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To cite this article: Clark, T. M., Flis, B. J., & Remold, S. K. (2004). pH tolerances and regulatory abilities of freshwater and euryhaline Aedine mosquito larvae. *Journal of Experimental Biology*, *207*(13), 2297. https://doi.org/10.1242/jeb.01021

The Journal of Experimental Biology 207, 2297-2304
Published by The Company of Biologists 2004
doi:10.1242/jeb.01021

pH tolerances and regulatory abilities of freshwater and euryhaline Aedine mosquito larvae

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Accepted 7 April 2004

Summary

The pH regulatory abilities of two members of the mosquito tribe Aedini, known to have dramatically different saline tolerances, are investigated. The freshwater mosquito *Aedes aegypti* and the euryhaline *Ochlerotatus taeniorhynchus* tolerate very similar pH ranges. Both species complete larval development in waters ranging from pH 4 to pH 11, but naïve larvae always die in water of pH 3 or 12. Across the pH range 4–11, the hemolymph pH of *O. taeniorhynchus* is maintained constant while that of *A. aegypti* varies by 0.1 pH units. The salt composition of the water (3.5 g l^{-1} sea salt, 3.5 g l^{-1} NaCl, or nominally salt-free) has no effect on the range of pH tolerated by *A. aegypti*. In both species, the effects of pH on larval growth and development are minor in comparison with the influence of species and sex. Acclimation of *A. aegypti* to pH 4 or 11 increases survival times in pH 3 or 12, respectively, and allows a small percentage of larvae to pupate successfully at these extreme pH values. Such acclimation does not compromise survival at the other pH extreme.

Key words: mosquito larvae, pH regulation, pH acclimation, life history, *Aedes aegypti*, *Ochlerotatus taeniorhynchus.*

Introduction

Maintenance of body fluid ionic composition and pH is of critical importance in homeostasis. Because of this, the ionic composition and pH of the environment are major physical factors limiting the distributions of organisms in aquatic habitats. For aquatic invertebrates in general, including larval insects such as Ephemeroptera and Trichoptera, species richness and population densities decline at lower habitat pH (Sutcliffe and Hildrew, 1989). Highly alkaline habitats also show limited biodiversity. Larval mosquitoes can tolerate ranges of ambient pH much greater than those tolerated by other aquatic animals. There is no evidence that pH ever limits the habitats of larval mosquitoes in nature (Clements, 2000), where reported pH values for larval habitats range from 3.3 to 8.1 (*Ochlerotatus taeniorhynchus*), 4.4–9.3 (*Aedes geniculatus*), 3.3–9.2 (*Psorophora confinnis*), and 4.4–9.3 (*Anopheles plumbeus*). In the laboratory, *Aedes flavopictus* has been reared in waters ranging from pH 2–9, and *Armigeres subalbatus* in the pH range of 2–10 (Keilin, 1932; Kurihara, 1959; MacGregor, 1921; Peterson and Chapman, 1970).

Despite the great range of ambient pH values tolerated by larval mosquitoes in nature and in the laboratory, we have almost no information about the effects of pH on larval growth and development or about regulation of hemolymph pH in these animals. The predominant physiological challenges of life in acidic water are Ca^{2+} regulation, Na^+ regulation, and mobilization of toxic metals such as aluminum from the substrate (Wiederholm, 1984). Most aquatic insects that have been examined are able to maintain relatively constant hemolymph pH when exposed to acid waters. This is apparently achieved using ion exchange mechanisms, especially Na^+/H^+ exchangers, to move acid/base equivalents. Because of this, the mechanisms of low pH toxicity appear to relate more strongly to disruption of general ionic balance, especially Na^+, than to failure of pH homeostasis *per se* (Havas, 1981). Low pH is thus generally more harmful in oligotrophic, low-ionic strength water, where ions involved in coupled transport of acid or base equivalents are limited (Vangenechten et al., 1989). Survival in alkaline conditions has received less attention, but in larval mosquitoes it is known to involve Cl^-/HCO_3^- exchange occurring in the rectum (Stobbart, 1971; Strange et al., 1982, 1984; Strange and Phillips, 1985).

