae use an osmoregulatory strategy that differs from the majority of saline-tolerant species (Bradley, 1987b), they will not be considered in the present study.

A key organ responsible for ion regulation in larval mosquitoes is the rectum. As larvae depend on ion regulation for survival, the recta of several culicine genera (including *Aedes* and *Culex*) have been studied in detail. Saline-tolerant osmoregulating culicine species have a structurally distinct rectum compared with freshwater species (Meredith and Phillips, 1973). The recta of freshwater culicines are structurally uniform and selectively resorb ions, water and nutrients from the primary urine produced by the Malpighian tubules. Conversely, the recta of saline-tolerant culicines are structurally divided into distinct anterior and posterior regions. The structure and function of the anterior rectum (AR) is similar to the recta of freshwater culicines, whereas the function of the posterior rectum (PR) is to secrete a hyper-osmotic urine when larvae inhabit saline water (Meredith and Phillips, 1973; Bradley and Phillips, 1977). The idea that the recta of freshwater and saline-tolerant larvae are distinct in structure and function is supported by data from various culicine larval species (Ramsay, 1950; Asakura, 1970; Bradley and Phillips, 1975; Bradley and Phillips, 1977). To our knowledge, previous work on the mosquito larval rectum was restricted to the culicine sub-family with little data published describing the recta of anophelines. As anopheline mosquitoes

account for 100% of the world's human malaria vectors, in addition to being vectors for many other deadly diseases, it is critical to understand the processes necessary for their survival (including ion regulation and adaptation). In this way, we may develop techniques to reduce the population of these vectors both specifically and safely.

We recently discovered a sub-set of cells on the dorsal anterior rectum (DAR cells) of a freshwater anopheline, *Anopheles gambiae*, which were distinct from the rest of the rectum (non-DAR cells) in localization of carbonic anhydrase (CA) (Smith et al., 2007), H^+ V-ATPase and Na^+/K^+ P-ATPase (Okech et al., 2008). We observed similar protein localization patterns in both freshwater and saline-tolerant anophelines (K.E.S., unpublished data). This unique type of two-part rectum was originally detected in the saline-tolerant *Anopheles salbaii* by light microscopy (Bradley, 1987a; Bradley, 1994). These data led us to hypothesize that anopheline larvae are distinct from culicine larvae in rectal structure and, ultimately, in methods of ion regulation.

V-ATPase and Na^+/K^+-ATPase are well-known membrane energizers important for ion regulation (e.g. Skou, 1990; Wieczorek et al., 1990) and recent work has established the presence of one or both of these proteins in the recta of culicines (Patrick et al., 2006) and anophelines (Okech et al., 2008). Additionally, CA plays a major role in HCO_3^- secretion in the larval rectum (Strange and Phillips, 1984; Corena et al., 2002), and CA protein localized to the DAR cells of all anophelines examined including *An. gambiae*, *Anopheles albimanus*, *Anopheles farauti*, *Anopheles quadrimaculatus*, *Anopheles aquasalis* and *Anopheles stephensi* (K.E.S., unpublished data) making it an excellent marker for these cells.

In the present study, we compare the localization of three ion-regulatory proteins (CA, Na^+/K^+-ATPase and V-ATPase) in the recta of *Aedes aegypti* (freshwater culicine), *An. gambiae* (freshwater anopheline), *Ochlerotatus taeniorhynchus* (saline-tolerant culicine) and *An. albimanus* (saline-tolerant anopheline) reared in freshwater and saline water. Additionally, we determine protein localization in larvae either reared in freshwater and exposed to saline water or reared in saline water and exposed to freshwater for 24, 48 and 72 h to determine the effects of short-term exposure to either of these conditions. From these analyses, we conclude that anophelines differ from culicines in larval rectal structure as well as in the regulation of protein expression. Additionally, we suggest putative functions for the DAR and non-DAR cells of anopheline larvae under both freshwater and saline water conditions.

MATERIALS AND METHODS

Artificial seawater (ASW)

100% artificial seawater (ASW): 420 mmol l^{-1} NaCl; 9 mmol l^{-1} KCl; 12 mmol l^{-1} $CaCl_2{\cdot}H_2O$; 23 mmol l^{-1} $MgCl_2{\cdot}6H_2O$; 26 mmol l^{-1} $MgSO_4{\cdot}7H_2O$; and 2 mmol l^{-1} $NaHCO_3$ in Milli-Q water; pH 8.1; osmolarity 860 osmol l^{-1} as measured using a 5500 vapor pressure osmometer (Wescor, Logan, UT, USA). All dilutions of the 100% ASW stock were made using Milli-Q water (Millipore, Billerica, MA, USA).

Mosquito rearing

Anopheles albimanus (Wiedemann) (STECLA), *Anopheles gambiae* (Giles) (SS G3), *Anopheles farauti* (Laveran) (FAR1) and *Anopheles stephensi* (Liston) (STE2) were hatched from eggs supplied by MR4 (The Malaria Research and Reference Reagents Resource Center) at the Centers for Disease Control and Prevention in Atlanta, GA, USA (http://www.malaria.atcc.org) and reared as described in the supplier manual (www2.ncid.cdc.gov/vector/vector.html).

Ochlerotatus taeniorhynchus (Wiedemann), *Aedes aegypti* (Linnaeus), *Aedes albopictus* (Skuse) and *Anopheles quadrimaculatus* (Say) were hatched from eggs supplied by the USDA (United States Department of Agriculture) in Gainesville, FL, USA.

Anopheles aquasalis (Curry) were hatched from eggs and reared to 4th-instar in 10% ASW by Dr Luciano Moreira at The Oswaldo Cruz Institute in Rio de Janeiro, Brazil.

Unless otherwise stated, all larvae were reared in identical freshwater conditions (Milli-Q water) at a density of approximately 100 larvae per 200 ml water. Additionally, certain species were hatched and reared in dilutions of ASW: *An. albimanus* (50% ASW); *An. gambiae* (10, 20, 30 and 40% ASW, and acclimated to 60% ASW from Milli-Q water by increasing the salinity by 10% each day beginning day 1 post-hatch), *Oc. taeniorhynchus* (100% ASW); and *Ae. aegypti* (40% ASW). Unless otherwise stated, all larvae were used at the early 4th-instar stage. Anopheline larvae were fed every other day with a dusting of ground TetraMin™ (Tetra; Melle, Germany) fish flakes. Culicine larvae were fed every other day with a mixture of brewers yeast and liver powder (2:3) (MP Biomedicals, Solon, OH, USA).

We evaluated the freshwater species *Ae. aegypti* (N=2 egg batches) and *An. gambiae* (N=3 egg batches) for larval size and mortality rates when reared in freshwater, 30 and 40% ASW. Mortality rates were determined by isolating 100 newly hatched 1st-instar larvae into a separate container and counting the surviving larvae daily. This continued until larvae reached 4th-instar (*An. gambiae*) or until the first pupa was observed (*Ae. aegypti*).

Acute saline/freshwater challenges

To determine protein localization after acute exposures to freshwater or dilute saline water, *An. albimanus* larvae were hatched in either freshwater or 25% ASW and reared individually in 1 ml of freshwater or 25% ASW, respectively, in 24 well plates. Larvae were carefully monitored every 24 h for molting. Newly molted larvae (within 24 h) at the 2nd-, 3rd- or 4th-instar stage were transferred from either freshwater to 25% ASW or from 25% ASW to freshwater. After 24, 48 and 72 h larvae were removed and prepared for immunohistochemistry. Fourth-instar larvae could not be observed 72 h post media transfer due to pupation. A minimal concentration of ASW was used which was shown to elicit Na^+/K^+-ATPase shift (25% ASW rather than 50% ASW). N>5 larvae for each experimental group. Each experiment was performed in triplicate.

