The Journal of Experimental Biology 212, 3728-3736
Published by The Company of Biologists 2009
doi:10.1242/jeb.032086

Effects of serotonergic agents on survival and hemolymph composition of the larval mosquito *Aedes aegypti* (Diptera: Culicidae, L.) *in vivo*: does serotonin regulate hemolymph acid–base homeostasis?

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Accepted 20 August 2009

SUMMARY

The role of serotonin in the regulation of larval *Aedes aegypti* hemolymph composition was investigated *in vivo* using two reuptake inhibitors (SSRIs), alaproclate HCl and 6-nitroquipazine maleate, and the receptor antagonist methiothepin mesylate. Larvae were placed in media differing in pH and salinity in the presence and absence of serotonergic agents. The toxicity of each agent was strongly influenced by ambient pH. For each agent, toxicity was negligible in acidic media, intermediate in neutral media and greatest in alkaline media. By contrast, toxicity of all agents was independent of salinity. No effects on mass-specific body water or hemolymph volume were observed whereas hemolymph osmotic pressure, Na^+ concentrations and pH differed significantly among treatments. 6-nitroquipazine caused a decrease in Na^+ from 115±1.7 to 103±0.9 mmol l^{-1}, and alaproclate caused alkalosis of the hemolymph from pH 7.55±0.026 to pH 7.72±0.044. Methiothepin decreased hemolymph osmotic pressure from 329±9.9 to 304±8.8 and showed the greatest overall toxicity. Control larvae excreted net base in pH 4 media (1.4 μmol g^{-1} h^{-1}) and net acid in pH 7 (1.2 μmol g^{-1} h^{-1}) and pH 11 (5.1 μmol g^{-1} h^{-1}) media. In pH 4 media, alaproclate and methiothepin caused a shift to net H^+ excretion (1.1 and 1.5 μmol g^{-1} h^1, respectively) whereas these agents did not influence acid excretion rates in pH 7 or pH 11 media. The hypothesis that serotonin is involved in hemolymph acid–base balance is discussed.

Key words: *Aedes aegypti*, serotonin, hemolymph, acid–base regulation, SSRI.

INTRODUCTION

Disruption of endocrine regulation of developmental programs is a useful insect control strategy, with insecticides such as s-methoprene used in fighting mosquitoes and other insects (Russell and Kay, 2008). By contrast, effective control practices targeting endocrine regulation of homeostasis have proved elusive. A potential target for such strategies is disruption of excretory function or acid–base homeostasis. Pharmacological disruption of hemolymph water and ion balance could kill insects directly, and, in addition, pharmacological interference with clearance of pesticides and their metabolites from the hemolymph could enhance the efficacy of existing pesticides. Serotonergic pathways are known to regulate ion and fluid transport properties, acid–base transport and/or muscular motility of multiple organs, including salivary glands, midgut, oviduct and Malpighian tubules (e.g. Farmer et al., 1981; Barrett et al., 1993; Clark and Bradley, 1997; Clark et al., 1999; Lange et al., 1989; Maddrell et al., 1991; Maddrell et al., 1993; Onken et al., 2004; Onken et al., 2008; Orchard et al., 1988). In the larval mosquito *Aedes aegypti*, serotonin stimulates fluid and ion transport by the Malpighian tubules and ion and acid–base transport across the midgut (Clark and Bradley, 1996; Clark and Bradley, 1997; Clark et al., 1999; Onken et al., 2004; Onken et al., 2008). In vertebrates, serotonin acts through a myriad of receptor types and subtypes, which, together with serotonin reuptake systems, produce nearly limitless signaling capabilities (Hoyer et al., 2002). Extensive studies in vertebrates have identified a number of agonists, antagonists and reuptake inhibitors that can be used to manipulate serotonergic pathways. Little is currently known about the number of distinct receptor and reuptake system subtypes in insects, their pharmacology, distributions among insect clades and structural and functional evolution. The pharmacology of insect serotonin receptors is likely to differ significantly from vertebrate serotonin receptors, as a single amino acid substitution between rodent and non-rodent $5HT_{1\beta}$ receptors alters their pharmacological profiles (reviewed in Hoyer et al., 2002). It is also not yet known to what extent peripheral serotonin regulates specific epithelial tissues by synaptic serotonin release or coordinates actions of multiple organs *via* global, endocrine release of serotonin. Can serotonergic pathways provide a weak link in the insect armor? If so, it may be possible to identify compounds with pharmacological activity on insect receptors that are blind to vertebrate receptors, or among insect taxa, providing a novel, specific, control strategy.

In the present work, we pharmacologically manipulate serotonin pathways in the larval mosquito *Aedes aegypti in vivo* in order to test the hypotheses that serotonin is involved in the regulation of hemolymph composition during both salinity and acid–base challenges, and that disruption of serotonergic pathways could contribute to control strategies targeting the larval mosquito. The toxicity of three serotonergic agents, and their effects on hemolymph composition, was determined under different conditions of ambient salinity and pH *in vivo*. Methiothepin is a serotonin 1,7 receptor antagonist in vertebrates that acts as a 5-$HT_{2\beta}$ receptor antagonist in Crustacea (Spitzer et al., 2008). Alaproclate and 6-nitroquipazine (DU 24565) are serotonin reuptake inhibitors in vertebrates (Classen et al., 1984; Rawlow et al., 1983). Of these agents, only methiothepin has previously been used in studies of insect systems (Troppmann et al., 2007; Clark and Bradley, 1997). In the larval mosquito *A. aegypti*, methiothepin blocks stimulation of Malpighian tubule fluid and ion secretion by serotonin (Clark and Bradley, 1997).

The results of this investigation show that serotonergic agents can be used to disrupt hemolymph composition *in vivo*, and provide the first evidence for the regulation of hemolymph pH by an identified neurotransmitter or neurohormone in any insect. They also suggest that targeting endocrine regulation of excretion may provide more effective control under some environmental conditions than others.

MATERIALS AND METHODS

Mosquitoes

A colony of *Aedes aegypti* (L.) was established using eggs from the Florida Medical Entomology Laboratory, in Vero Beach, FL, USA. Eggs were hatched and larvae were maintained in deionized water on a 16 h:8 h L:D photoperiod at 26°C. Larvae were reared on ground TetraMin flakes (TetraWerke, Melle, Germany).

