The Journal of Experimental Biology 210, 4359-4367
Published by The Company of Biologists 2007
doi:10.1242/jeb.010694

Strategies for regulation of hemolymph pH in acidic and alkaline water by the larval mosquito *Aedes aegypti* (L.) (Diptera; Culicidae)

Thomas M. Clark*, Marcus A. L. Vieira, Kara L. Huegel, Dawn Flury and Melissa Carper
Indiana University South Bend, South Bend, IN 46615, USA
*Author for correspondence (e-mail: tclark2@iusb.edu)

Accepted 27 September 2007

Summary

The responses of larval *Aedes aegypti* to media of pH 4, 7 and 11 provide evidence for pH regulatory strategies. Drinking rates in pH 4 media were elevated 3- to 5-fold above those observed in pH 7 or 11. Total body water was elevated during acute exposure to acidic media. During chronic exposure, total body water was decreased and Malpighian tubule mitochondrial luminosity, quantified using Mitotracker Green FM, increased. Malpighian tubule secretion rates and energy demands thus appear to increase dramatically during acid exposure. In alkaline media, drinking rates were quite low. Larvae in pH 11 media excreted net acid (0.12 nequiv H^+ g^{-1} h^{-1}) and the pH indicators azolitmin and bromothymol blue revealed that the rectal lumen is acidic *in vivo* at all ambient pH values. The anal papillae (AP) were found to be highly permeant to acid–base equivalents. Ambient pH influenced the length, and the mass-specific length, of the AP in the presence of NaCl (59.9 mmol l^{-1}). In contrast, the length and mass-specific length of AP were not influenced by ambient pH in low NaCl conditions. Mitochondrial luminosity was reduced in AP of larvae reared in acidic media, and was not elevated in alkaline media, relative to that of larvae reared in neutral media. These data suggest that the AP may compromise acid–base balance in acidic media, and may also be an important site of trade-offs between H^+ homeostasis and NaCl uptake in dilute, acidic media.

Key words: hemolymph pH, pH homeostasis, Malpighian tubules, rectum, mitochondrial density, mosquito, larva, *Aedes aegypti*.

Introduction

Maintenance of extracellular fluid pH is critically important for cellular function. For aquatic animals, ambient pH has direct effects on survival due to challenges to extracellular fluid ion and pH homeostasis, and indirect effects due to changes in the population dynamics of other species (Vangenechten et al., 1989). pH is thus an extremely important physical factor limiting the distribution and abundance of aquatic animals. Larval mosquitoes are remarkably tolerant of extreme pH, allowing them to exploit a wide variety of habitats in nature (Clements, 2000; Clark et al., 2004). Larvae of the mosquitoes *Aedes aegypti* and *Ochlerotatus taeniorhynchus* complete development in waters ranging from pH 4 to pH 11 in the laboratory. Across this range, hemolymph pH of acclimated larvae varies by only 0.1 pH units or less (Clark et al., 2004). They show phenotypic plasticity in pH tolerance in which acclimation to pH 4 or 11 increases their ability to tolerate subsequent exposure to more extreme pH (i.e. pH 3 or 12, respectively).

In larval endopterygotes, pH is regulated in two major extracellular compartments, the hemolymph and the midgut lumen. The cellular mechanisms involved in the generation of highly alkaline conditions within the midgut of lepidopteran and mosquito larvae have received considerable attention. In contrast, the physiology of acid–base homeostasis of insect hemolymph continues to be a neglected area of study. In fact, the specific contributions of each organ of the alimentary and excretory systems to elimination of a hemolymph acid or base challenge are known only for a single insect, the locust *S. gregaria*. In the locust, an acid load injected into the hemocoel was cleared primarily by the hindgut. The Malpighian tubules secreted acidic fluid, but their contributions to acid–base homeostasis were minor (Harrison et al., 1992; Stagg et al., 1991; Phillips et al., 1993; Harrison, 2001). Responses of locusts to alkaline challenges have not been described. Little is known about the regulation of hemolymph pH in other insects (Cooper, 1994; Harrison, 2001).

Aquatic insects rely primarily on epithelial transport across renal systems for acid–base homeostasis (Cooper, 1994; Harrison, 2001). The ion transport mechanisms relevant to acid–base homeostasis are expected to be located in ion-transporting epithelia including the midgut, the renal system (consisting of the Malpighian tubules and hindgut) and extrarenal ion-transporting organs. The Malpighian tubules form the primary urine, while the rectum recovers water and ions as necessary, and secretes additional ions. Aquatic insects frequently possess extrarenal organs such as anal papillae (AP), or clusters of mitochondria-rich chloride cells, that provide increased surface area for mechanisms involved in ionic homeostasis (Komnick et al., 1972). The Malpighian tubules

and hindgut of *Ochlerotatus taeniorhynchus* possess inducible specific transport mechanisms for secretion of a variety of ions (Bradley and Phillips, 1977; Maddrell and Phillips, 1978). Most inducible ion transport systems are located in the rectum, with the exception of inducible SO_4^{2-} transport, which occurs in the Malpighian tubules (Maddrell and Phillips, 1978). Induction of H^+ transport was not investigated.

The limited work on larval mosquito hemolymph pH homeostasis in alkaline media emphasizes the role of the rectum. Larvae of the mosquito *Aedes dorsalis* inhabit alkaline lakes containing high concentrations of bicarbonate, and under these conditions bicarbonate is excreted into the rectum *via* HCO_3^-/Cl^- exchange (Strange and Phillips, 1984; Strange et al., 1982; Strange et al., 1984). The AP of larval mosquitoes and related Diptera consist of four elongated gill-like structures attached to the last abdominal segment. They are covered with cuticle, contain a cellular syncetium, and can be isolated from the hemolymph by the actions of a ring of muscles at their base (Clements, 2000). The AP of larval mosquitoes are known to be important sites of ion uptake, and are the region of the cuticle most permeant to water (Wigglesworth, 1933; Wigglesworth, 1938; Koch, 1938). Larval *Aedes aegypti* reared in dilute water have larger AP (Wigglesworth, 1938), with greater mitochondrial densities (Edwards and Harrison, 1983), than larvae from less dilute media. These increases have been attributed to increased energetic requirements for active uptake of NaCl from the medium (Koch, 1938; Wigglesworth, 1938; Edwards and Harrison, 1983). The role of these organs in acid–base balance has not been investigated. Mechanisms used by larval mosquitoes to maintain hemolymph pH homeostasis in acidic media have not been addressed.

In the present study, responses of larval *Aedes aegypti* to acidic (pH 4), neutral and alkaline (pH 11) media allowed the elucidation of the overall physiological strategies of hemolymph pH homeostasis in this insect. This study focused on the roles of the Malpighian tubules, rectum and AP in the regulation of hemolymph pH. Evidence suggests that the larvae utilize novel and unexpected regulatory strategies to maintain homeostasis at each end of the tolerable pH range. It appears that the Malpighian tubules play a major role in acid excretion in acidic media. No evidence was obtained for an active role of any organ in acid–base homeostasis during alkaline challenges.