Antibody production

CA9 chicken antibody was generated by Aves Labs (Tigard, OR, USA) against the *An. gambiae* BSA-conjugated peptide: KEPIEVSHEQLELFREMRC and was affinity-purified using the immunogen peptide (Smith et al., 2007).

Na^+/K^+-ATPase monoclonal antibodies 'α5' that had been raised against the α–subunit of avian Na^+/K^+-ATPase in mice were obtained from the Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, IA, USA) in the form of hybridoma tissue culture supernatant (Lebovitz et al., 1989).

A polyclonal V-ATPase antiserum raised against the B-subunit of the V-type H^+-ATPase of *Culex quinquefasciatus* (Filippova et al., 1998) was obtained from Professor Sarjeet Gill at the University of California, Riverside, USA.

Immunolocalization

Paraffin sectioned preparations: primary fixation was achieved by injection into the hemocoel of a 4% formaldehyde solution diluted from ultrapure 16% formaldehyde (PolySciences, Warrington, PA, USA) with Tris-buffered saline (TBS), and larvae were immersed in 4% formaldehyde overnight at 4°C. Larvae were transferred to Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 90 min on ice and washed twice with 100% ethanol for 30 min each. Larvae were then cleaned with aniline:methylsalicylate (1:1) overnight, followed by 100% methylsalicylate overnight and embedded in paraffin. Sections, 6 μm thick, were cut using a microtome and mounted on gelatin-coated slides. Sections were deparaffinized through successive 5 min incubations in 100% xylene (twice) and xylene:ethanol (1:1), rehydrated through a series of 5 min washes of graded ethanols: 100% (twice), 95% (twice), 80%, 70%, 50%, and finally washed three times in TBS. The slides were then blocked in pre-incubation (pre-inc) buffer (TBS, 1% bovine serum albumin, 1% normal goat serum, 0.1% TritonX100) for 1–2 h at room temperature and incubated with primary antibodies to V-ATPase and CA9 at a dilution of 1:1000 and Na^+/K^+-ATPase at a dilution of 1:10 in pre-inc for 1 h at 37°C. The slides were washed three times in TBS and incubated with secondary antibodies [FITC-conjugated goat anti-rabbit, TRITC-conjugated goat anti-mouse and Cy5-conjugated donkey anti-chicken (Jackson ImmunoResearch, West Grove, PA, USA)] at a dilution of 1:250 in pre-inc for 1 h at 37°C. The slides were, again, rinsed three times in TBS and mounted in 60% glycerol in TBS with phenylenediamine (Sigma Chemical Co., St Louis, MO, USA) to diminish fluorescence quenching.

Whole-mount antibody localization was performed to visually distinguish the types of recta; those of freshwater culicines, saline-tolerant culicines, freshwater anophelines and saline-tolerant anophelines and, therefore, antibodies were used based on their ability to differentiate one rectal region from another in each species. Whole-mount preparations: larvae were dissected to separate the gut from the rest of the larva. The dissected tissue was fixed in a 1:1 solution of hemolymph-substitute solution (Clark et al., 1999) and 4% formaldehyde overnight at 4°C. The tissue was then washed twice with TBS for 30 min each at room temperature and incubated in pre-inc for 1–2 h at room temperature. The tissue was incubated with primary antibodies to Na^+/K^+-ATPase at a dilution of 1:10 and either CA9 at a dilution of 1:1000 or V-ATPase at a dilution of 1:1000 in pre-inc at 4°C overnight. The following day the tissue was washed approximately 10 times in pre-inc for 30 min each at room temperature. Secondary antibodies [FITC-conjugated donkey-anti-chicken (to detect CA9), TRITC-conjugated goat anti-mouse (to detect Na^+/K^+-ATPase) or FITC-conjugated goat-anti-rabbit (to detect V-ATPase)] were diluted 1:250 in pre-inc and incubated with the appropriate tissue(s) overnight at 4°C. The tissue was rinsed twice with pre-inc and once with TBS at room temperature for 30 min each, and then mounted in 60% glycerol in TBS with phenylenediamine (Sigma Chemical Co.) to diminish fluorescence quenching.

In all cases, multiple primary or secondary antibodies were added simultaneously rather than sequentially. All secondary antibodies purchased were specifically 'ML' grade (multiple label; Jackson ImmunoResearch). This grade is affinity purified and tested for minimal cross-species immunoreactivity. For each image, $N>10$ larval sections were observed.

All images were captured using a Leica LSCM SP2 laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL, USA). For each signal in each preparation, laser intensity and detector sensitivity were set to capture the full dynamic range of the fluorescence.

Quantification of signal intensity

Because sources of variability exist in the imaging system as well as in preparation of the samples, we were unable to quantitatively compare signal intensity between different samples; however, we were able to compare signal between rectal cell types (DAR and non-DAR or AR and PR) in the same sample. We therefore report the change in protein distribution as a change in the ratio of peak pixel intensity between the two rectal cell types in each sample. As rectal cell type distribution of CA and V-ATPase did not change between larvae reared in freshwater and those reared in saline water, we only report the pixel intensity ratios of Na^+/K^+-ATPase.

To quantify the immunostaining intensity between DAR and non-DAR cells (or AR and PR), the region of interest (ROI) function of the Leica Confocal 'Quantify' software (Leica Microsystems) was used to define all cells of either type (separately) in a given tissue section. Once we determined that no pixel intensities were beyond the dynamic range of the 8 bit gray scale, the peak pixel intensity of each ROI was calculated and used as the basis of comparison between DAR and non-DAR cells (or AR and PR).

Three representative recta were quantified from each group (including the recta presented in Fig. 1). For each rectum, the DAR/non-DAR (or AR/PR) peak Na^+/K^+-ATPase pixel intensity ratio was determined by dividing the peak pixel intensity of the DAR cells (or AR) by the peak pixel intensity of the non-DAR cells (or PR). The mean DAR/non-DAR (AR/PR) ratios of the three recta from each group were then calculated. Finally, the mean DAR/non-DAR (AR/PR) ratios of the freshwater-reared larvae were plotted against the saline water-reared larvae in each species using Graphpad Prism 3.0 graphing software (La Jolla, CA, USA). Standard deviation between the three recta from each group was indicated. For each graph, a value of '1' indicates that the peak pixel intensity was equal in the two cell types. A value greater than '1' indicates greater peak pixel intensity in the DAR cells (or AR) whereas a value less than '1' indicates greater peak pixel intensity in the non-DAR cells (or PR).

RESULTS

We compared the localization of three proteins with known roles in ion regulation in the larval rectum: a specific carbonic anhydrase (CA9) (Smith et al., 2007); Na^+/K^+-ATPase; and V-ATPase. The proteins were localized in longitudinal paraffin sections of recta from larval freshwater and saline-tolerant anophelines and culicines reared in varying osmotic conditions including freshwater and specific dilutions of ASW (the dilutions used were based on the upper tolerance limit of each species). We compared the freshwater mosquito larvae *Ae. aegypti* (culicine) and *An. gambiae* (anopheline) with the saline-tolerant mosquito larvae *Oc. taeniorhynchus* (culicine) and *An. albimanus* (anopheline). Additionally, we evaluated the change in Na^+/K^+-ATPase localization in *An. gambiae*, *Oc. taeniorhynchus* and *An. albimanus* as a ratio of Na^+/K^+-ATPase labeling intensity between the two rectal cell types in each species (either DAR cells *vs* non-DAR cells or AR *vs* PR).

The ultrastructure of both freshwater and saline-tolerant culicines is characterized by highly infolded apical lamellae and extensive basal infoldings (Meredith and Phillips, 1973). Preliminary electron micrographs (K.E.S., unpublished observations) indicate that the membranes of the DAR and non-DAR cells of anophelines reared in freshwater are folded in a similar way. For the present study, the cells of all recta examined are therefore assumed to possess apical lamellae and basal infoldings.