Serotonergic agent and control solutions

D,L-alanine, 2-[4-chlorophenyl]-1,1-dimethylethyl ester (henceforth called alaproclate) and 1-[10,11-dihydro-8-(methylthio) dibenzo[b,f]thiepin-10-yl[-4-piperazine mesylate (henceforth called methiothepin) were obtained from Sigma Aldrich (St Louis, MO, USA). 6-nitro-2-(1-piperazinyl)-quinoline maleate (henceforth called 6-nitroquipazine) was obtained from Research Biochemicals International (Natick, MA, USA). For assays across salinity and pH levels, stocks were prepared in 0.1% DMSO (dimethylsulphoxide) and assayed at final concentrations of 1×10^{-4} mol l^{-1}, except for methiothepin, which was assayed at 1×10^{-5} mol l^{-1} in 0.1% DMSO after early tests revealed high toxicity at 1×10^{-4} mol l^{-1}. Control larvae were assayed in 0.1% DMSO alone. For assays to determine the half-maximal lethal doses (LD_{50}s), all concentrations of 6-nitroquipazine were assayed in 0.1% DMSO. The other agents were dissolved directly in deionized water then added to the assay solutions to give the final concentrations.

Effects of serotonergic agents on total body water

Larvae were maintained in deionized water, without food, for 24 h in the presence of serotonergic agents or DMSO. Each larva was then removed, blotted dry and weighed to the nearest 10 μg using a Mettler Toledo AX 205 deltarange balance (Columbus, OH, USA). After drying the larva for 24 h at 65°C, it was weighed again. The difference between wet mass and dry mass constitutes body water. The percentage body water was arcsin-transformed, then single factor analysis of variance (ANOVA) was used to determine whether significant differences existed in body water among treatments.

Effects of serotonergic agents on hemolymph volume

The effects of serotonergic agents on hemolymph volume were determined in fourth instar larvae reared in deionized water. After 1, 3 and 24 h of exposure to treatment or control solutions, larvae were removed, blotted dry, then placed on tared pieces of Parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA) and weighed to the nearest 10 μg as before. They were then torn open under a dissecting microscope. The hemolymph was immediately blotted away from the carcass using absorbent paper and the larva was reweighed. The difference in mass before and after removal of hemolymph represents an estimate of the hemolymph volume, and the proportion of total mass that was hemolymph is henceforth called percentage hemolymph. The percentage hemolymph was arcsin-transformed, then single factor ANOVA was used to determine whether significant differences existed in hemolymph volume among treatments.

Effects of serotonergic agents on hemolymph osmotic pressure

Third and fourth instar larvae were placed into small Petri dishes containing deionized water. Larvae were then exposed to treatment or control solutions, as described above. They were removed after 24 h of exposure, blotted dry and torn open with fine forceps. The hemolymph was collected using 5 μl capillary tubing (Microcap, Drummond Scientific Co., Broomall, PA, USA). On average, somewhat less than 0.5 μl of hemolymph can be obtained from each larva, so samples were pooled as necessary. Osmotic pressure was determined using a Wescor model 5500 vapor pressure osmometer (Logan, UT, USA), as follows. In order to minimize the characteristic melanization reaction of hemolymph, the sample disc was initially wetted with 5 μl of antioxidant solution, consisting of (in mmol l^{-1}) KH_2PO_4 (125), EDTA (0.4), octoyl sulphate (0.16), containing 15% methanol and adjusted to pH 3.6 (Heslop et al., 1996). Five μl of hemolymph was then added to the disc. The hemolymph osmotic pressure was then determined by assuming that the measured osmotic pressure was the mean of that of the antioxidant solution (determined separately) and the hemolymph.

Effects of serotonergic agents on hemolymph pH and Na^+ concentrations

Third and fourth instar larvae were placed into small Petri dishes containing deionized water. Treatment or control solutions were added as above. Hemolymph pH and Na^+ concentrations were determined using pH or Na^+ selective microelectrodes. Electrodes were made from double-barreled borosilicate omega dot capillary tubing [1.5 mm outer diameter (OD), 0.75 mm inner diameter (ID), FHC, Brunswick, ME, USA]. The glass tubing was washed with nitric acid, then with nanopure water (R>17 MΩ) and oven dried. The reference barrel was cut short and capped with parafilm, and the resin barrel was inserted into a small hole in the top of a jar containing silanizing agent. Electrodes were pulled on a Kopf Model 720 needle/pipette puller (David Kopf Instruments, Tujunga, CA, USA). The resin tips were sylanized using 5% dimethyldichlorosilane (Sylon CT, Supelco, St Louis, MO, USA) and dried with gentle heat on a hot plate. The pH resin used was H^+ ionophore 1 (Sigma Aldrich) whereas the Na^+ resin was Na^+ ionophore II (Sigma Aldrich). The tip of the silanized barrel was filled with resin and the barrel was backfilled with 0.5 mol l^{-1} KCl. The reference (ground) barrel was also filled with 0.5 mol l^{-1} KCl. The electrode tip was broken to reduce electrical resistance. Ag/AgCl electrodes were inserted into each barrel.

To determine hemolymph pH or Na^+ concentrations, a larva was rinsed in deionized water, blotted dry and torn open on Parafilm using fine forceps. A pH or Na^+ microelectrode with a grounded reference barrel was then immediately inserted into the drop of hemolymph. The voltage across the resin was measured using a high impedance amplifier (Iso-DAM8; World Precision Instruments, Sarasota, FL, USA), coupled to a Sable Systems Data Acquisitions System (Sable Systems, Henderson, NV, USA). Only those measurements that reached stable values within one minute were accepted. Voltage was converted to pH or Na^+ by reference to a standard curve. Na^+ standards consisted of 100 and 200 mmol l^{-1} NaCl while H^+ standards consisted of commercial pH 7 potassium phosphate buffer and pH 10 potassium carbonate, borate and hydroxide buffer solutions (Fisher Scientific, Pittsburgh, PA, USA). These methods do not completely avoid evaporative losses. Similarly, a portion of the differential signal between 100 and 200 mmol l^{-1} Na^+ standards is due to the difference in ionic strength

of the standards rather than the difference in Na^+ concentration. Our main objective was comparison among treatments rather than determining absolute values, and thus we would not be surprised at small overestimations of Na^+ concentrations. However, such biases are likely to be consistent among measurements. Evaporative losses and influences of total ionic strength of standard solutions across ion selective resins should therefore not impinge on the ability to detect differences among treatments.