The present work investigates for the first time the effects of ambient pH on growth and development of larval mosquitoes. We compare the effects of ambient pH on two mosquito species with very different saline tolerances within the subfamily Aedini, *Aedes aegypti* (L.) and *Ochlerotatus taeniorhynchus* (Wiedemann) (these species were considered congeneric until recently; Reinert, 2000). *Aedes aegypti* is an obligately freshwater species that inhabits open containers

such as tin cans and discarded tires, and is unable to survive in waters of salinity greater than about 40% seawater (14 g l^{-1}). The euryhaline *O. taeniorhynchus* can complete development in waters ranging from fresh to those more concentrated than full-strength seawater (35 g l^{-1}) (for the salinity tolerances of the larvae used in these experiments, see Clark et al., 2004). Because of the known interactions between pH regulation and ionoregulation, we were interested to ascertain whether the distinct saline tolerances of these two species were correlated with differences in pH regulatory abilities. In addition, we wished to determine whether mosquito larvae possess the capacity for acclimatory responses to pH, and whether there are physiological trade-offs inherent in the mechanisms used to deal with acid or base loads.

Data presented here demonstrate that (1) *O. taeniorhynchus* and *A. aegypti* have very similar pH tolerances, completing larval development in buffered waters ranging from pH 4 to pH 11 in the laboratory. (2) Larvae of both species are highly effective pH regulators, maintaining hemolymph pH within narrow limits across the entire tolerable pH range. (3) The effects of pH on larval growth and development are quite similar in the two species, and are minor in comparison with the influences of sex, and species. (4) Further studies in *A. aegypti* alone showed that the ionic composition of the water (3.5 g l^{-1} NaCl, 3.5 g l^{-1} sea salt, no added salt) had no effect on the range of pH tolerated. (5) *Aedes aegypti* can acclimate to either acidic or basic conditions without interfering with survival at the other extreme of pH. This demonstrates that the mechanisms used to regulate body pH in acidic and alkaline conditions are not mutually exclusive and thus may be physically or temporally separated, as in the acid- and base-secreting intercalated cells of the mammalian nephron (Brown et al., 1992).

Materials and methods

Mosquitoes

Colonies of *Aedes aegypti* L. and *Ochlerotatus taeniorhynchus* Wiedemann were established using eggs from the colonies of the Florida Medical Entomology Laboratory, in Vero Beach, FL, USA. Eggs were hatched in distilled water. The next day, 20 larvae were placed in 50 ml of buffered water (236 ml total container volume; water 1 cm deep) with the appropriate pH and salt composition (see below) and fed ground TetraMin flakes (TetraWerke, Melle, Germany). Larvae were maintained on a 16 h:8 h L:D photoperiod at 26°C. In all cases, water was replaced and larvae were fed daily, beginning 2 days after transfer when larvae were large enough to begin noticeably altering water conditions and continuing until death or pupation occurred.

Solutions

Three different rearing solutions were used. All rearing solutions contained 2.5 mOsm l^{-1} Trizma base (Tris[hydroxymethyl]aminomethane), and 2.5 mOsm l^{-1} Hepes (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), and were adjusted to the appropriate pH using HCl or NaOH. One rearing solution, subsequently referred to as sea salt, contained in addition to these buffers 3.5 g l^{-1} (83 mOsm l^{-1}) artificial sea salt (Instant Ocean; Aquarium Systems, Mentor, Ohio, USA). A second rearing solution, referred to hence as NaCl, contained in addition to the buffers 3.5 g l^{-1} NaCl (59.9 mOsm l^{-1}), while in a third rearing solution the added ions were limited to the NaOH or HCl used to adjust the pH and those present in the food. pH was determined using a Perphect LogR meter model 330 (Orion, Beverly, MA, USA), with an Orion Perphect gel-epoxy triode electrode.

pH tolerance and the influence of pH on life history parameters

Larvae were reared as described above, in batches of 20 larvae per 50 ml, until all larvae had died or pupated. Live pupae were collected each morning, blotted dry, and weighed to the nearest 0.01 mg using a high precision analytical balance (Mettler Toledo AX 205 Deltarange; Columbus, OH, USA). Dry mass was determined after drying at 65°C for 24 h. Masses of dead larvae or pupae were not determined (mosquitoes have pupae that are highly mobile, and dead pupae can be readily distinguished by their failure to remain within the water column, swim to the surface to obtain air, or respond to the presence of the investigator).

Transfer experiments

Larvae were reared for 3 days in buffered, 3.5 g l^{-1} NaCl solution, at pH 4, 7 or 11. Larvae were acclimated in batches of 10 larvae, and after 3 days of acclimation survivors of each batch were either maintained in the rearing pH (4 to 4 or 11 to 11) or transferred to another pH (7 to 12, 7 to 3, 4 to 11, 4 to 3, 11 to 4, 11 to 12), with the first number representing the acclimation pH and the second the pH in which they completed development. Water was changed and larvae were fed each day. Live pupae appearing during the previous 24 h were collected each morning, weighed and sexed.