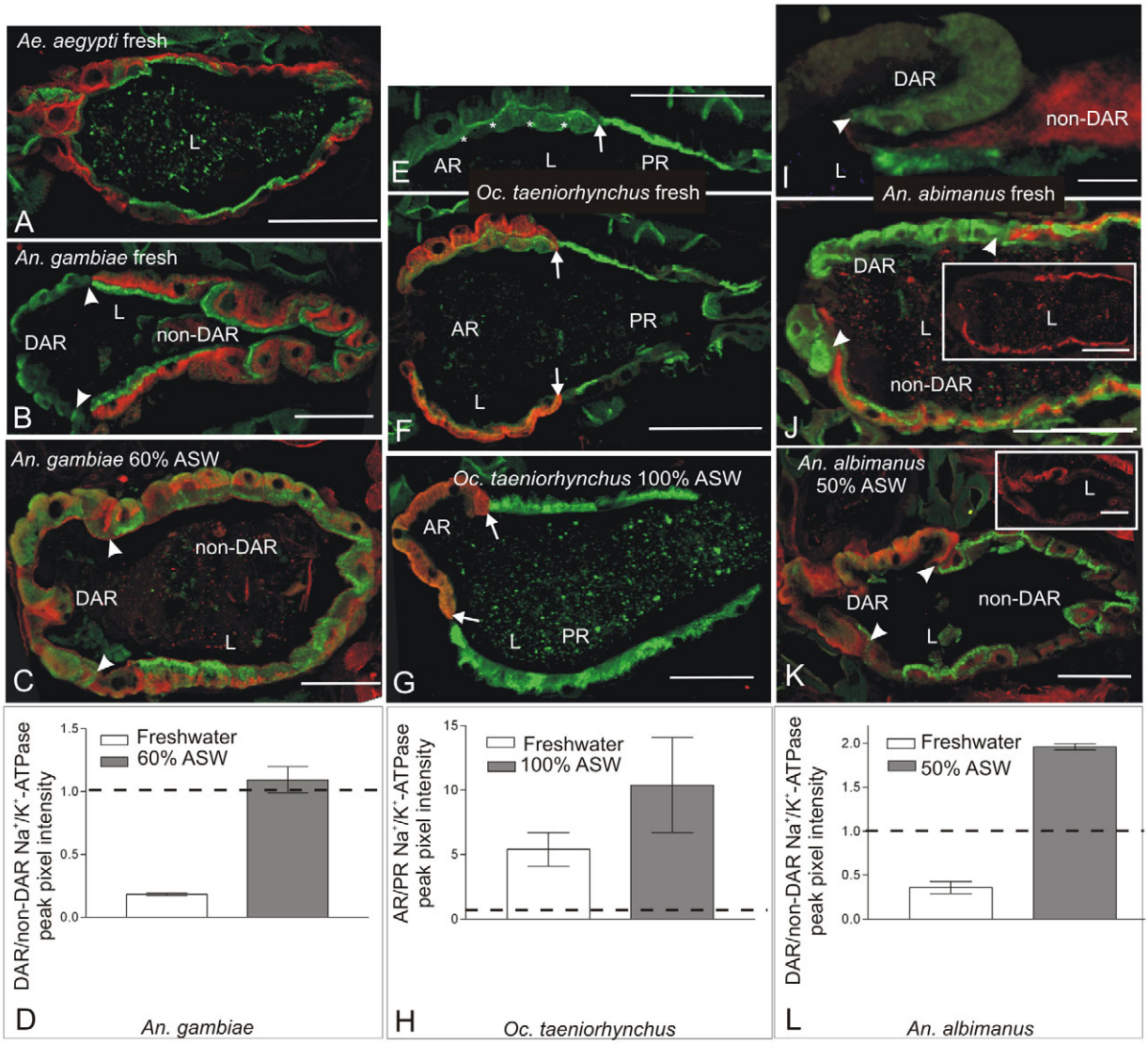

Fig. 1. Immunohistochemical detection of Na^+/K^+-ATPase (red) and V-ATPase (green) protein localization in longitudinal sections of the recta of *Ae. aegypti* (A), *An. gambiae* (B,C), *Oc. taeniorhynchus* (E,F,G) and *An. albimanus* (I,J,K) reared in either freshwater or saline water. Distribution of Na^+/K^+-ATPase is indicted as a ratio of Na^+/K^+-ATPase peak pixel intensity in the DAR cells (or AR) *vs* the non-DAR cells (or PR) (D,H,L). Arrowheads demark the junction between DAR and non-DAR cells in anophelines and arrows demark the junction between AR and PR in culicines. The inset images in panels J and K are identical to the corresponding panel but lack V-ATPase staining signal thereby giving a clearer view of Na^+/K^+-ATPase localization. *Ae. aegypti* protein localization did not change between larvae reared in freshwater or saline water: Na^+/K^+-ATPase localized to the basal infoldings and V-ATPase to the apical lamellae (A). In freshwater-reared *An. gambiae* and *An. albimanus* Na^+/K^+-ATPase localized to the basal infoldings of the non-DAR cells and V-ATPase to the apical lamellae of the non-DAR cells and cytoplasm of the DAR cells (B,I,J). The apparent cytoplasmic localization of V-ATPase in *An. albimanus* is shown in higher magnification (I). When acclimated to 60% ASW, *An. gambiae* showed Na^+/K^+-ATPase on the basal infoldings of both DAR and non-DAR cells, and V-ATPase appeared cytoplasmic in all cells as well as apical in the non-DAR cells (C). The change in Na^+/K^+-ATPase distribution can be quantified as a change in Na^+/K^+-ATPase peak pixel intensity from being significantly greater in the non-DAR cells (in larvae reared in freshwater) to being approximately the same in the DAR and non-DAR cells (in larvae acclimated to 60% ASW) (D). In saline-reared *An. albimanus*, Na^+/K^+-ATPase exhibited a drastic shift in localization to the DAR cells (J inset *vs* K inset). This shift can be quantified as a change in Na^+/K^+-ATPase peak pixel intensity from being significantly greater in the non-DAR cells (in larvae reared in freshwater) to being significantly greater in the DAR cells (in larvae reared in 50% ASW) (L). V-ATPase remained the same (K). In freshwater reared *Oc. taeniorhynchus*, Na^+/K^+-ATPase localized to the basal infoldings of the AR whereas V-ATPase localized to the apical lamellae of the PR (F). However, in most larvae, a weak V-ATPase signal was evident on the apical lamellae of the AR (E, asterisks). When reared in 100% ASW, protein localization did not change drastically, although AR apical V-ATPase signal was not evident (G). The absence of a change in Na^+/K^+-ATPase localization is apparent in (H) as larvae reared in both freshwater and 100% ASW have significantly more Na^+/K^+-ATPase pixel intensity in the AR compared with the PR. AR, anterior rectum; ASW, artificial seawater; DAR, dorsal anterior rectum; L, lumen; PR, posterior rectum. Scale bars: A, 150 μm; B,J,J inset, 75 μm; C, 86.13 μm; E,F, 149.36 μm; G,K,K inset, 99.32 μm; and I, 12 μm. Error bars indicate means ± s.e.m.