Materials and methods

Mosquitoes

The *Aedes aegypti* (L.) colony was derived in 1999 from a colony maintained at the Florida Medical Entomology Laboratory (Vero Beach, FL, USA). Eggs were hatched in deionized water. The next day, larvae were placed in rearing solutions of the appropriate pH and NaCl concentrations (see below). Larvae were maintained on a 16 h:8 h L:D photoperiod at 26°C. In all cases, rearing solutions were replaced and larvae were fed ground TetraMin flakes (TetraWerke, Melle, Germany) daily. Rearing solutions (RS) contained 2.5 mmol l^{-1} Trizma base, 2.5 mmol l^{-1} Hepes and 59.9 mmol l^{-1} NaCl unless stated otherwise (chemicals were obtained from Sigma, St Louis, MO, USA). RS were adjusted to the appropriate pH (3, 4, 7, 9 or 11) using HCl or NaOH, giving RS 3, 4, 7, 9 and 11, respectively. NaCl was used in initial studies so that adjustment of pH did not cause proportionally large differences in Na^+ or Cl^- concentrations in the acidic and alkaline water (Clark et al., 2004). This allowed the observed physiological changes to be attributed with greater certainty to differences in pH. In the present study, rearing solutions low in NaCl were also prepared at pH 4, 7 and 11 (low-NaCl RS 4, 7 and 11). These low-NaCl RS contained the same buffers, and were adjusted to pH in the same way, but any NaCl present consisted only of that present in the food and the Na^+ or Cl^- added while adjusting the pH of the solution.

Mitochondrial luminosity

To isolate Malpighian tubules, the head and seventh abdominal segment of late-stage fourth instar larvae were severed under cold mosquito hemolymph substitute solution (HSS) (Clark et al., 2005). Fine forceps were then used to grasp the esophagus or the gastric caeca and pull the gut from the body using care to avoid stretching the tissue. To label mitochondria, guts with attached Malpighian tubules and terminal segments with AP were incubated for 30 min in cold HSS containing 50 nmol l^{-1} Mitotracker Green FM (Molecular Probes Inc., Eugene, OR, USA). They were then placed on a coverslip with a drop of glycerol. Mitotracker Green FM only fluoresces when bound to a mitochondrial membrane, and does not require respiring mitochondria (product literature, Molecular Probes Inc.). Mitotracker Green fluorescence was then recorded in whole mounts using a Zeiss Axioskop microscope (Oberkochen, Germany), with a Kubler Codex ebq100 lamp source (Gena, Germany). The excitation and emission wavelengths of Mitotracker Green are 490 and 516 nm respectively; an Omega Optical XF100-2 filter set (Brattleboro, VT, USA) was used. Images were captured at ×100 magnification using a Pixera Penguin 150CL digital camera (Los Gatos, CA, USA) under manual control (exposure 1/8 s). Because the same exposure time and magnification were used for all images, it was possible to quantify changes in the mitochondrial signature of the tissues. Images were imported into Adobe Photoshop where luminosity was quantified from the intensity of the fluorescence in boxes of uniform pixel numbers.

Drinking rates

The rates at which acclimated larvae drink RS were determined at each pH by placing larvae in 1 ml of their respective RS containing 0.5 g l^{-1} fluorescein isothiocyanate conjugated to dextran (FITC-dextran, average molecular mass 4.3 kDa; Sigma) for a period of between 1 and 3 h. The treatment groups were sampled in repeated series during assays to avoid artifacts from possible time-dependent changes in drinking rates. Controls consisted of larvae ligated at the neck, and assayed alongside treatment groups at each pH. Following exposure to FITC-dextran, larvae were rinsed, blotted dry, weighed to the nearest 10 μg, and homogenized in 200 μl of cold Tris-buffered saline. This step ensured that the fluorescence of FITC-dextran was always determined at the same final pH. Following centrifugation for 1 min at 18 000 ***g***, the fluorescence of the supernatant was quantified in a Turner Designs TD-700 Fluorometer with minicell adaptor (Sunnyvale, CA, USA). The FITC-dextran content of each larva

was determined by comparison with a standard curve. The fluorescence of ligated controls, due to a combination of autofluorescence and low rates of transcuticular FITC-dextran entry, was subtracted from treatment values assayed at the same pH. This corrected for possible artifacts such as effects of pH on transcuticular FITC-dextran permeability.

Effects of pH challenges on total body water

The effects on total body water of the transfer of acclimated larvae to more acidic media were determined in the presence and absence of NaCl. Tests performed in the presence of NaCl involved rearing larvae in RS 7 or RS 4. Fourth instar larvae were then either maintained at the rearing pH or transferred to RS 3 for 2 h. In similar experiments without NaCl, larvae reared in low-NaCl RS 7 were assayed in either low-NaCl RS 7 or low-NaCl RS 3. Following exposure, larvae were blotted dry, and wet mass was determined to the nearest 1×10^{-5} g using a Mettler-Toledo AX205 deltarange balance (Columbus, OH, USA). The larvae were then dried overnight in a drying oven at 96°C, and reweighed. The decrease in mass represents total free body water while the ratio of body water to total mass represents the percentage body water or body water ratio, calculated as: [(wet mass – dry mass)/wet mass].

pH of the rectum in vivo

Larvae reared in RS 4, 7 and 11 were exposed to kaolin and azolitmin (1 g per 50 ml) for 24 h. Kaolin is an inert silicate that is ingested by larvae, displacing the food column and providing a background against which the color of ingested pH indicators can be established (Dadd, 1975). They were then transferred to RS 4, 7 or 11. Each combination of rearing and acute pH exposure was performed. The larvae were photographed after 24 h of exposure to the assay pH using an Olympus C-3040 digital camera through a Leica Stereozoom 6 microscope (Wetzlar, Germany).

pH of the AP in vivo

It was noted that bromothymol blue enters the AP in acidic media allowing the investigation of the acid–base permeability of the AP. Bromothymol blue was initially dissolved in dimethyl sulfoxide (7.6 mg per 10 ml). This mixture was vortexed, then centrifuged. The supernatant was diluted 1:1000 in RS 4. Larvae were exposed to this solution for 24 h. They were then placed into RS lacking bromothymol blue. The pH of the AP was noted, and the larvae were photographed within 5 min and at 24 h of exposure to the assay pH using an Olympus C-3040 digital camera through a Leica Stereozoom 6 microscope. Bromothymol blue was also visible in the rectum of several of these larvae allowing confirmation of the azolitmin results.

Morphology of the AP

Lengths of AP were determined for larvae reared in RS 4, 7 and 11, in low-NaCl RS 4, 7 and 11, and in deionized water, 5.25 g l^{-1} artificial seawater and 10.5 g l^{-1} artificial seawater (Instant Ocean, filtered; Aquarium Systems, Mentor, OH, USA). Lengths of AP of larvae fixed in 2% glutaraldehyde in PBS were determined with a Leica Stereozoom 6 with ocular micrometer. Larvae were oven dried for 24 h at 96°C, then weighed to the nearest 1×10^{-5} g in order to determine the mass-specific length of the AP.