Measurement of hemolymph pH

pH electrodes were made from double-barreled borosilicate omega dot capillary tubing (1.5 mm o.d., 0.75 mm i.d.; FHC, Brunswick, ME, USA). The glass tubing was washed with nitric acid, then with nanopure water (R >17 MΩ) and oven dried. Electrodes were pulled on a Kopf (Tujunga, CA, USA) Model 720 Needle/Pipette puller. The tips to contain pH resin were sylanized using 5% dimethyldichlorosilane (Sylon CT; Supelco, Bellefonte, PA, USA), and dried with gentle heat on a hot plate. The tip of the silanized barrel was filled with resin (Hydrogen Ionophore 1; Sigma, St Louis, MO, USA) 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.

Larvae reared in buffered, 3.5 g l^{-1} NaCl solutions of different pH (see above) were rinsed in deionized water,

blotted dry, and torn open on Parafilm using fine forceps. A pH microelectrode with grounded reference barrel was then immediately inserted into the drop of hemolymph. The voltage was determined using a high impedence amplifier (Iso-DAM8; World Precision Instruments; Sarasota, FL, USA), coupled to Sable Systems Data Acquisitions System (Sable Systems, Henderson, NV, USA). Voltage was converted to pH by reference to a standard curve.

Statistical analyses

Mortality rates of the two species were compared using Fisher's exact test (SAS frequency procedure; SAS Institute Inc. 1997). Mortality across salt types within pH were investigated using single factor analysis of variance (ANOVA). The dependence of larval hemolymph pH on rearing pH was analyzed for each species using a one-factor analysis of variance, with rearing pH as a categorical predictor (Microsoft Excel 2002). Effects of pH on life history parameters were modeled using mixed linear models (SAS mixed procedure; SAS Institute Inc. 1997). Models testing for effects of species, sex, pH and their interactions, included as categorical variables, were further explored with separate models fitted for each species. Significant pH×sex interaction effects were further explored using sub-models in which each sex was considered separately. Acclimation experiments were analyzed using Student's *t*-tests in Microsoft Excel.

Results

pH tolerances

No substantive differences are observed in pH tolerance between larval *A. aegypti* and *O. taeniorhynchus* under the conditions of this study (Table 1; $P>0.05$, Fisher's exact test). High survival percentages are observed in both species following hatching when placed into waters ranging from pH 4 to pH 11, while larvae of both species always die when placed instead into waters of pH 3 or pH 12 (Table 1). The range of pH tolerated by *A. aegypti* is 4–11, whether in buffered 3.5 g l^{-1} NaCl or in buffered 3.5 g l^{-1} sea salt (Table 1). Larval *A. aegypti* also pupate successfully when reared in pH 4, 7 and 11 in buffered water containing no added salt, and survival rates are no different under these nominally salt-free conditions than in the presence of NaCl or sea salt (Table 1; $P>0.05$, single factor ANOVAs testing differences among salt treatments at each pH value for this species).

Hemolymph pH

Larvae of both species reared in waters ranging from pH 4 to pH 11 show relatively constant hemolymph pH values, measuring above 7.5 across the entire pH range (Fig. 1). Hemolymph pH values of *A. aegypti* and *O. taeniorhynchus* are comparable (pH 7.7) in animals reared at pH 11. Hemolymph pH of *O. taeniorhynchus* is independent of ambient pH ($P>0.9$; single factor ANOVA), measuring around pH 7.7 at all ambient pH values. Hemolymph pH of *A. aegypti* is influenced by ambient pH ($P<0.005$; single factor ANOVA), decreasing by approximately 0.1 pH units to pH 7.6 in larvae reared in pH 7 or 4 (Fig. 1). Hemolymph pH of *A. aegypti* is thus more acidic than that of *O. taeniorhynchus* at pH 4 and 7 (pH 4: $P<0.05$, pH 7: $P<0.002$, two-tailed *t*-test, Microsoft Excel).