Anopheline rectal structure

The immunolocalization patterns of CA9 and Na^+/K^+-ATPase were used to identify anopheline DAR and non-DAR cells, respectively, in larvae reared in freshwater. No obvious differences in protein localization were observed between the recta of freshwater anophelines (*An. gambiae*, *An. stephensi* and *An. quadrimaculatus*) and saline-tolerant anophelines (*An. albimanus*, *An. farauti* and *An. aquasalis*). In all larvae, CA9 localized to DAR cells and Na^+/K^+-

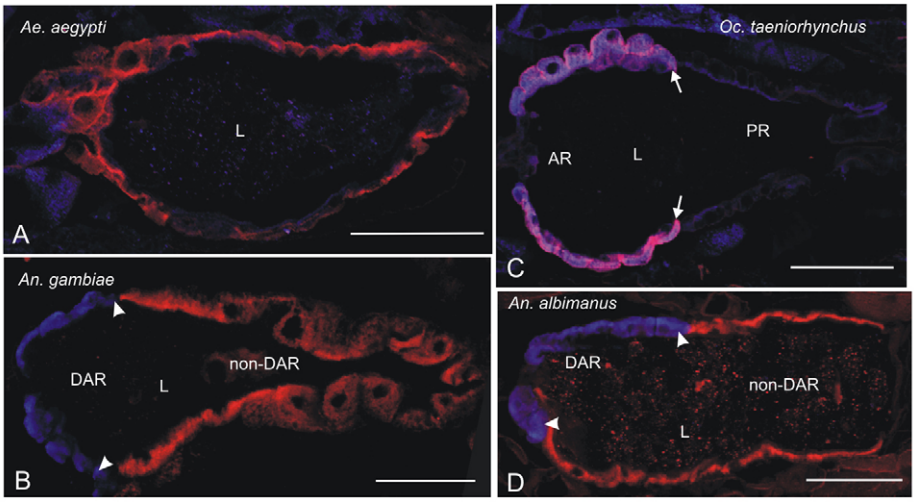

Fig. 2. Immunohistochemical detection of carbonic anhydrase (CA9) (blue) protein localization in longitudinal sections of the recta of freshwater-reared *Ae. aegypti* (A), *An. gambiae* (B), *Oc. taeniorhynchus* (C) and *An. albimanus* (D). Na^+/K^+-ATPase (red) was used as a counterstain. CA localization did not change in larvae reared in saline water; therefore, only images of freshwater-reared larvae are shown. CA localized to the dorsal anterior rectum (DAR) cells in *An. gambiae* and *An. albimanus* and to the anterior rectum (AR) of *Oc. taeniorhynchus*. CA was not detected in *Ae. aegypti* rectum. Arrowheads indicate the junction between DAR and non-DAR cells. Arrows indicate junction between anterior and posterior recta. L, lumen; PR, posterior rectum. Scale bars: A, 150 μm; B, 75 μm; C, 149.36 μm; and D, 99.32 μm.

ATPase localized to non-DAR cells (i.e. Fig. 1B,J, Fig. 2B,D and Fig. 4C) as described for *An. gambiae* in Smith et al. (Smith et al., 2007). This similarity suggests that all anopheline larvae, regardless of saline-tolerance, have DAR and non-DAR cells.

Ae. aegypti: obligate freshwater culicine

Ae. aegypti developed to 4th-instar in concentrations of ASW up to a maximum of 40% (~350 omol l^{-1}). After seven days post-hatch, 53% of larvae survived in 40% ASW, 82% survived in 30% ASW and 63% survived in freshwater. Protein localization did not differ between larvae reared in freshwater *vs* 40% ASW: Na^+/K^+-ATPase was present on the extensive basal infoldings whereas V-ATPase localized to the apical lamellae (Fig. 1A). CA9 was not detectable in the non-segmented rectum of *Ae. aegypti* (Fig. 2A).

An. gambiae: obligate freshwater anopheline

An. gambiae developed to 4th-instar in ASW concentrations up to 40% but could not survive in higher salinities. After six days post-hatch 16% of larvae survived 40% ASW but were developing much slower than those in lower salinities, 87.3% survived in 30% ASW and 73.6% survived in freshwater. However, the larvae could be acclimated from freshwater to higher ASW concentrations by slowly increasing the ASW concentration by 10% each day (up to 60% ASW for a maximum of 24 h).

The localization of Na^+/K^+-ATPase, V-ATPase and CA9 in *An. gambiae* reared in freshwater has been described previously (Rheault et al., 2007; Smith et al., 2007; Okech et al., 2008). Na^+/K^+-ATPase is restricted to the basal infoldings of the non-DAR cells (Fig. 1B and Fig. 2B) whereas CA9 protein is restricted to the cytoplasm of the DAR cells (Fig. 2B). V-ATPase localizes to the apical lamellae of the non-DAR cells and appears to be cytoplasmic in the DAR cells (Fig. 1B). The localization pattern of all three proteins was identical in another obligate freshwater anopheline species, *An. stephensi*, when reared in freshwater (results not shown).

Localization patterns of the three proteins did not change in *An. gambiae* larvae reared in 10 or 20% ASW. However, the recta of larvae reared in 30% ASW or acclimated to 60% ASW showed subtle changes in the localization of Na^+/K^+-ATPase and V-ATPase compared with the recta of larvae reared in freshwater. Na^+/K^+-ATPase shifted from being undetectable in the DAR cells (Fig. 1B) to being detectible on the basal infoldings of both DAR and non-DAR cells (Fig. 1C). This change can be seen graphically in Fig. 1D. When reared in freshwater, the DAR cells have significantly less Na^+/K^+-ATPase peak pixel intensity than the non-DAR cells. When acclimated to 60% ASW, there is no significant Na^+/K^+-ATPase difference between the DAR and non-DAR cells. In many larvae, this signal appeared reduced compared with that of those reared in freshwater. Additionally, V-ATPase appeared to localize to the cytoplasm of the non-DAR cells in addition to the apical lamellae (Fig. 1C). Localization of V-ATPase and CA9 in the DAR cells did not change.

Oc. taeniorhynchus: saline-tolerant culicine

The rectum of *Oc. taeniorhynchus* is composed of regionalized anterior (AR) and posterior (PR) segments in contrast to DAR and non-DAR cells, and protein localization in these regions did not appear to change drastically between larvae reared in freshwater and those reared in 100% ASW. In both cases, Na^+/K^+-ATPase and CA9 localized to the AR; and Na^+/K^+-ATPase localized to the basal infoldings (Fig. 1F,G and Fig. 2C) and CA9 localized to the cytoplasm (Fig. 2C). The consistency of Na^+/K^+-ATPase distribution can be seen graphically in Fig. 1H. When reared in both freshwater and 100% ASW, there is significantly more Na^+/K^+-ATPase in the AR than the PR. Conversely, V-ATPase localized mainly to the apical lamellae of the PR and appeared absent from the AR (Fig. 1F,G). However, many larvae reared in freshwater, but not 100% ASW, exhibited a low level of V-ATPase on the apical lamellae of the AR (Fig. 1E, asterisks). In all cases, this signal was less intense than that in the PR.

An. albimanus: saline-tolerant anopheline

Protein localization in the recta of *An. albimanus* was identical to that in *An. gambiae* when larvae were reared in freshwater; Na^+/K^+-ATPase appeared to be restricted to the basal infoldings of the non-DAR cells (Fig. 1J, inset and Fig. 2D), CA9 protein was evident only in the cytoplasm of the DAR cells (Fig. 2D) and V-ATPase localized to the apical infoldings of the non-DAR cells and the cytoplasm of the DAR cells (Fig. 1J). This apparent cytoplasmic localization is better seen at a higher magnification in Fig. 1I. The localization pattern of all three proteins was identical in another saline-tolerant anopheline species, *An. farauti*, when reared in freshwater (results not shown).