Rates of acid–base excretion

Larval acid–base excretion rates were determined under controlled conditions by rinsing fed, acclimated larvae twice in fresh RS, and placing them individually into 2.0 ml of fresh RS 4 or RS 11. RS aliquots without larvae were run alongside larvae and served as paired negative controls. After a period of 1–2 h, the pH of the experimental and control solutions was determined. Titration of the RS allowed calculation of the rate of acid excretion from the difference in pH of the experimental and control solutions.

Results

Effects of ambient pH on drinking rates, total body water and excretion rates

The effects of ambient pH on drinking rates were determined in animals reared in the presence (RS) and absence (low-NaCl RS) of NaCl. Larvae in acidic media (RS 4 or low-NaCl RS 4) drank the medium at much higher rates than larvae in neutral or alkaline media (Fig. 1A; $P<1\times10^{-8}$, $F=18.59$, d.f.=47). The lowest rates observed were in highly alkaline media (pH 11), where drinking rates were 0.28±0.051 ml g^{-1} day^{-1} in the presence of NaCl (RS 11) and 0.88±0.168 ml g^{-1} day^{-1} in the absence of NaCl (low-NaCl RS 11). Thus, even in the most alkaline media the drinking rates were substantial, representing 28–88% of total body volume each day. In acidic media, larvae consumed 500–700% of their total body volume each day. These values are similar to those reported for mosquito larvae under other conditions (Bradley, 1987; Clements, 2000). The presence or absence of NaCl did not influence the effect of pH on drinking rates [$P>0.05$, single factor ANOVA followed by Student–Newman–Keuls (SNK) *a posteriori* test with $\alpha=0.05$].

Total body water was influenced by both ambient pH and NaCl (Fig. 1B; $P<1\times10^{-8}$, $F=18.59$, d.f.=47, single factor ANOVA of arcsine-transformed body water ratios, followed by SNK with $\alpha=0.05$) (Sokal and Rohlf, 1969). Total body water was reduced in larvae reared in RS 4 relative to RS 7. Total body water increased when larvae reared in RS 4 were transferred to RS 3, and when larvae reared in RS 7 or low-NaCl RS 7 were transferred to RS 3 or low-NaCl RS 3, respectively. Because chronic exposure to acidic water resulted in increased drinking rates, but decreased percentage body water, fluid excretion rates must increase as well. In the presence of NaCl, fluid ingestion and excretion rates in acidic water (pH 4) were approximately 20 times those in alkaline water (pH 11; 5.9 *vs* 0.3 ml g^{-1} day^{-1}). In the absence of NaCl, they were 7.9-fold greater in acidic water than in alkaline water (7.1 *vs* 0.9 ml g^{-1} day^{-1}). Drinking rates were similar in neutral and alkaline media and in the presence and absence of NaCl (Fig. 1).

Effects of ambient pH on acid–base excretion

Larvae in RS 4 did not change the pH relative to controls over 1–2 h time intervals. Larvae in RS 11 excreted net acid (0.12±0.017 nequiv g^{-1} h^{-1}, mean ± s.e.m.) relative to paired larva-free controls (larvae *vs* larva-free controls, $P<1\times10^{-9}$, two-factor ANOVA without replication, EXCEL).

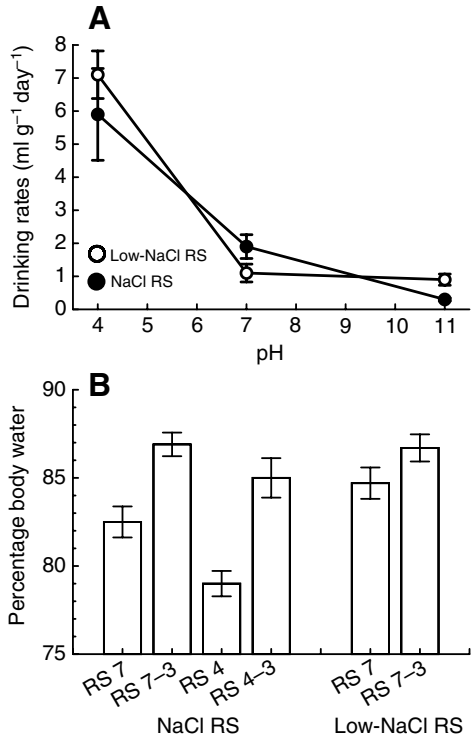

Fig. 1. Effects of acid–base exposure on drinking rates and total body water. Animals were reared and assayed in media containing NaCl (RS; 59.9 mmol l^{-1}), or in media in which the only added NaCl was that present in the food (low-NaCl RS). (A) Effects of pH on drinking rates in the presence and absence of NaCl. Drinking rates of acclimated larvae were determined using FITC-dextran ingestion rates (average molecular mass 4.3 kDa). Drinking rates were highest in acidic rearing solutions (column effects; $P<1\times10^{-8}$, F=50.8, d.f.=47). The presence or absence of NaCl did not influence the effect of pH on drinking rates (column effects; F=1.37, P>0.22). Rearing solutions containing NaCl are indicated by ●, while those without added NaCl are indicated by ○. (B) Effects of ambient pH on percentage body water. Body water was reduced in larvae chronically exposed to acidic media, and was elevated when larvae were acutely exposed to more acidic media (single factor ANOVA of arcsine-transformed body water ratio). The presence or absence of NaCl did not influence the effects of acute pH challenges on body water. RS 7 represents larvae reared and assayed in RS 7, RS 7–3 represents larvae reared in RS 7 and assayed in RS 3, RS 4 represents larvae reared and assayed in RS 4, and RS 4–3 represents larvae reared in RS 4 and transferred to RS 3. For larvae reared and assayed in low NaCl conditions, low-NaCl RS 7 represents larvae reared and assayed in low-NaCl RS 7, while low-NaCl RS 7–3 represents larvae reared in low-NaCl RS 7 and assayed in low-NaCl RS 3. Data are presented as means ± s.e.m.

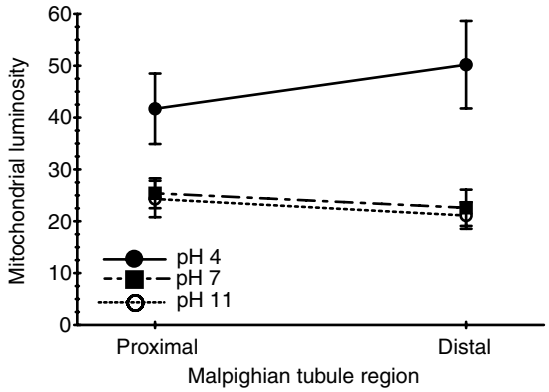

Fig. 2. Mitochondrial luminosity within the Malpighian tubules of animals reared in acidic (pH 4), neutral or alkaline (pH 11) media. Mitochondrial densities were influenced by environmental pH within the proximal tubule (P<0.05, F=4.24, d.f.=17) and within the distal tubule (P<0.003, F=8.92, d.f.=17, single factor ANOVA). N=6/pH value. All measurements were made with an exposure time of 1/8 s and at the same magnification (×100). Data are presented as means ± s.e.m.