Table 1. *The range of pH tolerated by larval* Aedes aegypti *and* Oclerotatus taeniorhynchus

	O. taeniorhynchus	*A. aegypti*		
pH	NaCl	NaCl	Sea salt	No salt
3	0	0	0	
4	78±7.3	75±8.7	87±1.7	80
7	80±5.8	93±1.7	93±4.4	100
11	86±6.8	85±7.6	80±7.6	75
12	0	0	0	

NaCl, survival of larval *O. taeniorhynchus* and *A. aegypti* (%) in buffered, 3.5 g l^{-1} NaCl. *A. aegypti* (N=3 runs; 420 larvae), *O. taeniorhynchus* (N=5 runs; 250 larvae).

Sea salt, survival of *Aedes aegypti* larvae (%) in 3.5 g l^{-1} artificial sea salt (Instant Ocean). N (sea salt)=30–50 larvae / pH unit in 3–5 runs of 10 larvae each.

*N*o salt, survival of larval *Aedes aegypti* (%) in buffered water of pH 4, 7 and 11 in the absence of added salt. N (no added salt)=20 larvae/pH unit in 1 run. Larvae were not tested in pH 3 and 12 in the absence of added salt.

No significant differences were detected between any of the treatments at any pH ($P>0.05$; single factor ANOVA between salt types run independently at pH 4, 7 and 11).

Effects of pH on growth and development of A. aegypti *and* O. taeniorhynchus

When grown in water containing buffered 3.5 g l^{-1} NaCl, larval stage duration and growth rate (wet mass) but not pupal

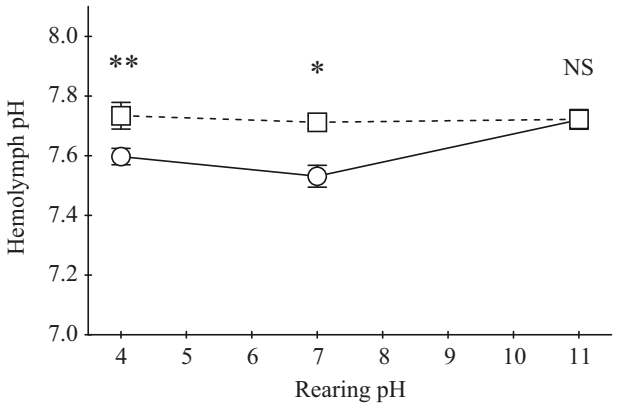

Fig. 1. Hemolymph pH of larval *Aedes aegypti* (solid line) and *Ochlerotatus taeniorhynchus* (broken line) is highly regulated across the entire tolerable pH range from pH 4 to 11. Data are raw means ± S.E.M., sample size=6 replicates per species and pH value. **$0.001<P<0.01$; *$0.01<P<0.05$; NS, $P>0.1$ for *t*-tests comparing pH of the two species at each ambient pH.