Effects of alaproclate and methiothepin on acid excretion rates

Acid excretion rates were determined for larvae in media of pH 4, 7 and 11. Rearing solutions (RS) consisted of 3.5 g l^{-1} NaCl, buffered with 2.5 mmol l^{-1} Hepes and 2.5 mmol l^{-1} Tris. This RS was then adjusted to pH 4, pH 7 and pH 11 using HCl or NaOH, forming RS 4, RS 7 and RS 11, respectively (Clark et al., 2004; Clark et al., 2007). Larvae were assayed at the rearing pH. Fed, acclimated larvae were rinsed twice in fresh RS and then placed individually into 2.0 ml of fresh RS. Controls for each replicate of RS 4 and RS 7 consisted of a larva exposed to 0.1% DMSO; the pH of RS 11 was found to change spontaneously, presumably due to uptake of CO_2 from the atmosphere, and an additional control consisting of 0.1% DMSO in RS 11, without larvae, was performed for these samples. Treatments consisted of alaproclate (1×10^{-4} mol l^{-1}) in 0.1% DMSO and methiothepin (1×10^{-5} mol l^{-1}) in 0.1% DMSO. Addition of the agents to the media did not alter pH. After a period of 1–2 h, the pH of the solutions was determined. Titration of the RS allowed calculation of the rate of acid excretion under these conditions. The differences in pH changes of the controls and the treatments allowed for the determination of the acid–base excretion rates of the larvae. Wells containing injured larvae, as determined by abnormal swimming behavior, were discarded. 6-nitroquipazine was not tested for effects on acid excretion.

Effects of methiothepin on posterior ventriculus (stomach) serotonin receptors

Methiothepin is known to block Malpighian tubule fluid secretion but its actions on other epithelia are not known. We determined the effects of methiothepin on the characteristic hyperpolarization by serotonin of the posterior midgut transepithelial potential *in vitro* (Clark et al., 1999). Isolated sections of posterior stomach were perfused with symmetrical hemolymph substitute solution (HSS), following procedures modified from Clark et al. (Clark et al., 1999). In brief, glass perfusion pipettes were hand-forged from 100 µl capillary tubes (VWR International, West Chester, PA, USA). Inflow and outflow pipettes were connected to a programmable perfusion/withdrawal syringe pump (WPI SP 260P) and perfused at a rate of 20 µl h^{-1} with HSS, in a bath also containing HSS. HSS consisted of the following (in mmol l^{-1}): NaCl, 42.5; KCl, 3.0; $MgSO_4$, 0.6; $CaCl_2$, 5.0; dextrose, 10; succinic acid, 5.0; malic acid, 5.0; Hepes, 5.0; sucrose, 100; L-proline, 5; L-glutamine, 5; L-histidine, 5; L-arginine, 5; L-alanine, 5; and $NaHCO_3$, 10. All salines were adjusted to pH 7.5 using NaOH. An Ag/AgCl electrode was inserted into the outflow pipette, and voltage between ground and lumen collected using a World Precision Instruments Iso-DAM8 high impedance amplifier. The data were digitized and imported into spreadsheets using Sable Systems data acquisition software. Perfused gut preparations were exposed to hemolymph-side methiothepin (10^{-6} mol l^{-1}) or control solutions for a minimum of 15 min, then serotonin was added to the bath for a final concentration of 10^{-7} mol l^{-1}.

Salinity- and pH-dependence of 6-nitroquipazine, alaproclate and methiothepin toxicity

Two-day-old larvae were transferred in batches of 10 to small Petri dishes containing 4 ml of media of appropriate pH or salinity. All drugs are assayed in 0.1% DMSO: 6-nitroquipazine and alaproclate are assayed at 1×10^{-4} mol l^{-1}, methiothepin at 1×10^{-5} mol l^{-1}. Controls consisted of 0.1% DMSO. For each drug and control 10 replicates were performed for each of the three environmental conditions of salinity or pH. The pH series consisted of RS4, RS 7 and RS11, described above. The media used in the salinity series consisted of deionized water (0 g l^{-1} sea salt) or artificial sea salt (Instant Ocean, Aquarium Systems, Mentor, OH, USA) at concentrations of 5.25 or 10.5 g l^{-1} sea salt. Larvae were maintained on a 16 h:8 h L:D photoperiod at 26°C, without food, during experiments. The numbers of surviving larvae were counted at 24 h intervals for 72 h. Larvae that did not move when disturbed were counted as dead.

Determination of LD_{50}s

Mosquito larvae were raised in 1 mmol l^{-1} NaCl solution. Five 1–3-day-old larvae were placed into a well containing 1 ml of either RS 4, RS 7 or RS 11. Alaproclate and methiothepin were dissolved in deionized water and added to the wells to produce the desired final concentration. Deionized water was added to control wells. 6-nitroquipazine was initially dissolved in DMSO and assayed at the desired final concentration in media containing 0.1% (v/v) DMSO. For 6-nitroquipazine controls, 0.1% DMSO was used. Larvae were maintained on a 16 h:8 h L:D photoperiod at 26°C, without food, during experiments. The numbers of live, dead and missing mosquitoes (assumed to have been consumed by survivors) were recorded at approximately 24, 48 and 72 h following the drug treatment.

RESULTS

Effects of serotonergic agents on percentage body water, percentage hemolymph and hemolymph osmotic pressure

Serotonergic agents had no effect on percentage body water ($P>0.2$, $F=1.59$, d.f.=59, arcsin-transformed data) or percentage of body mass that is hemolymph ($P>0.67$, $F=0.68$, d.f.=23, arcsin-transformed data) (Table 1). By contrast, a significant effect on hemolymph osmotic pressure was observed (Table 1) (treatment effects: $F=5.0$, $P<0.05$, row effects: $F=9.99$, $P<0.001$, d.f. (total)=44, two factor ANOVA) (Table 1). This effect was attributed to a decrease in osmotic pressure in animals exposed to methiothepin.

Effects of serotonergic agents on hemolymph pH

Hemolymph pH was significantly influenced by treatment (Table 1) ($P<0.002$, $F=6.28$, d.f.=46). This effect was due to an increase in hemolymph pH of 0.2 pH units in animals exposed to alaproclate. Hemolymph pH was not altered in the presence of 6-nitroquipazine or methiothepin (Table 1).

Effects of serotonergic agents on hemolymph Na^+ concentrations

Serotonergic agents significantly influenced hemolymph Na^+ concentrations. This effect was attributed to a significant decrease in Na^+ in larvae exposed to 6-nitroquipazine relative to other treatment groups (Table 1) ($P<0.002$, $F=6.75$, d.f.=30, single factor ANOVA). In control larvae, hemolymph Na^+ was measured at 114.8±1.72 mmol l^{-1} compared with 103.1±0.85 mmol l^{-1} in 6-nitroquipazine-treated larvae, a decrease of nearly 10% (Table 1).