Effects of ambient pH on mitochondria

Changes are observed in the fluorescence of the mitochondrial dye Mitotracker Green FM in response to chronic pH exposure. Mitochondrial luminosity was higher in both proximal and distal Malpighian tubule regions of animals reared in acidic water than in larvae reared in neutral or alkaline media, which did not differ in luminosity (Fig. 2; proximal tubule: P<0.05, F=4.24, d.f.=17; distal tubule: P<0.005, F=8.92, d.f.=17; followed by SNK) (Sokal and Rohlf, 1969). In larvae reared in acidic media (RS 4), the distal Malpighian tubule showed significantly greater luminosity than the proximal region (P<0.05, two-tailed, paired Student's t-test, N=6 tubules). No differences in luminosity were observed within pH between proximal and distal regions of the Malpighian tubules of larvae reared in RS 7 or RS 11 (RS 7, P>0.5; RS 11, P>0.09, two-tailed, paired t-test, N=6 tubules/RS). The influence of pH on hindgut mitochondrial luminosity was not determined due to difficulties experienced in the removal of the rectal contents, which autofluoresce.

Mitotracker Green FM produced uneven fluorescence within the AP. Luminosity was always greatest in the proximal and distal regions, and lowest in the central region (Fig. 3; by region, pH 4: P<0.0001, F=12.646, d.f.=65; pH 7: P<0.01, F=5.137, d.f.=56; pH 11: P<0.0005, F=8.777, d.f.=62; single factor ANOVA followed by SNK). Changes were observed in Mitotracker Green FM fluorescence, within the AP, in response to chronic pH exposure. Animals reared in RS 4 showed reduced luminosity across regions compared with animals reared in RS 7 or 11 (Fig. 3; P<0.05, F=3.161, d.f.=61, single factor ANOVA followed by SNK.

In the absence of Mitotracker Green FM no fluorescence was observed at the exposure time of 1/8 s used in these experiments.

The pH of the rectal lumen

The pH indicator azolitmin revealed that larvae always have an acidic rectal lumen (pH <6.2), even when chronically exposed to RS 11 (Fig. 4; N=12 each in RS 4, RS 7 and RS 11). Similar results were obtained using bromothymol blue (transition from yellow to blue at pH 6.8; Fig. 5). Obtaining similar results with two separate pH indicators provides increased confidence that the excretory system of larvae exposed to highly alkaline media eliminates fluid more acidic than the hemolymph.

Effects of ambient pH on the pH within the AP

The pH indicator bromothymol blue entered the AP of larvae in RS 4, but not in RS 7 or RS 11. In RS 4, the bromothymol

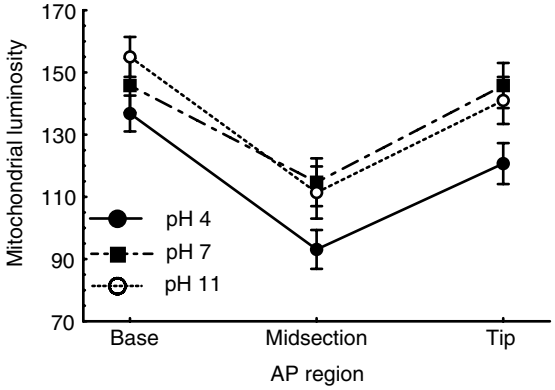

Fig. 3. Mitochondrial luminosity of anal papillae (AP) of animals reared in acidic (pH 4), neutral or alkaline (pH 11) media. All measurements were made with an exposure time of 1/8 s and at the same magnification. Luminosity was determined for the base, midsection and tip of one AP from each animal. Luminosity was always greatest in proximal and distal regions, and lowest in the central region. Larvae reared in acidic media (RS 4) showed reduced mitochondrial densities in the midsection and tip, and mitochondrial densities averaged across regions were also reduced in RS 4 (P<0.05).

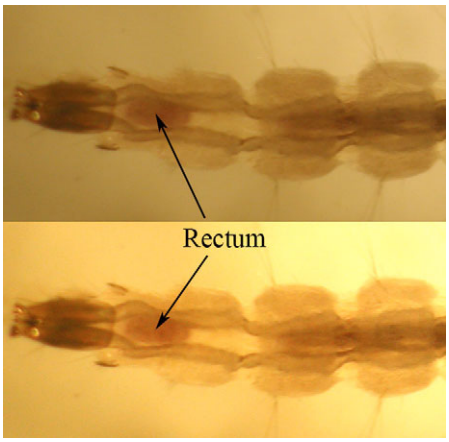

Fig. 4. The rectum of a larva that had ingested azolitmin *in vivo*, reared and assayed in alkaline water (pH 11). Azolitmin is blue in alkaline water (pH >6.2), and red in acidic water (pH <6.2). A total of 36 larvae were assayed (12 larvae each in RS 4, RS 7 and RS 11). The rectal contents were red (pH <6.2) in all larvae. Top, the original image. Bottom, the same image adjusted for brightness (+39) and contrast (+35) using Adobe Photoshop.

blue within the AP was yellow, revealing that the pH was <6.8 (Fig. 5A). When these larvae were transferred to RS 11, the bromothymol blue within the AP changed from yellow to blue within minutes. The blue color remained within the AP for at least 24 h following transfer to RS 11, revealing (1) diffusion trapping of the ionized form upon alkalization, and (2) that the pH within the AP remained >6.8 during that time (Fig. 5B). These data reveal a high degree of transparency of the AP to acid–base equivalents, and failure to recover the initial acidic pH within the AP in the face of alkalization of the ambient media.

Effects of salinity and ambient pH on larval mass and the size of the AP

The size of AP is influenced by ambient salinity and by pH (Figs 6 and 7). The AP were longest in dilute water, and length decreased with salinity (Fig. 6A; P<0.05, F=3.97, d.f.=25, single factor ANOVA). The dry mass of larvae used to determine the effects of environmental conditions on the length of AP differed in both the salinity and pH experiments. Significant differences in dry mass were recorded among salinities (P<0.05, F=4.66, d.f.=25). Among media differing in pH values, significant differences were recorded in the presence of NaCl (P<0.0005, F=11.25, d.f.=29) but not in the absence of NaCl (P>0.21, F=1.64, d.f.=29; Table 1). However, because salinity also influences mass, the mass-specific lengths of the AP were also determined. No differences were observed in mass-specific lengths (mm mg^{-1} dry mass) of the AP across salinities (Fig. 6B; P>0.27, F=1.37, d.f.=25, single factor ANOVA) although larvae reared at the highest salinities possessed the smallest AP.

In contrast to salinity, pH influenced both the length and mass-specific length of the AP of larvae reared in RS containing 59.9 mol l^{-1} NaCl (Fig. 7A,B; length: P<0.01, F=6.96, d.f.=29; mass specific length: P<0.0001, F=15.34, d.f.=29, 2-way P values, single factor ANOVA). Under these conditions, the AP of larvae reared in RS 4 were reduced in both length and mass-specific length relative to those reared in RS 7, while those reared in RS 11 possessed the longest AP. The decrease in mass-specific length in acidic media was proportionally much greater than the decrease in length. In contrast, pH had no effect on the length, or mass-specific length, of the AP of larvae reared in low-NaCl RS (Fig. 7A,B; length: P>0.27, F=1.39, d.f.=29; mass-specific length: P>0.87, F=0.14, d.f.=29, 2-way P values, single factor ANOVA).