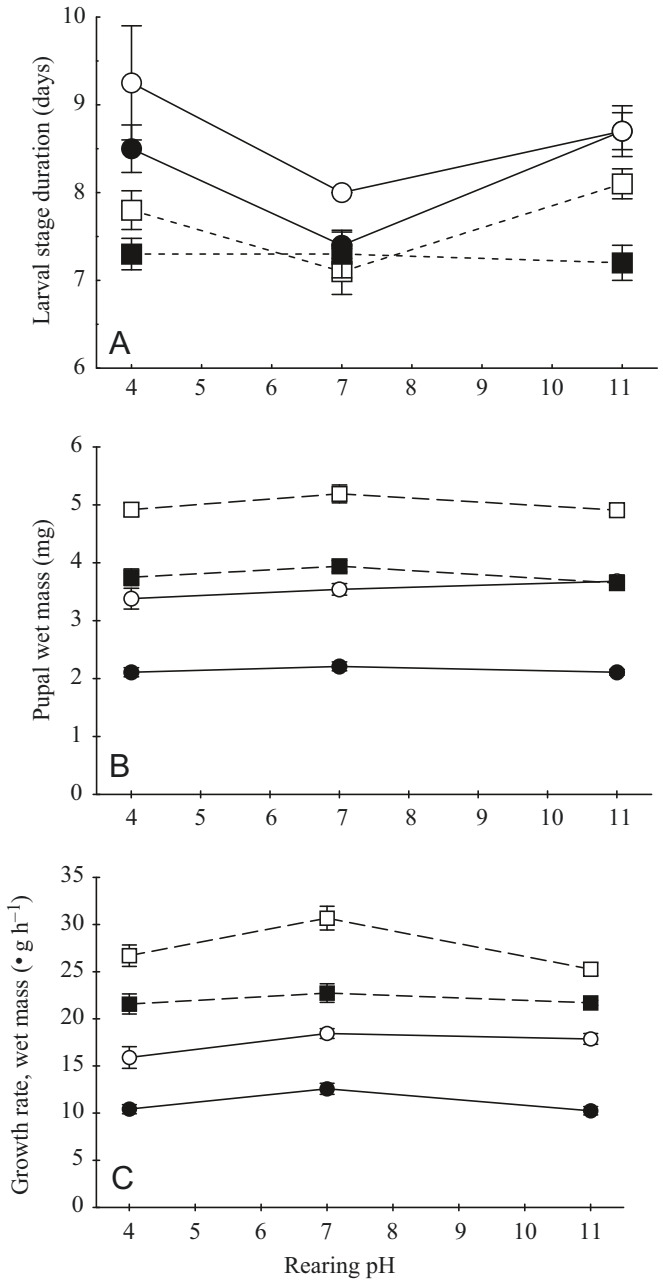

Fig. 2. Effects of rearing pH on life history parameters of larval *Aedes aegypti* (solid line) and *Ochlerotatus taeniorhynchus* (broken line) reared in buffered 3.5 g l^{-1} sea salt (Instant Ocean). Only larvae pupating successfully are shown. Effects of pH on (A) duration of the larval stage, (B) pupal wet mass and (C) growth rate. Males, filled symbols; females, open symbols. Data are raw means ± S.E.M.; sample size (*A. aegypti*)=148, sample size (*O. taeniorhynchus*)=71.

wet mass are differentially affected by pH in the two species (Fig. 2, Table 2). Nevertheless, for all three life history parameters, the influence of pH, even when statistically significant, is small in magnitude when compared with the much larger influences of species and sex.

Table 2. *Best-fit mixed linear models describing the effects of species, sex, pH and their interactions on life history parameters of* Aedes aegypti *and* Ochlerotatus taeniorhynchus *from mixed linear models*

Effect	n.d.f.	d.d.f.	*F* value
Days to pupation			
Species	1	119	34.97***
Sex	1	119	7.58**
Species×sex	1	119	0.04NS
pH	2	119	8.25***
Species×pH	2	119	2.46†
Sex×pH	2	119	0.61NS
Species×sex×pH	2	119	3.97*
Wet mass			
Species	1	119	521.32***
Sex	1	119	369.38***
Species×sex	1	119	1.42NS
pH	2	119	2.37†
Species×pH	2	119	1.92NS
Sex×pH	2	119	0.74NS
Species×sex×pH	2	119	0.31NS
Growth rate, wet mass			
Species	1	119	387.23***
Sex	1	119	122.79***
Species×sex	1	119	0.52NS
pH	2	119	8.87***
Species×pH	2	119	1.17NS
Sex×pH	2	119	0.83NS
Species×sex×pH	2	119	3.00†

n.d.f., numerator degrees of freedom; d.d.f., denominator degrees of freedom.

***P<0.001, **0.001<P<0.01; *0.01<P<0.05; †0.05<P<0.1; NS, P>0.1.

Developmental time

Developmental time differs significantly between the two species, with *A. aegypti* taking longer overall to pupate than *O. taeniorhynchus* (Fig. 2A, Table 2). However the difference between species in development time depends significantly on both pH and sex (indicated by the significant species×sex×pH interaction; Table 2). A separate model exploring this interaction detects significant differences between the sexes in the effect of rearing pH in *O. taeniorhynchus* (significant sex×pH interaction, Table 3, Fig. 2A). Further submodels run for each sex show that developmental time of female *O. taeniorhynchus* is significantly lower at intermediate pH (4 *vs.* 7, P<0.05; 7 *vs.* 11, P<0.005) whereas male developmental time does not fluctuate much across a wide pH range (Fig. 2A; P>0.05 for effect of pH). Averaged across both sexes, the overall effect of pH on development time is not statistically significant (Table 3). In *A. aegypti*, both males and females pupate significantly earlier at pH 7 than in pH 4 or in pH 11, but do not differ in development time in pH 4 and 11 (P>0.001,

Table 3. *Best-fit mixed linear models describing the effects of sex, pH and their interactions on life history parameters of* Aedes aegypti *and* Ochlerotatus taeniorhynchus *from mixed linear models*