Table 1. Effects of serotonergic compounds on hemolymph composition

	Control	6-nitroquipazine	Alaproclate	Methiothepin	Stats
Osmotic pressure (mosmol l^{-1})	329±9.9 (11)	329±9.2 (11)	314±13.7(12)	304±8.8 (12)	***P*<0.01**
% Body water	85±0.6(15)	87±0.7 (15)	85±1.0 (15)	85±1.3 (15)	*P*>0.2
% Hemolymph	45±4.3 (6)	48±2.5 (5)	50±2.4 (5)	46.5±2.1 (6)	*P*>0.6
Hemolymph pH	7.5±0.04 (12)	7.6±0.03 (12)	7.7±.04 (11)	7.6±0.03 (12)	***P*<0.002**
Hemolymph Na^+ (mequiv. l^{-1})	115±1.7 (8)	103±0.9 (8)	115±3.2 (7)	111±2.3 (8)	***P*<0.002**

Significant differences are in bold. Values in parentheses are *N* values.

Effects of alaproclate and methiothepin on acid excretion rates

The effects of alaproclate and methiothepin on rates of acid excretion were investigated in RS 4, RS 7 and RS 11. Significant effects of treatment on acid–base excretion rates were observed in RS 4 but not in RS 7 and RS 11 (Table 2). Larvae in RS 4 were found to produce net base under control conditions (i.e. they alkalized the media) but larvae treated with either alaproclate or methiothepin showed a shift to net acid excretion. Overall acid excretion rates were much greater in alkaline media than in acidic or neutral media in all treatment and control groups. All larvae excreted net acid in pH 11 whereas some larvae excreted base in all other conditions of pH and treatment. Effects of 6-nitroquipazine on acid excretion were not tested.

Effects of pH and salinity on the toxicity of serotonergic agents

Survival of larvae in both the treatment and control groups was influenced by environmental pH and salinity (Figs 1 and 2). Two-way ANOVA with replication (Sokal and Rohlff, 1981) showed that survival within pH and salinity was influenced by both treatment and time of exposure (Table 3). Survival of controls was similar across salinities. All three agents tested showed toxicity in all salinities, with methiothepin showing the greatest toxicity even though it was assayed at 0.1× the concentration of the others. For all ambient salinities, both *P* (treatment) and *P* (time) were highly significant. Interaction terms were only significant in 10.5 g l^{-1} sea salt. In the pH series, survival of controls was reduced in RS 4 compared with RS 7 or RS 11. For all ambient pH values, both *P* (treatment) and *P* (time) were significant, and interaction terms were only significant in pH 11. In RS 4, none of the agents showed toxicity. Instead, the difference among treatments is attributed to enhanced survival in the presence of alaproclate. By contrast, alaproclate was toxic in RS 7 and highly toxic in RS 11 (Fig. 3A,B). 6-nitroquipazine and methiothepin showed moderate toxicity at neutral pH and high toxicity in RS 11, with methiothepin again showing the greatest toxicity. We then determined the mortality due to serotonergic agents by subtracting survival at 72 h of exposure in the presence of serotonergic agents from survival of controls within each run. No differences were detected in toxicity of any of the agents across salinity (alaproclate: $P>0.67$; 6-nitroquipazine: $P>0.17$, methiothepin: $P>0.71$, two factor ANOVA) (Fig. 3A). By contrast, strong pH-dependent effects on survival were detected (alaproclate: $P<0.00001$, 6-nitroquipazine: $P<0.0001$, methiothepin: $P<0.0001$; single factor ANOVA across pH values) (Fig. 3B).

LD_{50}s

LD_{50}s were strongly influenced by ambient pH. For 6-nitroquipazine at the highest concentration tested, 1×10^{-3} mol l^{-1}, survival after 48 h in RS 4 was 78%, in RS 7 64% and in RS 11 24%. Alaproclate showed no toxicity in acidic media (92% survival) but 82% and 100% mortality in neutral and alkaline media, respectively, at the highest concentration tested, 1×10^{-3} mol l^{-1}. Similarly, in the presence of 1×10^{-3} mol l^{-1} methiothepin in acidic media survival was 75% whereas no survival was observed at this concentration in neutral and alkaline media. All three agents thus showed greatest toxicity in alkaline media and lowest in acidic media (Fig. 4). A comparison of mortality in control solutions (deionized water in alaproclate and methiothepin assays, 0.1% DMSO in quipazine assays) revealed that the presence of DMSO did not influence survival in RS 4 ($P>0.15$) or RS 11 ($P>0.80$) but increased mortality in RS 7 ($P<0.05$).

Effects of methiothepin on stimulation of posterior ventriculus (stomach) by serotonin

Methiothepin (1×10^{-6} mol l^{-1}) was found to prevent the characteristic hyperpolarization of the transepithelial potential of the posterior ventriculus caused by serotonin (1×10^{-7} mol l^{-1}) (Fig. 5).

DISCUSSION

In insects, the actions of alaproclate and 6-nitroquipazine have not been established, and the receptor specificity of methiothepin is unknown. Until this information is available it is not known conclusively that the observed actions of these drugs are mediated by their effects on serotonin pathways. Nevertheless, with no evidence to the contrary, with strong evidence for regulation of midgut, salivary gland and Malpighian tubule ion transport pathways by serotonin (Clark and Bradley, 1996; Clark and Bradley, 1997; Clark et al., 1999; Onken et al., 2004; Onken et al., 2008) and disruption of hemolymph composition by serotonergic agents (present work), we will assume for the present discussion that the observed effects of these agents are due to interactions with serotonin receptors or reuptake transporters, as they are in vertebrates.

The results of this study demonstrate toxicity of serotonergic compounds *in vivo* but suggest that serotonergic agents may provide

Table 2. Effects of serotonergic compounds on acid excretion rates in μmol g^{-1} h^{-1}

pH	Control	Alaproclate	Methiothepin	
4	–1.4±0.56 (2/10)	1.1±0.72 (4/7)	1.5±0.61 (7/9)	***P*<0.005**
7	1.2±0.84 (8/10)	0.4±0.63 (8/10)	1.1±0.33 (9/10)	*P*>0.65
11	5.1±0.66 (9/9)	6.5±0.67 (9/9)	5.1±0.50 (9/9)	*P*>0.2

Significant differences are in bold. Values in parentheses are (first number/second number) = (number of larvae excreting net acid/total number of larvae sampled).