The distributions of CA9 and V-ATPase proteins remained unchanged in larvae reared in 50% ASW compared with those reared in freshwater but Na^+/K^+-ATPase underwent a dramatic shift and appeared to localize mainly to the basal infoldings of the DAR cells

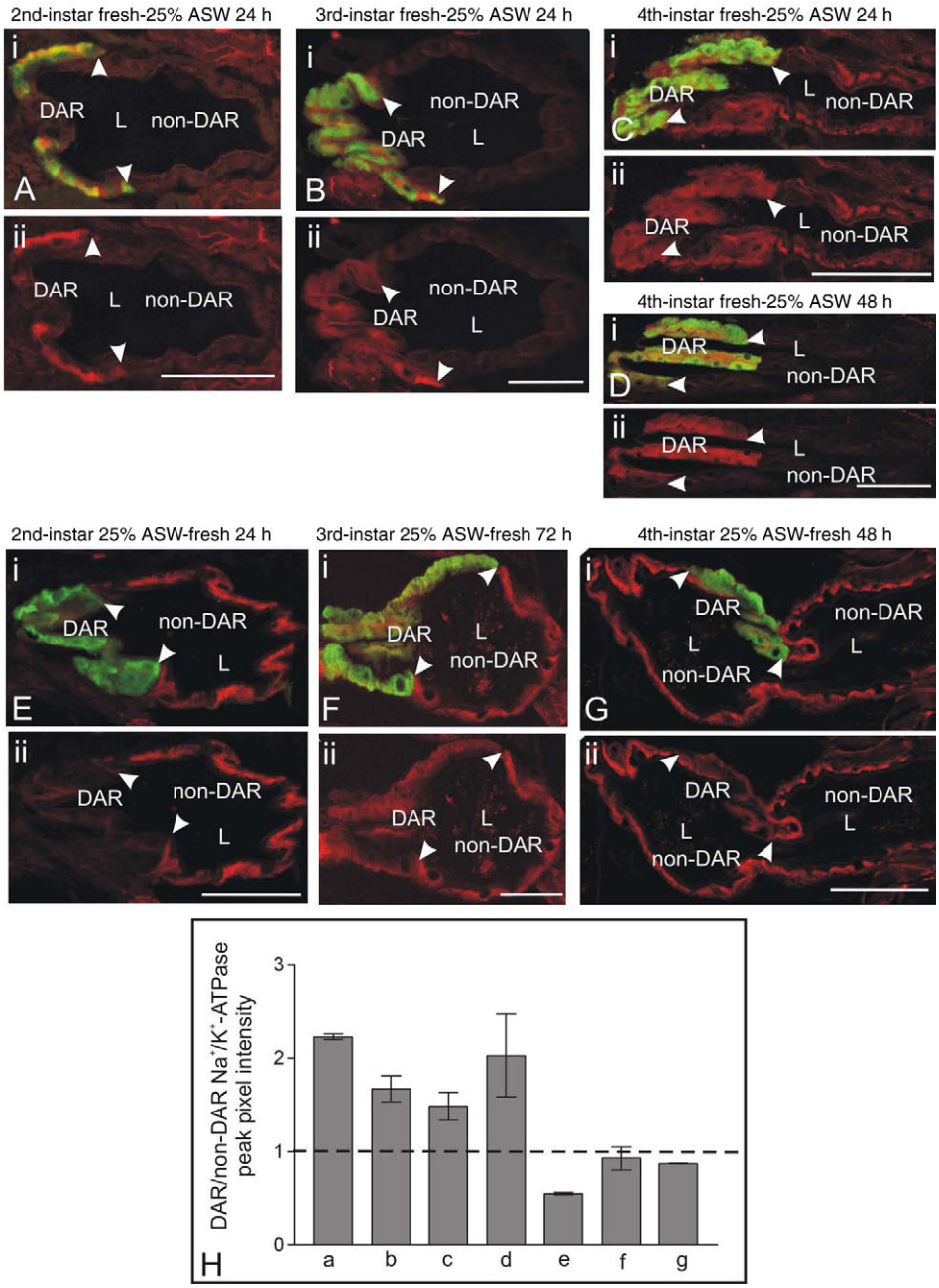

Fig. 3. Immunohistochemical detection of Na^+/K^+-ATPase (red) and CA9 (green) in *An. albimanus* larvae reared in freshwater and transferred to 25% ASW during the 2nd-instar stage for 24 h (A), during the 3rd-instar stage for 24 h (B) and during the 4th-instar stage for 24 h (C) and 48 h (D). Also shown are larvae reared in 25% ASW and transferred to freshwater during the 2nd-instar stage for 24 h (E), during the 3rd-instar stage for 72 h (F) and during the 4th-instar stage for 48 h (G). Distribution of Na^+/K^+-ATPase in each sample is indicted as a ratio of Na^+/K^+-ATPase peak pixel intensity in the DAR cells *vs* the non-DAR cells (H). Lowercase bar labels in H correspond to the uppercase letters in Fig. 3 panels (i.e. Fig. 3A corresponds to Fig. 3H bar 'a'). Localization of CA9 is indicative of DAR cells. Na^+/K^+-ATPase and CA9 co-localization is indicated in yellow. In each set of images, (i) shows both Na^+/K^+-ATPase and CA9 localization whereas (ii) is the same picture showing only Na^+/K^+-ATPase localization. If reared in freshwater and exposed to 25% ASW during the 2nd- (A,H bar a) or 3rd- (B,H bar b) instar stages, a change in Na^+/K^+-ATPase localization from the non-DAR cells to DAR cells was evident within 24 h. Fourth-instar larvae exposed only for 24 h expressed Na^+/K^+-ATPase in both DAR and non-DAR cells, as if in an intermediate stage (C,H bar c). However, a change in Na^+/K^+-ATPase localization from the non-DAR cells to DAR cells was evident after 48 h (D,H bar d). When larvae were reared in 25% ASW and transferred to freshwater slightly different results were found. Whereas 2nd-instar larvae shifted Na^+/K^+-ATPase localization from DAR to non-DAR cells within 24 h (E,H bar e), 3rd- (F,H bar f) and 4th- (G,H bar g) instar larvae did not fully shift Na^+/K^+-ATPase localization after 72 h or 48 h, respectively, and expressed the protein in both DAR and non-DAR cells. ASW, artificial seawater; L, lumen. Scale bars: A,B,C,E, 75 μm; D, G, 150 μm; and F, 65.17 μm. Error bars indicate means ± s.e.m.

(Fig. 1K, inset), presenting a drastic increase of this protein in the DAR cells and a contrasting reduction in the non-DAR cells (compare Fig. 1J,K insets). The change in Na^+/K^+-ATPase distribution is shown graphically in Fig. 1L. When reared in freshwater, Na^+/K^+-ATPase peak pixel intensity was significantly greater in the non-DAR cells, whereas when reared in 50% ASW it was significantly greater in the DAR cells.

To determine if the Na^+/K^+-ATPase protein shift is a reversible event, larvae were reared in either freshwater or 25% ASW to 2nd-, 3rd- or 4th-instar and were transferred to 25% ASW or freshwater, respectively, for 24, 48 or 72 h. For each image in Fig. 3, the data are presented graphically as the ratio of Na^+/K^+-ATPase peak pixel intensity in the DAR cells *vs* the non-DAR cells (Fig. 3H). Lowercase bar labels in Fig. 3H correspond to the experimental group indicated by the uppercase letters (i.e. Fig. 3A corresponds to bar 'a' in Fig. 3H). When larvae were reared in freshwater and exposed briefly (for 24 to 48 h) to 25% ASW, the ability for larvae to shift rectal Na^+/K^+-ATPase localization depended on the larval stage at which the exposure occurred. If exposed to ASW during the 2nd- or 3rd-instar stages, a change in Na^+/K^+-ATPase peak signal intensity from the non-DAR cells to the DAR cells was evident within 24 h (Fig. 3A,B,H bars a,b). Fourth-instar larvae exposed only for 24 h expressed Na^+/K^+-ATPase in both DAR and non-DAR cells as if in an intermediate stage (Fig. 3C,H bar c). However, a change in Na^+/K^+-ATPase localization from the non-DAR cells to DAR cells was evident after 48 h (Fig. 3D,H bar d). In most cases, Na^+/K^+-ATPase shift was most dramatic in larvae exposed during the 2nd larval stage. Most 3rd- and 4th-instar larvae retained some Na^+/K^+-ATPase signal in the non-DAR after exposure to 25% ASW.