	A. aegypti			*O. taeniorhynchus*		
Effect	n.d.f.	d.d.f.	*F* value	n.d.f.	d.d.f.	*F* value
Days to pupation						
Sex	1	54	2.59NS	1	65	5.52*
pH	2	54	7.44**	2	65	1.18NS
Sex×pH	2	54	0.87NS	2	65	4.51*
Wet mass						
Sex	1	54	219.55***	1	65	159.58***
pH	2	54	0.96NS	2	65	3.44*
Sex×pH	2	54	1.05NS	2	65	0.09NS
Growth rate, wet mass						
Sex	1	54	121.58***	1	65	41.60***
pH	2	54	5.25**	2	65	5.53**
Sex×pH	2	54	1.48NS	2	65	2.38NS

n.d.f., numerator degrees of freedom; d.d.f., denominator degrees of freedom.
***P< 0.001, **0.001<P<0.01; *0.01<P<0.05; †0.05<P<0.1; NS, P>0.1.

P<0.005, P=0.59, respectively for contrasts within the one-species model). Under the conditions of this study, males and females of this species do not differ from one another overall in their development times nor do they differ in their response to pH (Fig. 2A, Table 3).

Pupal wet mass

Pupal wet mass differs significantly between *A. aegypti* and *O. taeniorhynchus* (Fig. 2B, Table 2). The full model including data on both species detects a marginally significant maximum wet mass at neutral pH, with lower mass at the pH extremes (Fig. 2B, Table 2). The models run separately for each species detect significant differences in pupal mass across pH values in *O. taeniorhynchus* but not in *A. aegypti* (Table 3). However, even for *O. taeniorhynchus* the effect of pH is small (maximum difference among pH values are: males; 9%, females 5%). In both species, females are substantially larger at pupation than males (Fig. 2B), regardless of rearing pH. This significant effect is detected in all three models (Tables 2, 3).

Growth rate of wet mass

The growth rate of wet mass is higher for *O. taeniorhynchus* than for *A. aegypti*, and higher in females than in males in both species (Fig. 2C, Tables 2, 3). Across species and sexes, this rate differs significantly across the pH range, demonstrating a maximum at pH 7 (Fig. 2C, Table 2), but this effect of pH depends marginally significantly both on the larva's sex and the species (species×sex×pH interaction; Table 2). Separate models run for each species further investigating this interaction detect the overall effect of sex and pH in both species, further supporting the results of the single-species model, but fail to detect any significant interdependence (Table 3).

Acclimation of A. aegypti *larvae to extreme pH*

Transfer experiments using larval *A. aegypti* demonstrate the capacity for acclimatory responses to extreme pH in mosquito larvae. Acclimation of larval *A. aegypti* to extreme pH increases the tolerable range of pH, rather than shifting it (Fig. 3A,B). Larvae always die rapidly when placed directly into water of pH 3 or 12 on the day following hatching (see Table 1). Larvae acclimated to pH 4 or 11 for several days survive significantly longer upon transfer to pH 3 or 12, respectively, than do controls reared in pH 7 before transfer (Fig. 3A; P<0.05, t-tests). Acclimation also allows survival of a few larvae to pupation at each pH extreme. One of 27 larvae transferred to pH 12 after acclimation in pH 11 survived to pupation, as did 2 of 32 transferred to pH 3 following acclimation in pH 4. In contrast, larvae reared for 3 days in pH 7 always die rapidly upon transfer to either pH 3 or 12 [Fig. 3A, N(pH 3)=24, N(pH 12)=10], demonstrating that it is acclimation and not larval age that extends life at these extreme pH values.

Larvae reared initially in pH 4 for 3 days, then transferred to pH 11, showed no decrease in the rate of successful pupation compared to larvae maintained in pH 4 (Fig. 3B; P>0.05, t-test). Similarly, larvae acclimated to pH 11, then transferred to pH 4, showed the same proportion of successful pupation as those maintained in pH 11 (Fig. 3B; P>0.05, t-test). Among males and females transferred from pH 11 to 4 and pH 4 to 11 following 3 days of acclimation, there were no significant differences in duration of the larval stage, pupal mass or growth rates when compared with controls, with the exception of reduced growth rates of females transferred from pH 11 to 4 (Fig. 3C, P<0.05, t-test). The reduced growth rates of females transferred from pH 11 to 4 were due to a combination of a small but non-significant increase in larval stage duration and a similar non-significant

decrease in pupal mass (data not shown). These two non-significant effects summed to produce a significant decrease in growth rates by female larvae transferred from pH 11 to 4 (Fig. 3C, $P<0.05$; *t*-test). No such decrease was observed in males.