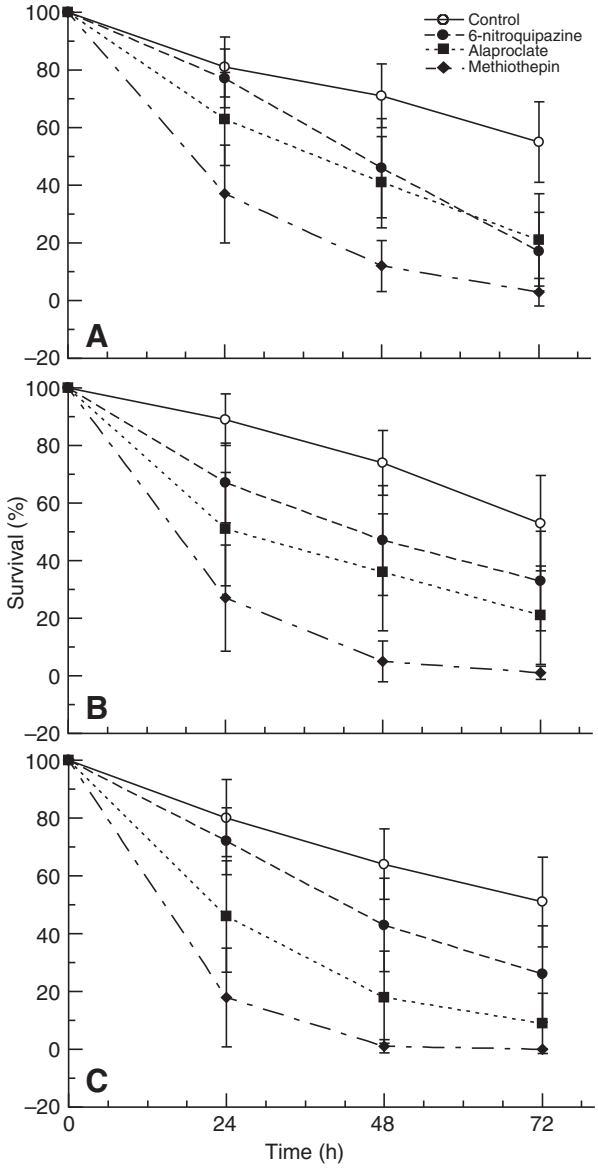

Fig. 1. The effects of serotonergic agents on percentage survival over time in media differing in salinity. Serotonergic agents are assayed in (A) 0 g l^{-1}, (B) 5.25 g l^{-1} and (C) 10.5 g l^{-1} artificial sea salt. 6-nitroquipazine and alaprocolate are assayed at a concentration of 1×10^{-4} mol l^{-1}; methiothepin at 1×10^{-5} mol l^{-1}. All treatment and control groups contained 0.1% DMSO (dimethylsulphoxide). *N*=10 (methiothepin) or *N*=15 (control, 6-nitroquipazine, alaprocolate) replicates, each replicate consisting of 10 larvae. Data are presented as means ± s.e.m.

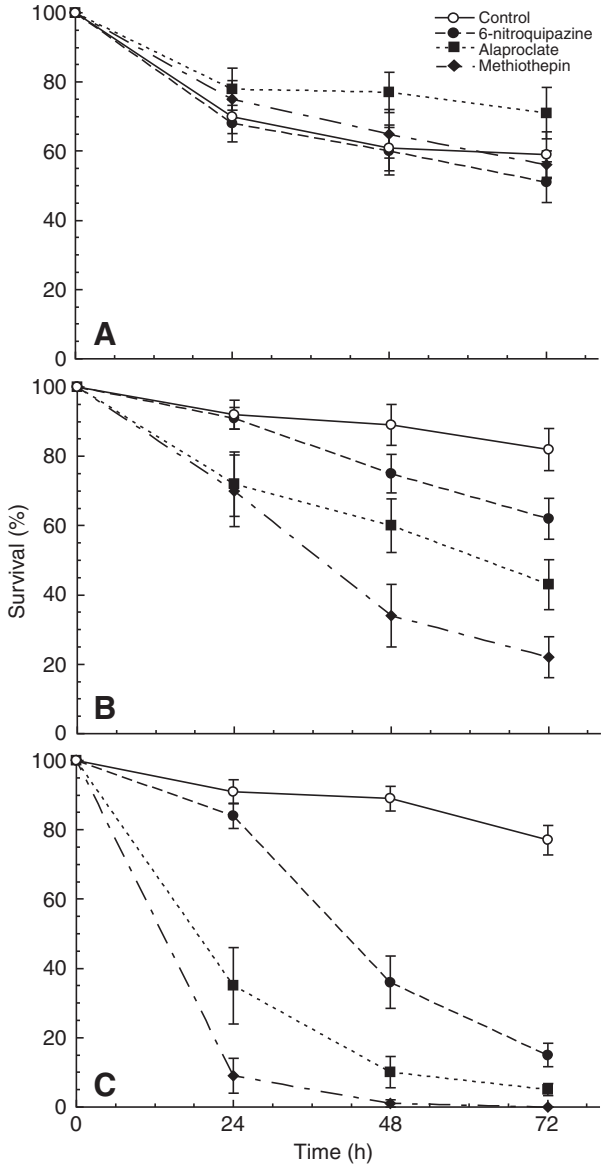

Fig. 2. The effects of serotonergic agents on percentage survival across ambient pH. Serotonergic agents are assayed in (A) pH 4, (B) pH 7 and (C) pH 11 rearing solution. 6-nitroquipazine and alaprocolate are assayed at 1×10^{-4} mol l^{-1}; methiothepin at 1×10^{-5} mol l^{-1}. Controls consisted of 0.1% DMSO (dimethylsulphoxide). *N*=10 replicates, each consisting of 10 larvae. Data are presented as means ± s.e.m.

effective control under some environmental conditions and not others. The agents tested showed negligible toxicity in acidic conditions, and toxicity was relatively low in neutral conditions, suggesting that they would provide little control in the habitats normally experienced by the larvae of this species. Serotonergic pathways thus may not prove to be viable stand-alone targets for insecticides in larval mosquitoes. However, it remains possible that disruption of serotonergic pathways could potentiate the actions of insecticides targeting other systems. For example, inhibition of excretion using serotonin receptor antagonists could reduce clearance rates of established pesticides, leading to lower LD_{50}s and thus safer and more effective control.