Slightly different results were found for larvae reared in 25% ASW and exposed to freshwater (for 24 to 72 h). While 2nd-instar larvae shifted Na^+/K^+-ATPase localization from DAR to non-DAR

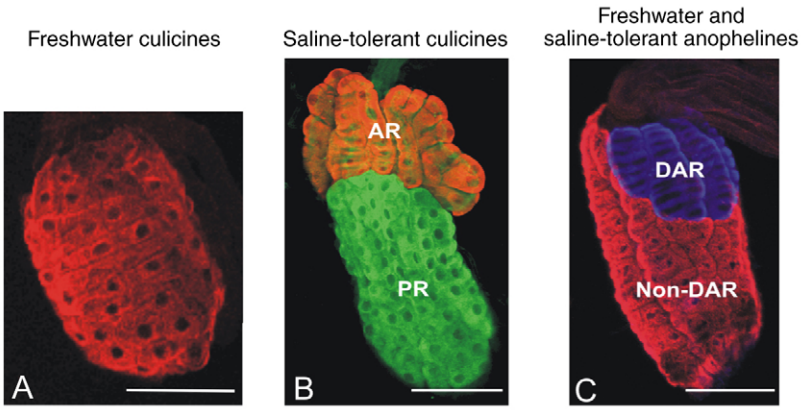

Fig. 4. Comparison of the rectal structure of the freshwater culicine *Ae. aegypti* (A), the saline-tolerant culicine *Oc. taeniorhynchus* (B), and the freshwater anopheline, *An. gambiae* (C) using confocal microscopy of whole mount immunohistochemical preparations. Similar data have previously been reported (Patrick and Gill, 2003; Smith et al., 2007; Okech et al., 2008). Protein localizations are shown for visual distinction of rectal segments in each species. Freshwater culicine larvae possess a structurally uniform rectum as shown by Na^+/K^+-ATPase (red) localization (A) whereas saline-tolerant culicine larvae possess a rectum structurally divided into distinct anterior (AR) and posterior (PR) regions as shown by the localization of Na^+/K^+-ATPase (red) to the AR and V-ATPase (green) to the PR (B). By contrast, both freshwater and saline-tolerant anopheline larvae possess a unique type of segmented rectum, which differs from culicines and consists of a patch of cells on the dorsal anterior region of the rectum (DAR cells), which are distinguishable from the rest of the rectum (non-DAR cells) as shown by the localization of Na^+/K^+-ATPase (red) to the non-DAR cells and CA9 (blue) to the DAR cells (C). AR, anterior rectum; DAR, dorsal anterior rectum; PR, posterior rectum. Scale bars: A,C, 150 μm; and B, 186 μm.

cells within 24 h (Fig. 3E and Fig. 3H bar e), 3rd-instar (Fig. 3F,H bar f) and 4th-instar (Fig. 3G,H bar g) larvae did not fully shift Na^+/K^+-ATPase localization after 72 h or 48 h, respectively, and expressed the protein in both DAR and non-DAR cells.

DISCUSSION

Ion regulation by the rectum helps mosquito larvae to survive and adapt to a constantly changing environment. A wealth of literature has focused on the structure and function of the larval culicine rectum and the distinction between its role in freshwater and saline-tolerant species (e.g. Wigglesworth, 1972; Meredith and Phillips, 1973). However, the discovery that the anopheline rectum is structurally distinct from any culicine species described (Bradley, 1987a; Bradley, 1994; Smith et al., 2007) suggested that anophelines may utilize a unique method of ion regulation. To test this hypothesis, we compared the localization patterns of three proteins involved in ion regulation (CA9, Na^+/K^+-ATPase and V-ATPase) in the recta of anopheline and culicine larvae (including freshwater larvae and saline-tolerant larvae) reared in freshwater *vs* saline water.

Three key points regarding the comparison of anopheline and culicine recta emerge from these data: (1) in contrast to obligate freshwater and saline-tolerant culicines, which have structurally distinct recta, all anophelines examined (regardless of saline-tolerance) possess a similarly structured rectum consisting of DAR and non-DAR cells (Fig. 4); (2) anopheline larvae undergo a dramatic shift in rectal Na^+/K^+-ATPase localization when reared in freshwater *vs* saline water. This shift is not seen in any culicine larvae examined; and (3) with the exception of *Ae. aegypti*, CA9 consistently localized to an anterior region of the rectum in both culicine and anopheline larvae regardless of the salinity of the rearing water. The first two key points, along with the localization patterns of Na^+/K^+-ATPase and V-ATPase (discussed below), will be used to suggest putative functions for the anopheline rectal regions. Localization of CA9 will be discussed in a separate section. Figs 4 and 5 summarize our results; Fig. 4 illustrates the differences between anopheline and culicine recta and Fig. 5 summarizes protein localization in anophelines and culicines in a schematic form.

Freshwater–reared larvae: V-ATPase and Na^+/K^+-ATPase localization

When reared in freshwater, the protein localization patterns in *An. gambiae* and *An. albimanus* recta were identical; non-DAR cells contained an apical V-ATPase and basal Na^+/K^+-ATPase whereas the DAR cells were enriched in cytoplasmic V-ATPase. The protein localization pattern of the non-DAR cells in *An. gambiae* and *An. albimanus* was similar to that of the freshwater culicine, *Ae. aegypti*, which also expressed an apical V-ATPase and basal Na^+/K^+-ATPase in its non-segmented rectum. The detection of V-ATPase in *Ae. aegypti* larval rectum confirms the finding of Filippova et al. (Filippova et al., 1998) but conflicts with the findings of Patrick et al. (Patrick et al., 2006) who detected V-ATPase mRNA in the rectum but did not detect protein when using the same antibody against the B-subunit, which was used in this study. However, ultrastructural studies of the larval *Ae. aegypti* rectum identified a particulate coat on the apical lamellae (Meredith and Phillips, 1973) suggesting the presence of portasomes (Clements, 1992), which correspond to the V_1 portion of the V-ATPase (Harvey, 1992; Radermacher et al., 1999; Zhuang et al., 1999).

Ae. aegypti is a freshwater culicine that can acclimate to 50% ASW by osmoconforming in water hyper-osmotic to their hemolymph [average hemolymph of *Ae. aegypti* reared in freshwater ~250 osmol l^{-1} (Clements, 1992; Edwards, 1982)]. Larvae possess a non-segmented rectum, which selectively resorbs ions from the primary urine. Like *Ae. aegypti*, anophelines reared in freshwater actively resorb ions from the environment (*via* the anal papillae) and from their urine (*via* their rectum) to maintain constant ionic and osmotic hemolymph concentrations (Bradley, 1994). The combination of basal Na^+/K^+-ATPase and apical V-ATPase in the rectum is ideally suited for this task. The polarity is similar to that in frog skin (Ehrenfeld and Klein, 1997) where the V-ATPase hyperpolarizes the apical membrane, which drives Na^+ into the cell from the Na^+-deficient environment. The Na^+/K^+-ATPase then