Discussion

Conclusion

In acidic media, larval *Aedes aegypti* utilize novel strategies for the maintenance of hemolymph acid–base homeostasis involving increased rates of ingestion of the medium followed by fluid and acid clearance in which the Malpighian tubules play a prominent role. The mechanisms of homeostasis in alkaline media remain obscure and appear to be largely passive.

The role of the Malpighian tubules in acid–base homeostasis

The Malpighian tubules appear to be primarily responsible for acid–base homeostasis in acidic media. During exposure to acidic media, larvae increased drinking rates substantially. Naïve larvae became volume loaded whereas acclimated larvae maintained high drinking rates yet showed reduced body water. Exposure to acidic media thus results in increased rates of fluid flux through the organism. Acclimation to acidic media also resulted in an increase in fluorescence of a mitochondria-specific dye in the Malpighian tubules. The increase in Malpighian tubule mitochondria is presumably paralleled by upregulation of specific transport mechanisms driving increased rates of fluid secretion.

We have not yet established the pH of the fluid produced by the larval Malpighian tubules, or whether the pH of the secreted fluid changes during acid exposure. However, it is not necessary

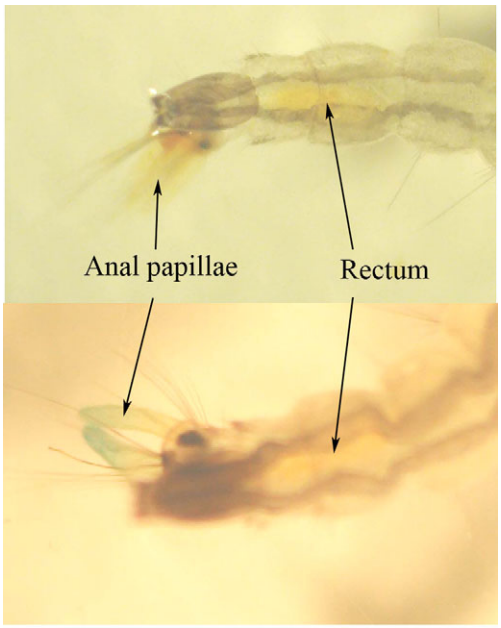

Fig. 5. The pH within the AP changes in response to changes in ambient pH *in vivo*. Top, larva exposed to bromothymol blue *in vivo* in pH 4 media (RS 4). Under these conditions bromothymol blue enters the AP. Bromothymol blue is yellow at pH <6.8 and blue at pH >6.8. In acid media, the interior of the AP is thus acidic (pH <6.8). Bottom, AP that have been 'loaded' by exposure of larvae to bromothymol blue in acidic media followed by transfer to pH 11 media (RS 11) for at least 24 h. Note that the rectal lumen is acidic under these conditions.

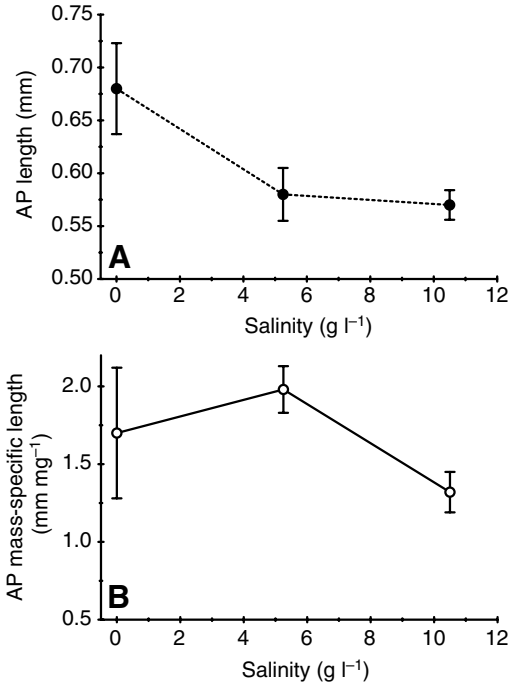

Fig. 6. Length (A) and mass-specific length (B) of the AP as a function of salinity. Larvae were reared in different concentrations of artificial seawater. (A) Length was significantly influenced by salinity (P<0.05, F=3.97, d.f.=25, single factor ANOVA). (B) Mass-specific length of the AP (relative to dry mass) of larvae reared at different salinities. No significant mass-specific effects of salinity were observed (P>0.27, F=1.37, d.f.=25).

for the H^+ concentration or buffer capacity of the excreted fluid to increase for overall Malpighian tubule H^+ excretion rates to increase, if the volume of secreted fluid increases. Malpighian tubules of adult *A. aegypti* secrete acidic fluid, and cyclic AMP increases the rate of fluid secretion while pH remains constant, resulting in elevated H^+ clearance rates (Petzel et al., 1999). The transport mechanisms of larval and adult Malpighian tubules differ (Clark and Bradley, 1996), however, and it remains to be determined whether the larval Malpighian tubules respond in a similar way during acid exposure. We also do not rule out an additional increase in H^+ excretion rates due to an increase in secretion of buffer or ammonia during acid challenges, as occurs in the mammalian kidney.

The role of the rectum in acid–base homeostasis

Evidence presented here suggests that the role of the larval mosquito rectum in alkaline media should be re-evaluated. A series of papers by Strange and coworkers (Strange and Phillips, 1984; Strange et al., 1982; Strange et al., 1984) showed that larval *Aedes dorsalis* exposed to alkaline media (pH 10.5) high in HCO_3^- (up to 250 mmol l^{-1}) eliminate this ion *via* rectal HCO_3^-/Cl^- exchange. It is not clear from the available data whether the rectum of larval *Aedes aegypti* plays a role in acid–base homeostasis. We have found that larval *A. aegypti* has a similar ability to tolerate highly alkaline media without ill effect, at least in media rendered alkaline using NaOH (Clark et al., 2004). Under these conditions, the pH within the rectal lumen of larval *A. aegypti* is always acidic *in vivo* (pH <6.2). The pK of H_2CO_3/HCO_3^- is 6.4 at 25°C (Weast et al., 1986). Most HCO_3^- excreted into the rectum at the observed pH <6.2 would form CO_2, assuming equilibrium is reached by the time the excretory product is eliminated. Any CO_2 so formed would then most likely diffuse through the tissues and out through the cuticle, resulting in net acid excretion (Wigglesworth, 1938). If the physiology of acid–base regulation is similar in larvae of *A. aegypti* and *A. dorsalis*, the high rates of HCO_3^- excretion observed in the rectum of *A. dorsalis* (Strange and Phillips, 1984; Strange et al., 1984) may have been induced by the high HCO_3^- concentration of the medium (250 mmol l^{-1}) rather than by its alkaline pH (pH 10.5). This deserves further study.

The relative contributions of the Malpighian tubules, midgut and hindgut in secretion of the acid observed in the rectal lumen were not determined. We could not measure mitochondrial luminosity of the hindgut due to interference from non-specific fluorescence of lumen contents, which were much more difficult to remove from this region of the alimentary canal. An increase in mitochondrial luminosity would not necessarily indicate a direct role in acid–base transport, however. Elevation of Malpighian tubule secretion rates, observed in acidic media, requires increased rates of rectal recovery of solutes such as K^+, Cl^-, etc., that drive the formation of Malpighian tubule secretions. Changes in energy demands of the rectum in response to ambient pH would therefore provide little information regarding acid–base transport by this organ.