As predicted, serotonergic agents proved toxic to larval mosquitoes, disrupted hemolymph composition *in vivo*, and their toxicity was influenced by ambient water chemistry. The results provide support for regulation of hemolymph composition by serotonin and, in addition, establish a novel experimental method for analysis of this regulation. The three agents have distinct effects on hemolymph composition, consistent with different mechanisms of action. Details of the results were unexpected, however, and highlight the complex physiology of serotonin in this animal. For example, hemolymph Na^+ and osmotic pressure were altered yet no salinity-dependent toxicity was observed whereas only one agent significantly altered hemolymph pH yet all three caused pH-dependent mortality. How do we reconcile

Table 3. Analysis of the effects of treatment and exposure time of controls and serotonergic agents 6-nitroquipazine, alaproclate and methiothepin for each ambient salinity and pH

Medium	Source of variety	Probability	*F*-values
Salinity 0 g l^{-1}	Treatment	**≪0.001**	37.38
	Time	**≪0.001**	50.77
	Interaction	n.s.	1.92
Salinity 5.25 g l^{-1}	Treatment	**≪0.001**	30.29
	Time	**≪0.001**	24.66
	Interaction	n.s.	0.69
Salinity 10.5 g l^{-1}	Treatment	**≪0.001**	49.28
	Time	**≪0.001**	45.99
	Interaction	**<0.05**	2.27
pH 4	Treatment	**<0.05**	3.53
	Time	**<0.01**	5.11
	Interaction	n.s.	0.28
pH 7	Treatment	**≪0.001**	24.69
	Time	**≪0.001**	17.38
	Interaction	n.s.	1.54
pH 11	Treatment	**≪0.001**	160.29
	Time	**≪0.001**	97.01
	Interaction	**<0.001**	8.33

Significant differences are in bold.

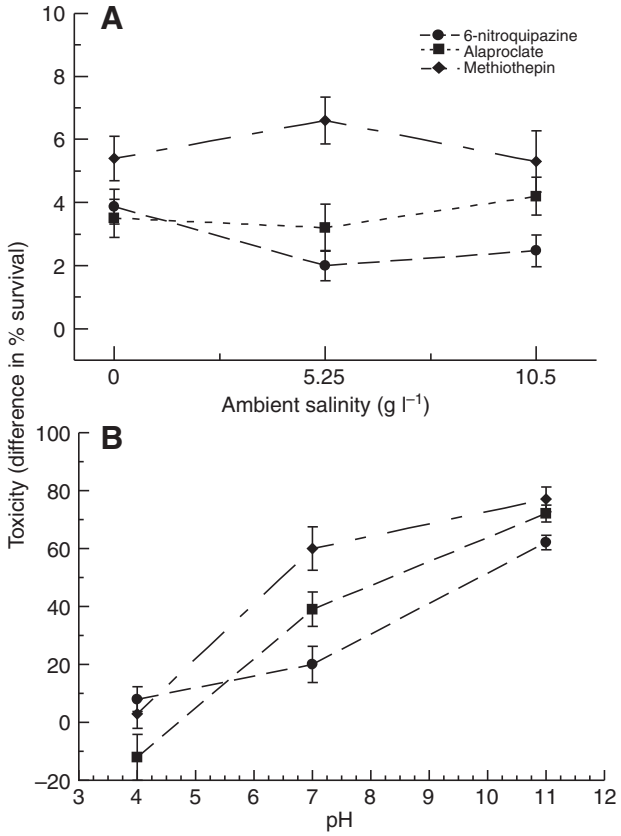

Fig. 3. The toxicity of serotonergic agents across salinities (A) and ambient pH (B). The toxicity of each agent was determined from the data presented in Figs 1 and 2, by subtracting the percentage mortality observed in controls at 72 h from the percentage mortality of each treatment group at 72 h. No significant effect of salinity was observed for any agent whereas all three agents showed significant pH-dependent mortality. Data are presented as means ± s.e.m.

this discrepancy? Possible explanations include: (A) serotonin may play a more important role in acid–base balance than in ionic homeostasis. (B) Ionic homeostasis may be more resistant to disruption due to greater redundancy in homeostatic pathways. (C) Larvae may have a greater ability to tolerate disruption of hemolymph ionic composition than acid–base balance. The agents chosen may by coincidence all disrupt acid–base tolerance, and other agents that have not yet been tested may prove to have salinity-dependent effects.

What do the results tell us about the role of serotonin in regulation of hemolymph pH?

It is surprising to us that agents with different and opposing mechanisms of action would show similar pH-dependent effects, each characterized by minimal toxicity in acidic conditions and greatest toxicity in alkaline conditions. The effects of alaproclate appear easiest to interpret. As a SSRI, actions of alaproclate are likely to mimic global release of serotonin into the hemolymph, stimulating all serotonin-regulated peripheral tissues. Larval *A. aegypti* survive in media ranging from pH 4 to pH 11, regulate hemolymph pH across this entire pH range during chronic exposures and survive abrupt transfer from pH 11 to pH 4, or *vice versa*, without apparent ill effect (Clark et al., 2004). The mechanisms involved in hemolymph pH homeostasis must be very flexible and highly regulated to maintain hemolymph composition in the face of such large and rapid changes in ambient pH. Ventilatory responses do not appear to play an important role in aquatic insect acid–base homeostasis, although they are involved in recovery from exercise in the locust (Cooper, 1994; Phillips et al., 1994; Harrison, 2001). Epithelial acid–base excretion rates thus appear to be rapidly adjusted by homeostatic mechanisms, and neural or endocrine control of acid–base excretion rates appears likely. However, no signaling molecule has ever been implicated in regulation of hemolymph pH in any insect. Alaproclate stimulates acid excretion, causing alkalization of hemolymph. It is non-toxic in acidic media but is toxic in neutral and alkaline media, and toxicity increases with ambient pH. Stimulation of acid excretion is not expected to be deleterious in highly acidic media, as homeostatic mechanisms of acid clearance have presumably already been activated under these conditions. Indeed, the observed increase in survival in RS 4 in the presence of alaproclate is consistent with stimulation of acid clearance. By contrast, inappropriate stimulation of acid excretion by larvae in neutral or alkaline water is expected to compromise acid–base homeostasis, increasing mortality. It remains necessary to measure serotonin levels in response to alaproclate, and under different conditions of ambient pH, to establish the role of serotonin in hemolymph acid–base homeostasis. However, this is the strongest evidence to date for regulation of hemolymph pH by any identified hormone or neurotransmitter in any insect.