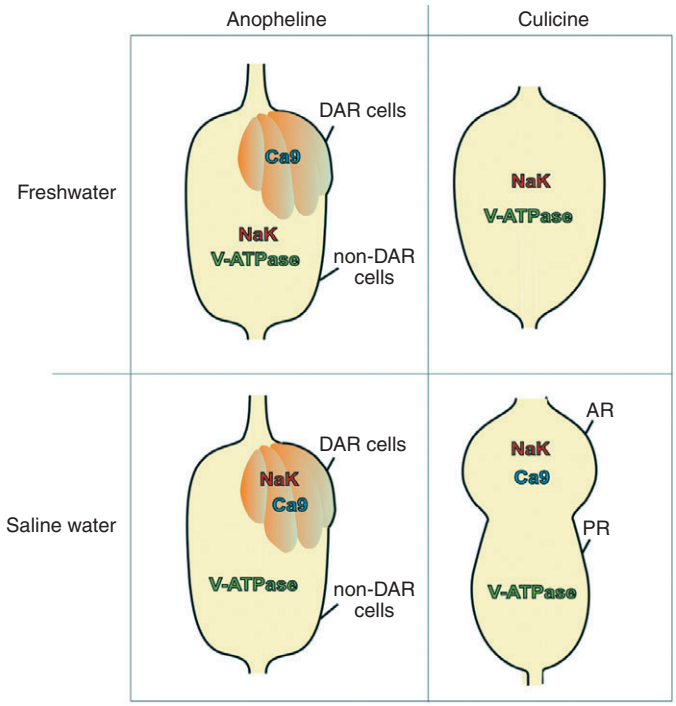

Fig. 5. A comparison of the major protein localization patterns found in the recta of freshwater-reared culicines and anophelines with those in saline water-reared culicines and anophelines. This figure allows an easy comparison of the proteins in the anopheline and culicine recta. In all cases CA9 localizes to the cytoplasm, Na^+K^+-ATPase (NaK) localizes to the basal membrane and V-ATPase localizes to the apical membrane. The non-DAR (dorsal anterior rectum) cells of freshwater-reared anophelines resembled the recta of freshwater culicines in protein localization as both expressed Na^+/K^+-ATPase and V-ATPase. When reared in saline water, the anopheline rectum shifted Na^+/K^+-ATPase localization and resembled the saline-water culicine rectum in protein localization; anopheline DAR cells and culicine AR possessed CA9 and Na^+/K^+-ATPase whereas anopheline non-DAR cells and culicine PR possessed V-ATPase.

transports the Na^+ across the basal membrane into the hemolymph replenishing ions, which are lost to the aquatic environment as first suggested by Koefoed-Johnsen and Ussing (Koefoed-Johnsen and Ussing, 1958).

One example of V-ATPase-mediated Na^+ transport into mosquito cells occurs in the alimentary canal of many species: Na^+ is driven into the cell by the electrical coupling of a V-ATPase and a Na^+:amino acid±nutrient amino acid transporter (NAT) (Boudko et al., 2005). Hyperpolarization of the apical membrane by V-ATPase drives electrophoretic Na^+:amino acid symport into the cells. The efflux of H^+ and influx of Na^+ driven by the coupling of these two proteins constitutes a Na^+/H^+ exchanger (NHE), $NHE_{V\text{-}NAT}$ (Okech et al., 2008). In addition to driving Na^+, V-ATPase hyperpolarization of the apical membrane may drive the absorption of numerous other essential ions.

In *Oc. taeniorhynchus* reared in freshwater, a similar synergy between V-ATPase (apical) and Na^+/K^+-ATPase (basal) is evident in the AR, a region that has a resorptive function (Bradley and Phillips, 1977). This finding provides further support that the physiological coupling between these ATPases is involved in resorbing essential ions from the primary urine. Importantly, when *Oc. taeniorhynchus* are reared in 100% ASW, the apical V-ATPase appears to localize to the cytoplasm, breaking the coupling with Na^+/K^+-ATPase, which may indicate that these cells decrease their resorptive function in the presence of high salinity.

The cytoplasmic localization of V-ATPase, a membrane protein, in the DAR cells of both *An. gambiae* and *An. albimanus* as well as in the AR of saline-reared *Oc. taeniorhynchus* was unexpected although there are several possible explanations. The rectal ultrastructure of the DAR cells has not been extensively studied but the AR of saline-tolerant culicines exhibits a highly infolded basal membrane which constitutes the major elaboration of the surface area (Meredith and Phillips, 1973). The apparent cytoplasmic V-ATPase may instead be localizing to these basal infoldings. Alternatively, this localization could represent V-ATPase protein on the membrane of vacuoles within the cells (Harvey, 1992) or subunits that have dissociated from their membrane-bound V_0 anchors (V-ATPase is composed of two domains, V_0, which consists of membrane bound subunits, or V_1, which consists of pheripherally associated subunits). Dissociation of V_1 from V_0 occurs during molting in caterpillars (Sumner et al., 1995) and during glucose deprivation in yeast (Kane and Parra, 2000) and indicates inactivation of the protein.

Saline water-reared larvae: V-ATPase and Na^+/K^+-ATPase localization

When anopheline larvae were reared in saline water, we observed a shift in Na^+/K^+-ATPase localization to the DAR cells (this protein was not abundant in the DAR cells of freshwater-reared larvae). Whereas in *An. albimanus* this shift was drastic and accompanied by a decrease in Na^+/K^+-ATPase in the non-DAR cells, in *An. gambiae* Na^+/K^+-ATPase was present in both DAR and non-DAR cells suggesting an intermediate condition. In *An. gambiae* we also noted a marked reduction in the overall Na^+/K^+-ATPase signal as well as a noticeable increase in V-ATPase signal in the cytoplasm of all rectal cells. This may indicate a breakdown in the ion-transporting functions of the rectal cells. As an obligate freshwater larva, *An. gambiae* lacks the ability to secrete a hyperosmotic urine and, in fact, cannot survive more than 24 h when acclimated to 60% ASW. It is possible that the stress incurred by exposure to saline water causes a breakdown in the cellular components of the ion-regulatory organs, which would lead to death.

In saline-tolerant larvae such as *An. albimanus*, we did not observe any decrease in Na^+/K^+-ATPase protein signal or any indications of protein degradation. *An. albimanus* larvae can survive to pupation in up to 75% ASW, with about 35% of the 1st-instar larvae reaching pupation (Hurlbut, 1943). The dramatic shift in Na^+/K^+-ATPase (upregulation in the DAR cells and downregulation in the non-DAR cells) appears to disrupt the physiological coupling between Na^+/K^+-ATPase and V-ATPase, which we predict to be responsible for Na^+ resorption in freshwater conditions. This shift renders the non-DAR cells similar in protein localization to the PR of the saline-tolerant culicine *Oc. taeniorhynchus*, and is likely to result in the ability of the non-DAR cells of *An. albimanus* to secrete a hyper-osmotic urine in a fashion similar to *Oc. taeniorhynchus* as discussed below.

Oc. taeniorhynchus larvae are highly saline-tolerant, able to survive up to 300% ASW by regulating their hemolymph to maintain a constant osmolarity of ~350 osmol l^{-1} (Nayar and Sauerman, 1974; Bradley, 1994). The rectum of this species differs from that of the freshwater *Ae. aegypti* by the presence of an additional segment (PR) which secretes a hyper-osmotic urine and rids the hemolymph of excess ions from a saline environment. The PR of *Oc. taeniorhynchus* expressed high levels of V-ATPase on the apical lamellae but did not appear to express Na^+/K^+-ATPase. The presence of an apical V-ATPase is supported by ultrastructural

studies of other saline-tolerant culicines, which described a particulate coat made up of spherical subunits on the cytoplasmic surface of the apical lamellae (Meredith and Phillips, 1973) indicating V-ATPase-containing portasomes. As mentioned above and discussed in greater detail by Harvey (Harvey, 1992), the hyperpolarizing action of V-ATPase can be used to drive many ion transporting processes against an otherwise unfavorable concentration gradient. We hypothesize that the V-ATPase on the apical lamellae of the PR in *Oc. taeniorhynchus* and in the non-DAR cells in *An. albimanus* is energizing one or more transporters also present on this membrane to translocate ions from the cells to the lumen in order to excrete excess ions from the hemolymph.