Differences in responses of species and sexes to pH

Despite the overall similarities in growth and developmental parameters between species and sexes, some differences are observed. The duration of the larval stage of *O. taeniorhynchus* is influenced by sex and not pH, whereas larval stage duration of *A. aegypti* is influenced by pH and not sex. Wet mass of *O. taeniorhynchus* is influenced by both sex and pH, whereas only sex influences wet mass of *A. aegypti.* In several instances we also detect evidence for differences between sexes within a species. Developmental time of male *O. taeniorhynchus* is more robust to changes in pH than is that of females. Growth rates are reduced by transfer of female but not male *A. aegypti* from pH 11 to pH 4.

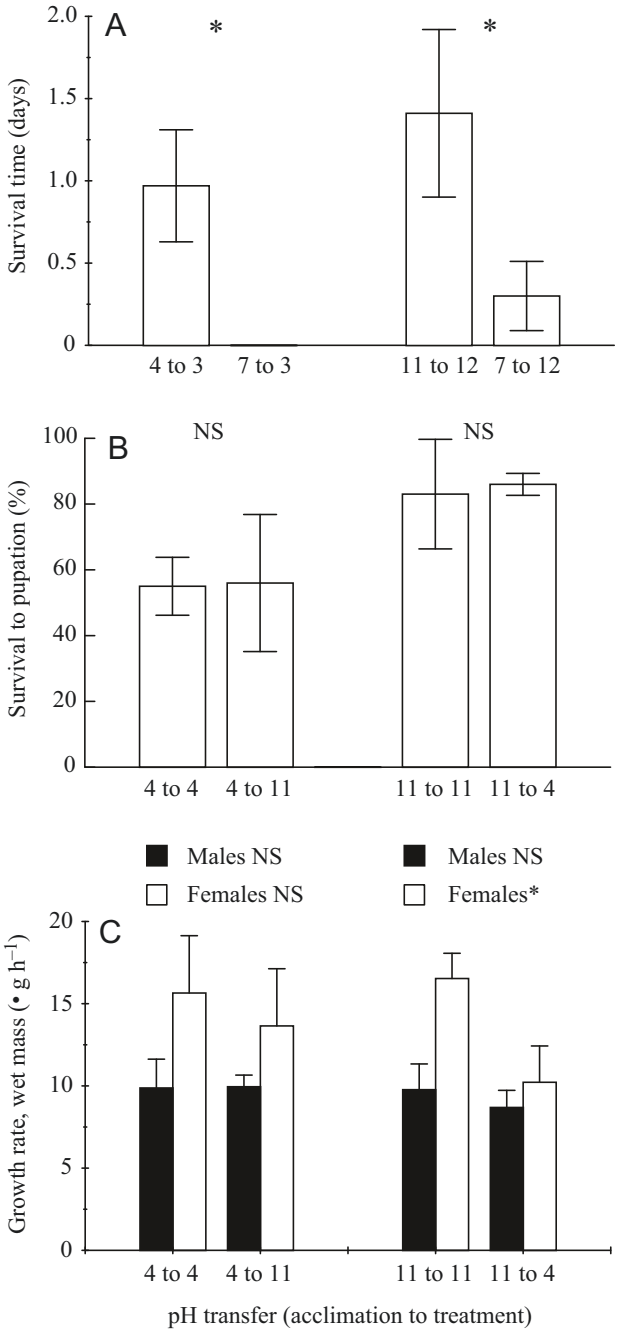

Fig. 3. Effects of acclimation on pH tolerance of larval *Aedes aegypti*. (A) Survival times in extreme pH are increased by prior acclimation. Bars represent mean ± S.E.M. of survival time following transfer from acclimation pH to test pH. Sample sizes: 4 to 3, 32; 7 to 3, 24; 11 to 12, 27; 7 to 12, 10. (B) Acclimation to extreme pH does not influence survival at the other pH extreme. Bars represent the mean ± S.E.M. of the proportion of larvae surviving to pupation following acclimation at pH 4 or 11, then either maintained in the acclimation pH or transferred to the test pH. Samples sizes are four runs for all groups except 11 to 4, where only three runs were performed. (C) Transfer of acclimated larvae from one pH extreme to the other has little influence on the growth rate. Larvae were acclimatized to pH 4 or 11 for 3 days, then transferred to the other pH extreme. Males, filled bars; females, open bars. Sample sizes are (for males; females): 4 to 4 (8;8); 4 to 11 (14;6); 11 to 11 (9;15); 11 to 4 (10;10). Significance of comparison of transfers to same test pH are indicated for A and B; significance of comparisons within males or females from the same acclimation pH are shown for C. *$P<0.05$; NS, $P>0.05$.