How might alaproclate exert its actions on hemolymph pH? Onken et al. have demonstrated that serotonin stimulates acid–base transport by the anterior stomach region of the midgut *in vitro* (Onken et al., 2008). The function of this stimulation is assumed to be maintenance of optimal conditions for digestion and absorption of nutrients rather than regulation of hemolymph acid–base homeostasis (Berenbaum, 1980; Clark, 1999; Onken et al., 2008). This interpretation is supported by the present study. Assuming transepithelial acid–base transport, stimulation of midgut alkalinization by serotonin would lead to acidification of the hemolymph rather than the alkalization observed in response to alaproclate. Serotonin thus appears to also stimulate acid clearance elsewhere, counteracting the effects on hemolymph pH of its stimulation of midgut alkalization. Serotonin is known to strongly stimulate Malpighian tubule fluid and ion secretion rates (Clark and Bradley, 1996; Clark and Bradley, 1997). Larvae chronically

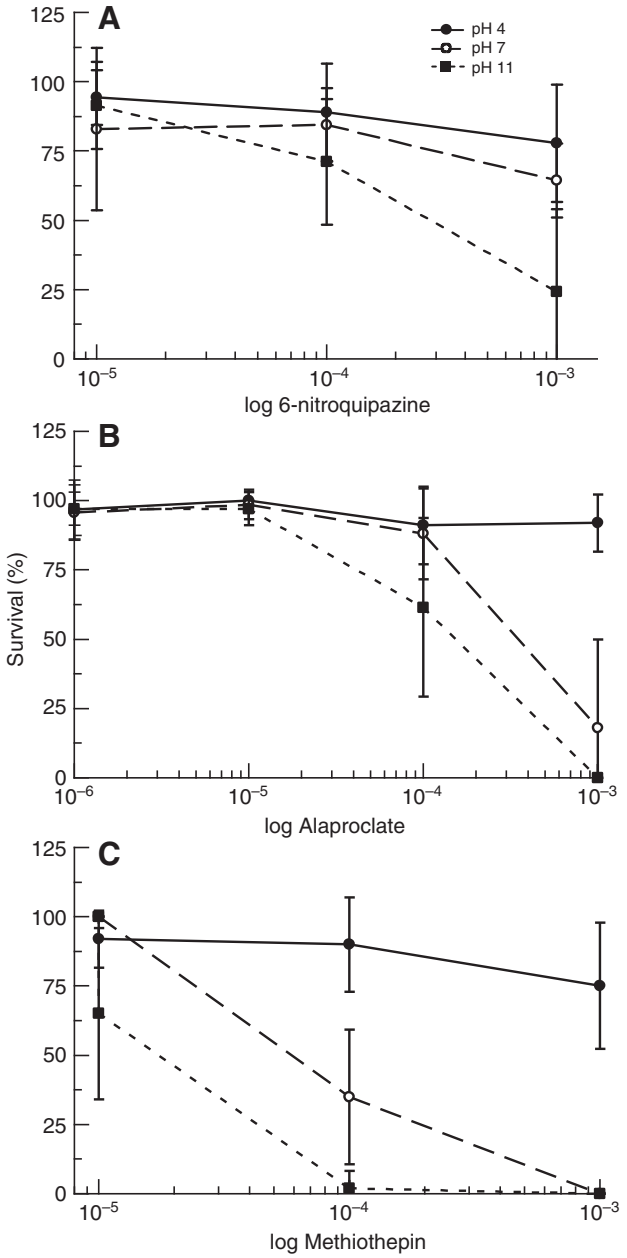

Fig. 4. Concentration dependence of the toxicity of (A) 6-nitroquipazine, (B) alaproclate, and (C) methiothepin. Survival of larvae during 48 h exposures are shown. Control groups (no drug) are not shown due to the low toxicity of the lowest concentrations tested. Data are presented as means ± s.e.m.

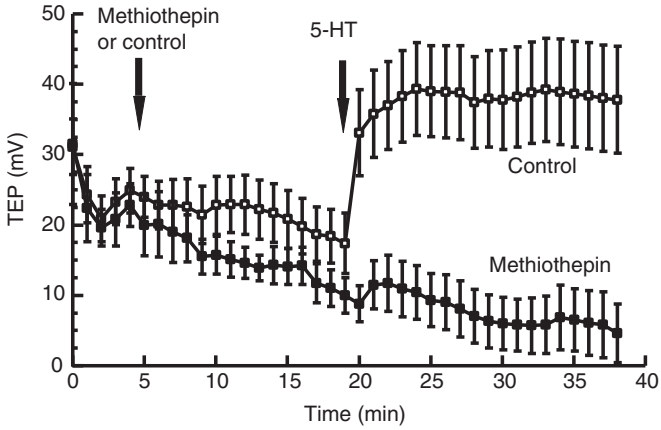

Fig. 5. Methiothepin blocks the effects of serotonin on the transepithelial potential (TEP) of the posterior midgut. Methiothepin (open symbols; 1×10^{-6} mol l^{-1}) or control solution (closed symbols) was added at the first arrow and serotonin (1×10^{-7} mol l^{-1}) was added to all preparations at the second arrow. *N* (methiothepin)=7; *N* (control)=6. Data are presented as means ± s.e.m.

exposed to acidic media show greatly increased drinking and excretion rates, and their Malpighian tubules show large increases in mitochondrial luminosity, suggesting greatly increased energy demands under these conditions (Clark et al., 2007). Petzel et al. (Petzel et al., 1999) found that cAMP stimulates acid excretion by the Malpighian tubules, and Cady and Hagedorn (Cady and Hagedorn, 1998; Cady and Hagedorn, 1999) found that serotonin stimulates cAMP levels, in Malpighian tubules of adult *Aedes*. Together, the studies of Cady and Hagedorn (Cady and Hagedorn, 1998; Cady and Hagedorn, 1999) and Petzel et al. (Petzel et al., 1999) suggest that serotonin may stimulate acid excretion by Malpighian tubules of adult *Aedes*. We therefore hypothesize that serotonin regulates hemolymph pH in larval *Aedes* primarily by stimulating Malpighian tubule secretion rates, leading to increased acid clearance. If serotonin is acting as a neurotransmitter under these conditions, it may thus coordinate the actions of the midgut and Malpighian tubules in larval *Aedes* as it does in *Rhodnius*, despite the apparently different stimuli leading to its release and its apparently distinct roles in the two systems (i.e. clearance of a NaCl and volume load in *Rhodnius* and stimulation of midgut alkalization and clearance of an H^+ and volume load from the hemolymph in larval *Aedes*). It is not yet known whether serotonin also acts on the larval rectum, although serotonin receptors are found on the adult hindgut (Lee and Pietrantonio, 2003). If it does, regulation of rectal transport by serotonin could also be involved in acid–base homeostasis.