The ability for *An. albimanus* larvae to shift Na^+/K^+-ATPase protein localization in response to salinity, as well as the reversibility of this event, may be dependent on the larval stage at which exposure occurs. Younger larvae (2nd- and 3rd-instars) reared in freshwater and exposed to 25% ASW exhibited a shift in Na^+/K^+-ATPase localization from non-DAR to DAR cells in as little as 24 h. More mature larvae (4th-instars) exposed to 25% ASW for 24 h expressed Na^+/K^+-ATPase in both non-DAR cells and DAR cells as if in an intermediate state between freshwater and saline water protein expression. After 48 h, the majority of Na^+/K^+-ATPase was expressed in the DAR cells of 4th-instar larvae. This could indicate that early instar larvae are far more plastic in terms of regulating gene and/or protein expression than late larvae.

Slightly different results were obtained when rearing larvae in 25% ASW and exposing them to freshwater. Whereas 2nd-instar larvae shifted Na^+/K^+-ATPase localization from the DAR to non-DAR cells within 24 h of exposure to freshwater, 3rd- and 4th-instar larvae consistently expressed the protein in both DAR and non-DAR cells even after 72 and 48 h, respectively. This may indicate that at the 2nd-instar larval stages, Na^+/K^+-ATPase protein shift is fully reversible, whereas more mature larvae cannot downregulate Na^+/K^+-ATPase expression in the DAR cells within the period of time observed. It is possible that the concentration of ASW was too low to cause a complete shift in localization in more mature larvae, and that larvae would respond differently if reared in 50% ASW *vs* 25% ASW. It is interesting that a protein shift is seen at all in 25% ASW (250 osmol l^{-1}) as the osmolarity is less than that of the *An. albimanus* hemolymph (~300 osmol l^{-1}). This may be due to the abruptness of the salinity change with larvae being transferred directly from freshwater to 25% ASW in the present study. Additionally, it could indicate that some event signals the rectum to shift Na^+/K^+-ATPase protein before it is actually necessary to produce a hyper-osmotic urine as if in preparation.

CA9 is involved in CO_2 excretion

CA9 consistently localized to the DAR cells of all anopheline mosquitoes examined (including *An. gambiae*, *An. albimanus*, *An. farauti*, *An. quadrimaculatus*, *An. aquasalis* and *An. stephensi*) as well as to the AR of *Oc. taeniorhynchus* regardless of rearing water salinity. Members of the CA family are implicated in both ion and pH regulation in a number of organisms (e.g. Henry, 1984; del Pilar Corena et al., 2004; Schewe et al., 2008) and catalyze the hydration of CO_2 to H_2CO_3 (carbonic acid), which instantaneously dissociates into H^+ and HCO_3^- (bicarbonate). CA within the epithelial cells can convert cellular CO_2 to HCO_3^-, which can then be excreted by the action of an electroneutral Cl^-/HCO_3^- exchanger (Smith et al., 2007). Because the localization of this protein did not change in any species in response to being reared in saline water compared with freshwater, we hypothesize that those rectal cells abundant in CA9 protein have at least one role that is independent of external water salinity.

One possible role for CA-expressing cells is the transport of HCO_3^- from blood to lumen (in preparation for excretion) or from lumen to blood (absorption). In this way, the cells may use metabolic CO_2 to regulate hemolymph HCO_3^- levels. Determining the presence and polarity of Cl^-/HCO_3^- exchangers in these cells would reveal the direction in which HCO_3^- was being transported. Alternatively, cells that are enriched in CA protein could be more metabolically active than neighboring cells, which produce less CA. Cells with a robust metabolism would produce higher levels of CO_2 that, in turn, could induce synthesis of high levels of CA protein to convert the toxic CO_2 to HCO_3^- prior to excretion. In support of this suggestion, there is evidence that the AR of *Aedes dorsalis* is involved in HCO_3^- secretion (Strange et al., 1984), which is mediated by a rectal CA (Strange and Phillips, 1984).

Although we were unable to detect CA9 in the recta of *Ae. aegypti* larvae, this does not indicate a lack of CA activity in the rectum of this species. There are 13 predicted CA genes in the *Ae. aegypti* genome (www.ensembl.org), any of which could catalyze the conversion of CO_2 to HCO_3^- in the rectal cells. Likewise, it is possible that specific CAs are present in the non-DAR cells of anophelines and the PR of culicines. In support of CA activity in *Ae. aegypti* recta, alkalization of the rearing medium by starved *Ae. aegypti* larvae was reported by Stobbart who suggested that they may be excreting K^+ HCO_3^- (Stobbart, 1971). A similar alkalization was reported by Corena et al., who noted that alkalization is blocked by global CA inhibitors (Corena et al., 2002). However, Clark et al. (Clark et al., 2007) reported that the rectal lumen of *Ae. aegypti* is acidic (pH<6.2), not alkaline, which would be expected for a larva excreting either K^+ or Na^+ and HCO_3^-. It is possible that HCO_3^- secreted into the lumen associates with H^+ ions provided by an apical V-ATPase to form H_2CO_3, which would dissociate into H^+ and HCO_3^- when excreted from the rectum. However, as Clark et al. (Clark et al., 2007) discuss, the pKa of H_2CO_3/HCO_3^- is 6.4 and in an environment more acidic than this pH, HCO_3^- would exist primarily in the form of CO_2. Another possibility is that CA is not present in the recta of *Ae. aegypti*. Neither Stobbart (Stobbart, 1971) nor Corena et al. (Corena et al., 2002) directly measured the pH of rectal excretions, and it is possible that the larvae alkalized their media by some other manner (i.e. gaseous respiration *via* the tracheal system). The role of CA in *Ae. aegypti* recta, as well as in other anopheline and culicine species, can be further examined by measuring the precise pH and the concentration of HCO_3^- directly from the rectal contents.

CONCLUSIONS

Based on the comparison of ion-regulatory protein localization in culicine and anopheline larval recta, we hypothesize that saline-tolerant anophelines secrete a hyper-osmotic urine by the same rectal cells that are present in freshwater anophelines. This is in contrast to saline-tolerant culicines, which have a separate rectal region to secrete a hyper-osmotic urine. When reared in freshwater, both saline-tolerant and freshwater anopheline larvae actively resorb water and nutrients from the primary urine without excreting salt. In support of this idea, the protein localization patterns of anopheline non-DAR cells resemble those of the freshwater culicine rectal cells, which are known to be active in resorbing ions. When exposed to saline water, saline-tolerant anophelines activate a region of the rectum (non-DAR cells) to secrete a hyper-osmotic urine by shifting protein localization of certain membrane energizing proteins such as Na^+/K^+-ATPase. This shift breaks the system of ion resorption in the non-DAR cells and the rectum functions in a way that is similar to that of a saline water culicine rectum, with the DAR cells

performing the task of the AR (resorption) and the non-DAR cells performing the task of the PR (excretion).

These data suggest that two sub-families of mosquitoes, anophelines and culicines, differ greatly in rectal structure. These data also suggest that anophelines regulate protein expression differently than culicines do when reared in saline water. The present study demonstrates that data obtained from one species of mosquito cannot necessarily be applied to all species. The majority of the currently available information concerning rectal structure and function, as well as ion regulation (a system crucial for larval survival), pertains to culicine species. These data, along with ultrastructural and physiological research currently underway, will expand that information to include the equally important anopheline sub-family.

LIST OF ABBREVIATIONS

AP	alkaline phosphatase
AR	anterior rectum
ASW	artificial seawater
CA	carbonic anhydrase
DAR	dorsal anterior rectum
PR	posterior rectum
Pre-inc	pre-incubation buffer
TBS	tris-buffered saline

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