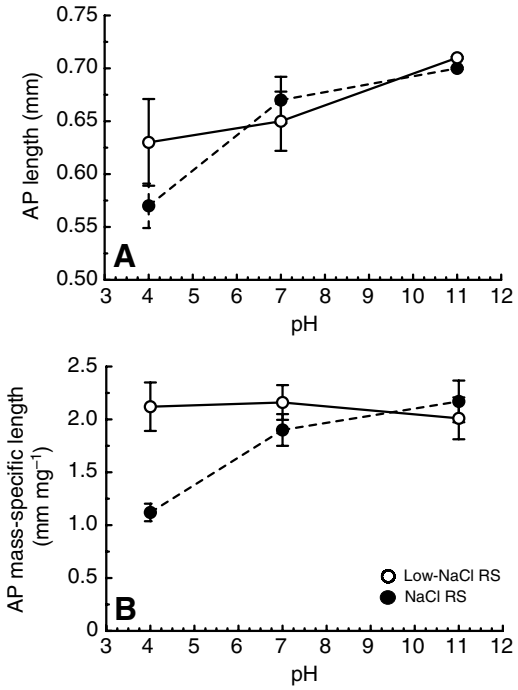

Fig. 7. Length (A) and mass-specific length (B) of the AP as a function of pH. The influence of pH was determined in the absence (○) and presence (●) of added NaCl. (A) Length was significantly influenced by pH in the presence (P<0.005, F=8.24, d.f.=29) but not the absence (P>0.19, F=1.75, d.f.=29) of NaCl. (B) In the presence of NaCl, pH had a highly significant influence on mass-specific length (P<0.0001, F=13.23, d.f.=29). In the absence of NaCl, no influence of pH on mass-specific length was observed (P>0.86, F=0.15, d.f.=29).

The role of the AP in acid–base homeostasis

Acid efflux has been shown in the AP of larval *Aedes aegypti* in neutral media (Donini and O'Donnell, 2005). In the present study, the changes observed in pH within the AP in response to changes in ambient pH are not consistent with an active role of these organs in acid–base homeostasis. Instead, the evidence suggests that acid–base flux across the AP is passive. First of all, one might expect the AP to become more alkaline, rather than acidic, if excreting acid, and to become more acidic if excreting base – in fact, the reverse occurs. Second, the change in pH within the papillae upon transfer from acidic to alkaline media is not reversed during at least 24 h of exposure, suggesting limited ability to regulate flux over this time scale. The passive role of the AP in acid–base homeostasis is supported by comparison of the phenotypic plasticity of the AP in response to pH and to NaCl. It is well established that active NaCl uptake from dilute media occurs in the AP (Wigglesworth, 1933; Wigglesworth, 1938; Koch, 1938). AP of larvae reared in dilute media show increased size and mitochondrial densities contributing to greater active NaCl absorption from the medium (Edwards and Harrison, 1983; Koch, 1938; Wigglesworth, 1938). In the present study, we demonstrated that the size and mitochondrial densities of the AP are all reduced, rather than increased, in acidic media relative to those from larvae reared in neutral and alkaline media (in the presence of NaCl). Reducing the relative surface area of the highly permeable cuticle appears to be a mechanism to reduce rates of passive transcuticular H^+ influx into the AP. The reduction in mitochondrial densities in acidic media is also not consistent with an active role of the AP in acid excretion. The hypothesis that the AP are important sites of active acid–base excretion in acidic media is therefore not supported.

No evidence was obtained supporting the AP as major sites of active acid–base excretion in alkaline media. Size and mitochondrial densities of the AP are similar in larvae reared in neutral and alkaline media.

The response of the AP to acidic media is influenced by the presence or absence of NaCl. The AP were reduced in size in acidic media in the presence of NaCl, but not in its absence. This suggests a trade-off between NaCl uptake and acid–base homeostasis occurring in the AP of larvae in dilute, acidic media. In a wide variety of aquatic organisms, death in acidic water is due primarily to loss of Na^+, rather than failure of pH homeostasis (Vangenechten et al., 1989; Havas, 1981; Havas and Advokaat, 1995; Lin and Randall, 1995). This appears to be related to Na^+/H^+ exchange mechanisms (Stobbart, 1971). In the fish gill, for example, Na^+ uptake is driven by active H^+ secretion. Increased ambient H^+ concentrations therefore increase the electrochemical gradients opposing H^+ secretion, reducing Na^+ uptake (Lin and Randall, 1995). Similarly, larvae of *Aedes aegypti* and *Culex quinquefasciatus* show decreased Na^+ uptake rates during acute exposure to acidic water (pH 3.5) (Patrick et al., 2002). It thus appears that AP surface area does not decrease in dilute media of low pH, because a reduction in size of the AP in dilute, acidic media would compromise NaCl uptake leading to the failure of Na^+ homeostasis. If such a trade-off exists, *A. aegypti* larvae appear to be able to minimize its effects (Clark et al., 2004). Intriguingly, the apical surface area of fish gill chloride cells, also involved in the uptake of ions from dilute media, is reduced during acid exposure (Goss et al., 1995). Could reduced surface area of permeable Na^+ uptake surfaces, a response driven by the deleterious consequences of high rates of passive H^+ influx, contribute to the trade-off between Na^+ and H^+ homeostasis in a variety of animals exposed to acidic water?

Table 1. *Dry mass of larvae used to determine the effects of salinity and pH on the relative size of anal papillae*

		RS with NaCl		RS no NaCl	
Salinity (g l^{-1})	Dry mass (mg)	pH	Dry mass (mg)	pH	Dry mass (mg)
0	0.48±0.052	4	0.55±0.053	4	0.32±0.027
5.25	0.31±0.03	7	0.36±0.019	7	0.31±0.022
10.5	0.46±0.045	11	0.34±0.019	11	0.38±0.034

Rearing solutions (RS) varied in salinity (columns 1, 2), pH in the presence of NaCl (columns 3, 4) and pH without NaCl (columns 5, 6).

It is highly unlikely that the decrease in size of the AP of larvae reared in acidic media containing NaCl is a response to the increased Cl^- concentrations resulting from the HCl added during adjustment of the pH of the rearing medium. The medium [Cl^-] was 59.9 mmol l^{-1}, and the small amounts of HCl required to adjust the pH to 4 were of the order of a few millimolar. In addition, the length and mass-specific length of the AP of larvae reared in acidic media in low-NaCl conditions (with pH adjusted using HCl) were not reduced compared with those of larvae reared in deionized water (0 g l^{-1}, see Fig. 2).