Effects of 6-nitroquipazine and methiothepin on hemolymph composition

Whereas the actions of alaproclate are consistent with the regulation of hemolymph pH by serotonin, the actions of 6-nitroquipazine and methiothepin are more difficult to interpret. Each has actions on hemolymph composition distinct from those of alaproclate, and from each other, and yet they show similar pH-dependent and salinity-independent toxicity. Unlike alaproclate, the SSRI 6-nitroquipazine did not significantly influence hemolymph pH. Instead, it decreased hemolymph Na^+ concentrations, although no effect on hemolymph osmotic pressure or volume was observed. It may thus stimulate exchange of hemolymph Na^+ for another solute. Perhaps alaproclate and 6-nitroquipazine act upon distinct serotonin transporters or differ in their specificity for serotonin transporters or have distinct dosage-dependent effects. An analysis of the effects of these agents on hemolymph serotonin levels will help to answer these questions.

Methiothepin, a known antagonist of Malpighian tubule (Clark and Bradley, 1997) and midgut (present study) serotonin receptors, had no effect on hemolymph Na^+, percentage body water, percentage hemolymph or H^+ but decreased hemolymph osmotic pressure. It also proved to be the most toxic of the agents tested. The transepithelial potential of posterior midgut responds to serotonin. Does stimulation of the posterior midgut by serotonin play a role in hemolymph alkalinization by stimulating transepithelial acid transport into the lumen? The serotonin receptors of the posterior stomach are completely blocked by methiothepin, suggesting either

a single receptor type or non-specific actions of methiothepin on multiple serotonin receptors. Methiothepin also acts on Malpighian tubule serotonin receptors (Clark and Bradley, 1997). The location and function of other peripheral receptors antagonized by methiothepin are not yet known.

The specific cause of the pH-dependent toxicity, and the effects of these compounds on hemolymph composition, must remain speculative until the pharmacology and distributions of serotonin transporters and serotonin receptor types on the epithelial organs of the larval mosquito are better known.

How do the responses of larval mosquitoes to increased acidity compare with their responses to increased salinity?

It is somewhat surprising to find minimal salinity-dependent toxicity of the agents tested, as Clark and Bradley (Clark and Bradley, 1997) found that hemolymph serotonin levels were increased in response to elevated salinity (20% seawater *vs* 2% seawater). Unlike the study by Clark and Bradley (Clark and Bradley, 1997), all animals tested in the present study were initially hatched in deionized water, and it is possible that circulating serotonin levels were near zero under these conditions. If so, reuptake inhibitors and receptor antagonists would be expected to show minimal effects as there would be no serotonin to block. However, the animals were subsequently exposed to different salt concentrations for 72 h, sufficient time to trigger release of serotonin, yet minimal salinity-dependent toxicity was observed. Furthermore, the agents proved toxic in deionized water, implying that serotonin was present under these conditions (assuming the actions were due to interference with serotonin pathways). It is possible that the relative changes in circulating serotonin levels in response to salinity are insignificant in the presence of the high doses of pharmacological agents used.

There is a direct mechanistic link between ionic and acid–base homeostasis, due to ubiquitous Na^+/H^+ and Cl^-/HCO_3^- exchangers (Stobbart, 1971). In many aquatic animals, uptake of Na^+ from dilute media is driven by H^+ gradients (Goss et al., 1995), and death in acidic media is ultimately caused by failure of Na^+, rather than H^+, homeostasis (Havas et al., 1981; Vangenechen et al., 1989). Similarly, in many animals, including larval *A. aegypti*, acclimation to decreased salinity is accompanied by alkalosis while acclimation to increased salinity often occurs with acidosis (Truchot, 1987; Donini et al., 2007). In this situation, disturbances of extracellular acid–base status in response to salinity changes may occur that are secondary to cellular processes such as cell volume regulation, which often involve Na^+/H^+ or Cl^-/HCO_3^- exchange (Truchot, 1987). Both elevated salinity and acidic media are known to stimulate drinking rates by larval mosquitoes (Clements, 2000; Clark et al., 2007). Increased drinking rates with elevated salinity are thought to be a mechanism to increase ionic clearance rates. According to this hypothesis, the Malpighian tubules can only excrete ions when the opposing electrochemical gradient is below a maximal value. By increasing drinking rates, the urine volume is increased but its concentration is decreased, leading to greater ion excretion rates (Clements, 2000). A similar explanation may hold for acid excretion. Peripheral release of serotonin may be a generalized response to increased fluid ingestion rates leading to the elimination of fluid loads and maintenance of body volume, resembling its role in volume regulation by *Rhodnius*. Additional regulatory mechanisms and signals may then fine-tune the excreted fluid in response to specific challenges. Alternatively, excretion rates may be stimulated due to acid or ionic loads, leading to a volume deficit that is corrected by drinking. The specific trigger for release of serotonin is thus unclear but may be relevant during both acidic and elevated salinity challenges.

Larvae in acidic media (pH 4) were found to excrete net base whereas those in neutral or alkaline (pH 11) media excreted net acid. Excretion of net base by larvae in pH 4 media requires an excretory product such as ammonia (protonated to NH_4^+ at pK_b=4.75). The observed excretion of net acid by larvae in alkaline media is consistent with the acidic rectum observed under these conditions (Clark et al., 2007). Excretion of CO_2 at a constant rate across salinities would result in apparently greater rates of acid excretion at increased alkalinity, because at pH 7 much of the released H_2CO_3 would dissociate to form H^+ and HCO_3^- (pKa_1=6.36) whereas at pH 11 CO_3^{2-} would be formed (pKa_2=10.25) and two H^+ ions released. Ammonium ion would also contribute to acidification in pH 11 media.

In summary, the data presented here demonstrate that serotonergic agents can be used to manipulate hemolymph composition of larval mosquitoes *in vivo*. The serotonin-selective reuptake inhibitor alaproclate increased rates of acid excretion, causing hemolymph alkalosis in neutral media and increasing survival in acidic media. These data suggest that a major role of peripheral serotonin in larval mosquitoes may be stimulation of acid excretion contributing to regulation of hemolymph pH. This regulatory pathway may contribute to the tolerance of larval mosquitoes for acidic media and thus the range of habitats that they can exploit. These data also demonstrate for the first time that pharmacological disruption of regulatory control of excretory function can kill a significant insect pest. Further experimentation is necessary in order to more fully understand the mechanisms of pH-dependent toxicity of these pharmacological agents, and the role of serotonin in hemolymph ion and acid–base balance. In particular, the distributions and pharmacology of serotonin receptors in the alimentary and excretory systems, the effects of ambient pH on serotonin release *in vivo*, the effects of alaproclate and 6-nitroquipazine on serotonin uptake and hemolymph serotonin concentrations, and the effects of serotonin on Malpighian tubule and rectal H^+ excretion must be determined.

The authors thank two anonymous reviewers for numerous thoughtful and helpful comments that have contributed to the final version of this article.

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