During the course of this investigation, we repeated classic studies documenting the influence of salinity on the size of the AP (Wigglesworth, 1938; Koch, 1938), in order to determine the relative magnitude of the effects of NaCl and pH on AP size. Those studies had found that the AP of larval mosquitoes reared in dilute media were larger than those of larvae reared at higher salinities, although AP of *Aedes aegypti* did not respond as strongly to ambient salinity as did those of several other species. As reported in those studies, we too found that AP are longest in larvae reared in dilute media. However, when scaled for mass, we did not observe any relationship between salinity and AP length. Under the conditions of this study, the effects of salinity on the length of the AP appear to be more strongly associated with allometric effects of salinity on overall growth, rather than changes in the relative sizes of ion uptake surfaces. We do not question the role of the AP in NaCl uptake. Note, however, that pH (in the presence of NaCl) has a much greater effect on AP size than does salinity in this species suggesting that acid–base flux into the AP in acidic media is of considerable physiological significance. It is possible that estimation of total surface area or a greater effort to eliminate NaCl (for example by feeding larvae NaCl-free food) may have resulted in a stronger relationship between mass-specific length and salinity in the present study.

Physiology of acid–base regulation in acidic media

In acidic media, larvae greatly increase drinking rates. This increase is likely to impose a considerable energy cost on the animal. The insect excretory system drives water movement by solute transport. Larvae in acidic media ingest much greater fluid volumes, the ingested fluid contributes to the acid load to be eliminated, and ions used to drive urine secretion must be recovered from the excreta prior to its elimination. Why then would larvae increase drinking rates in acidic media? Two hypotheses come to mind. According to one hypothesis, larvae exposed to acidic media increase drinking rates in order to reduce the transepithelial H^+ gradient opposing clearance of H^+. This would resemble the mammalian kidney, which can only excrete H^+ into filtrate of pH >4.5 but can increase clearance of H^+ by addition of buffers and NH_3 to the urine. We hypothesize that an increased volume of fluid of a given pH and buffer capacity allows a greater acid load to be cleared without increasing the driving force opposing H^+ excretion. For this to work, the increase in clearance capacity must compensate for the additional acid load ingested with the medium. A similar hypothesis was proposed to explain an increase in drinking rates observed in larvae exposed to elevated salinity (Clements, 2000).

A second hypothesis that might explain the increase in drinking rates in acid water is based on Na^+/H^+ exchange processes (discussed above). It is thus conceivable that the increase in drinking rates in acidic media functions to increase Na^+ ingestion, offsetting the reduction in Na^+ influx caused by elevated ambient H^+ concentrations. This hypothesis must be rejected in the present study, however. Larvae ingested the medium at similar rates in acidic media differing in NaCl concentration. Indeed, drinking rates increased approximately 5-fold between pH 4 and pH 7 in low-NaCl RS, but only by 2- to 3-fold in RS containing NaCl (59.9 mmol l^{-1}).

Physiology of acid–base regulation in alkaline media

We had expected to find that larvae exposed to alkaline media would excrete fluid more alkaline than the hemolymph, although not necessarily more alkaline than the environment, to remain in pH balance. We were surprised to find that the excretory system of larvae acclimated to highly alkaline media (pH 11) actually excreted fluid more acidic than the hemolymph. Animal metabolism always produces acids, due to the generation of CO_2 and/or lactic acid during metabolism. Additional acids are formed during processes such as triglyceride or protein catabolism. Depending on an animal's physiological state, however, excreted fluids may be either acidic or alkaline. Data presented by Stobbart (Stobbart, 1971; Stobbart, 1974) show that larval *Aedes aegypti* may either alkalize or acidify the surrounding medium, depending on their state of ionic homeostasis and the ionic composition of the medium. Vanatta and Frazier (Vanatta and Frazier, 1981) found that frogs rendered alkalotic by $NaHCO_3^-$ injection excreted base *via* transepithelial processes. Excretion of HCO_3^- would allow larval mosquitoes in highly alkaline media to excrete fluid that is more alkaline than the hemolymph yet more acidic than the environment. Because the pK of the transition from HCO_3^- to CO_3^{2-} is pH 10.25 at 25°C (Weast et al., 1986), HCO_3^- would act as an acid in media above this pH value. It is therefore not unexpected that the larvae show net acid excretion in highly alkaline media.

There are two ways in which larvae can excrete fluid more acidic than the hemolymph during steady-state exposure to alkaline media: (1) the larvae may neutralize ingested base-utilizing acids produced during metabolism, or (2) the larvae may excrete net base (or absorb acid) through the actions of extrarenal organs. If active excretion of base (either by the excretory system or by extrarenal organs) increases with ambient pH, then the energy demands of the organs involved are expected to increase as the rate of transport and the opposing concentration gradients increase. Larvae reared in high ambient pH did not possess elevated mitochondrial densities in any renal or extrarenal organ, and the AP were not increased in size in response to alkaline media. We have also found that the metabolic rates of larvae exposed to pH 11 media are the lowest observed under any conditions of pH or salinity (J. M. McLister and T.M.C., unpublished observation). Larvae thus appear to possess the remarkable ability to remain in pH balance in highly alkaline water without excreting base or, equivalently, absorbing acid. They appear to depend instead on metabolic acid production to neutralize ingested base. If so, the relatively low drinking rates observed in alkaline water may allow the larvae to remain in pH homeostasis by reducing the amount of base to be neutralized. To the best of our knowledge, this strategy, in which pH homeostasis is maintained in highly

alkaline media by excreting fluid more acidic than the hemolymph, has not been reported in any other animal. Few studies have addressed the physiology of acid–base homeostasis in alkaline media, however, and it is quite likely that this ability is not unique to the larval mosquito.

Larval *Aedes aegypti* develops well in highly alkaline media. Larvae of this species live in small containers of water, generally containing some plant materials. Such habitats are not highly alkaline. It is possible that the ability to survive under these conditions is an adaptation inherited from an ancestral species that is not currently useful. However, if this ability imposed a significant cost it is likely that it would have been lost. It therefore seems likely that the ability of larvae *Aedes aegypti* to survive in such media is a fortuitous consequence of physiological characteristics, such as low cuticular permeability, air breathing, and the generation of a highly alkaline midgut lumen, that evolved in other contexts.

We have earlier reported that high pH reduces growth rates, and increases developmental times, of larval *Aedes aegypti* (Clark et al., 2004). We have found in the present study that larvae acidify alkaline media. Larval mosquitoes inhabiting small volumes of alkaline water may thus make their surroundings more suitable for their own growth and development, and presumably also the survival of the organisms upon which they feed. Early instars appear to be more susceptible to extreme pH than later ones (T.M.C., unpublished observations). Neutralization of highly alkaline media by larval metabolic processes could therefore enhance the suitability of small volumes of water for future generations.

This work was supported by an IUSB Faculty Research Award to T.M.C. and by USDA NRICGP grant no. 99-35302-8371 to T.M.C. The authors thank Dr J. M. McLister and P. J. Bushnell for helpful comments and discussion of the manuscript.

References

Bradley, T. J. (1987). Physiology of osmoregulation in mosquitoes. *Annu. Rev. Entomol.* **32**, 439-462.

Bradley, T. J. and Phillips, J. E. (1977). Regulation of rectal secretion in saline-water mosquito larvae living in waters of diverse ionic composition. *J. Exp. Biol.* **66**, 83-96.

Clark, T. M. and Bradley, T. J. (1996). Stimulation of Malpighian tubules from larval *Aedes aegypti* by secretagogues. *J. Insect Physiol.* **42**, 593-602.

Clark, T. M., Flis, B. J. and Remold, S. K. (2004). pH tolerances and regulatory abilities of freshwater and euryhaline *Aedes* mosquito larvae (Insecta: Diptera, Culicidae). *J. Exp. Biol.* **207**, 2297-2304.

Clark, T. M., Hutchinson, M. J., Huegel, K. L., Moffett, S. B. and Moffett, D. F. (2005). Additional morphological and physiological heterogeneity within the midgut of larval *Aedes aegypti* (Diptera: Culicidae) revealed by histology, electrophysiology, and effects of *Bacillus thuringiensis* endotoxin. *Tissue Cell* **37**, 457-468.

Clements, A. N. (2000). *The Biology of Mosquitoes: Development, Nutrition and Reproduction, Vol. 1*. Wallingford: CABI Publishing.

Cooper, P. D. (1994). Mechanisms of hemolymph acid-base regulation in aquatic insects. *Physiol. Zool.* **67**, 29-53.

Dadd, R. H. (1975). Alkalinity within the midgut of mosquito larvae with alkaline-active digestive enzymes. *J. Insect Physiol.* **21**, 1847-1853.

Donini, A. and O'Donnell, M. J. (2005). Analysis of Na^+, Cl^-, K^+, H^+, and NH_4^+ concentration gradients adjacent to the surface of anal papillae of the mosquito *Aedes aegypti*: application of self-referencing ion-selective microelectrodes. *J. Exp. Biol.* **208**, 603-610.

Edwards, H. A. and Harrison, J. B. (1983). An osmoregulatory syncetium and associated cells in a freshwater mosquito. *Tissue Cell* **15**, 271-280.

Goss, G., Perry, S. and Laurent, P. (1995). Ultrastructural and morphometric studies on ion and acid-base transport processes in freshwater fish. In *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 257-284. San Diego: Academic Press.

Harrison, J. F. (2001). Insect acid/base homeostasis. *Annu. Rev. Entomol.* **46**, 221-250.

Harrison, J. F., Wong, C. J. H. and Phillips, J. E. (1992). Recovery from acute haemolymph acidosis in unfed locusts. 1. Acid transfer to the alimentary lumen is the dominant mechanism. *J. Exp. Biol.* **165**, 85-96.

Havas, M. (1981). Physiological response of aquatic animals to low pH. In *Effects of Acidic Precipitation on Benthos* (ed. R. Singer), pp. 49-65. Hamilton, NY: North American Benthological Society.

Havas, M. and Advokaat, E. (1995). Can sodium regulation be used to predict the relative acid-sensitivity of various life-stages and different species of aquatic fauna? *Water Air Soil Pollut.* **85**, 865-870.

Koch, H. J. (1938). The absorption of chloride ions by the anal papillae of dipteran larvae. *J. Exp. Biol.* **15**, 152-160.

Komnick, H., Rhees, R. W. and Abel, J. H., Jr (1972). The function of ephemerid chloride cells. Histochemical, autoradiographic and physiological studies with radioactive chloride on Callibaetis. *Cytobiologie* **5**, 65-82.

Lin, H. and Randall, D. (1995). Proton pumps in fish gills. In *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 229-255. San Diego: Academic Press.

Maddrell, S. H. P. and Phillips, J. E. (1978). Induction of sulphate transport and hormonal control of fluid secretion by Malpighian tubules of larvae of the mosquito *Aedes taeniorhynchus*. *J. Exp. Biol.* **72**, 181-202.

Patrick, M. L., Ferreira, R. L., Gonzalez, R. J., Wood, C. M., Wilson, R. W., Bradley, T. J. and Val, A. L. (2002). Ion regulatory patterns of mosquito larvae collected from breeding sites in the Amazon rain forest. *Physiol. Biochem. Zool.* **75**, 215-222.

Petzel, D. H., Pirotte, P. T. and Van Kerkhove, E. (1999). Intracellular and luminal pH measurements of Malpighian tubules of the mosquito *Aedes aegypti*: the effect of cAMP. *J. Insect Physiol.* **45**, 973-982.

Phillips, J. E., Thomson, R. B., Audsley, N., Peach, J. L. and Stagg, A. P. (1993). Mechanisms of acid-base transport and control in locust excretory system. *Physiol. Zool.* **67**, 95-119.

Sokal, R. R. and Rohlf, F. J. (1969). *Biometry: The Principles and Practice of Statistics in Biological Research* (1st edn). New York: W. H. Freeman.

Stagg, A. P., Harrison, J. F. and Phillips, J. E. (1991). Acid-base variables in malpighian tubule secretion and response to acidosis. *J. Exp. Biol.* **159**, 433-447.

Stobbart, R. H. (1971). Evidence for Na^+/H^+ and $Cl^-/HCO3^-$ exchanges during independent sodium and chloride uptake by the larvae of the mosquito *Aedes aegypti* (L.). *J. Exp. Biol.* **54**, 19-27.

Stobbart, R. H. (1974). Electrical potential differences and ionic transport in the larva of the mosquito *Aedes aegypti* (L.). *J. Exp. Biol.* **60**, 493-533.

Strange, K. and Phillips, J. E. (1984). Mechanisms of CO_2 transport in rectal salt gland of *Aedes dorsalis*. I. Ionic requirements of CO_2 secretion. *Am. J. Physiol.* **246**, R727-R734.

Strange, K., Phillips, J. E. and Quamme, G. A. (1982). Active HCO_3^- secretion in the rectal salt gland of a mosquito larva inhabiting $NaHCO_3$-CO_3 lakes. *J. Exp. Biol.* **101**, 171-186.

Strange, K., Phillips, J. E. and Quamme, G. A. (1984). Mechanisms of total CO_2 transport in the microperfused rectal salt gland of *Aedes dorsalis*. II. Site of Cl-/HCO_3^- exchange and function of anterior and posterior salt gland segments. *Am. J. Physiol.* **246**, R735-R740.

Vanatta, J. C. and Frazier, L. W. (1981). The epithelium of *Rana pipiens* excretes H+ and NH4+ in acidosis and HCO_3^- in alkalosis. *Comp. Biochem. Physiol.* **68A**, 511-513.

Vangenechten, J. H. D., Witters, H. and Vanderborght, O. L. J. (1989). Laboratory studies on invertebrate survival and physiology in acid waters. In *Acid Toxicity and Aquatic Animals* (*Society for Experimental Biology Seminar Series 34*) (ed. R. Morris, E. W. Taylor, D. J. A. Brown and J. A. Brown), pp. 153-169. Cambridge: Cambridge University Press.

Weast, R. C., Astle, M. J. and Beyer, W. H. (1986). *CRC Handbook of Chemistry and Physics* (67th edn). Boca Raton FL: CRC Press.

Wigglesworth, V. B. (1933). The function of the anal gills of the mosquito larva. *J. Exp. Biol.* **10**, 16-26.

Wigglesworth, V. B. (1938). The regulation of osmotic pressure and chloride concentration in the haemolymph of mosquito larvae. *J. Exp. Biol.* **15**, 235-247.