Discussion

A number of organisms are known that tolerate acidic or alkaline conditions, but mosquitoes appear to be the most proficient pH regulators yet described. *Aedes aegypti* and *O. taeniorhynchus* are unusual in their ability to survive and develop in waters ranging from pH 4 to 11. They regulate hemolymph pH quite well over this pH range. Even more remarkable is their ability to tolerate abrupt transfer from one extreme of this pH range to the other. The authors are unaware of any other organisms that can surpass these larvae in the breadth of their pH tolerances and their ability to tolerate sudden changes of this magnitude in ambient pH.

Prior reports have documented the remarkable abilities of larval mosquitoes to tolerate waters characterized by differences in H^+ concentrations of many orders of magnitude (Keilin, 1932; Kurihara, 1959; MacGregor, 1921; Peterson and Chapman, 1970). We extend those findings by demonstrating similar, broad pH tolerances in species with very different salinity tolerances. The distinct salinity tolerances of larval *Aedes aegypti* and *O. taeniorhynchus* are due to differences in their rectal structure and function. Freshwater species, including *A. aegypti*, have a rectum composed of a single segment that acts to recover ions from the rectal contents. The rectum of *O. taeniorhynchus* and other euryhaline osmoregulators consists of two segments, with an anterior segment that functions like the rectum of freshwater forms, and a salt-secreting posterior segment that allows these species to survive in saline water (Bradley and Phillips, 1975, 1977). Identical pH tolerances in related freshwater and

euryhaline species demonstrate that the salt gland, found only in euryhaline osmoregulators, is not involved in pH regulation. Survival of larvae of the mosquito *Aedes dorsalis* in highly alkaline lakes has been attributed to Cl^-/HCO_3 exchange occurring in the anterior rectal segment (Bradley and Phillips, 1977; Strange et al., 1982, 1984; Strange and Phillips, 1985). The data presented here are consistent with these observations, and demonstrate that pH regulation in acidic waters does not require a posterior rectal salt gland either. The organs involved in pH regulation in acidic waters have yet to be determined.

We were surprised to find that the pH tolerances of *Aedes aegypti* appear to be similar in nominally salt-free water and in water containing 3.5 g l^{-1} NaCl or sea salt. In aquatic animals, movement of acid/base equivalents is generally coupled with movements of strong ions such as Na^+ and Cl^- (Cooper, 1994; Truchot, 1987). In aquatic insects in general, failure to survive in acidic water appears to be due to failure to regulate Na^+ rather than failure to regulate hemolymph pH. This is presumably due to sacrifice of Na^+ to maintain hemolymph pH through Na^+/H^+ exchange (Wiederholm, 1984; Havas, 1981). In mosquitoes, ion exchange mechanisms have been investigated primarily in terms of Na^+ and Cl^- uptake (Stobbart, 1971; Strange et al., 1982, 1984; Strange and Phillips, 1985; Patrick et al., 2002a,b). However, based on the coupling of Na^+/H^+ and of Cl^-/HCO_3^- transport, one might expect the range of tolerable pH to be greater in the presence of NaCl; no such influence of NaCl was detected.

There are no major trade-offs between pH regulation in acidic and alkaline conditions in these larvae. We find that acclimation to low and high limits of the pH range allows larvae to tolerate even more extreme pH levels with no loss of ability to survive at the other pH extreme. The ability to tolerate sudden changes in pH from one extreme to the other suggests that major qualitative rearrangements of transporter expression are not necessary when faced with either a highly acidic or alkaline environment, while acclimatory expansion of the pH range suggests that qualitative changes in capacity are possible. We therefore hypothesize that the mechanisms of acid and base excretion are physically separate and independent, as in the acid- and base-secreting cells of the mammalian kidney (Brown et al., 1992). The ability to cope with rapid changes in pH may be a consequence of having separate mechanisms for acid and base secretion, rather than an adaptation providing the capacity to tolerate sudden changes in pH. However, these data also suggest that there exist mechanisms that allow rapid adjustments of the activity of existing transporters upon changes in ambient pH.

Extremes of ambient pH have little effect on growth and development of larval mosquitoes, whereas species and sex exert strong influences. These species thus exhibit life history parameters that are remarkably robust in the face of an enormous range of ambient pH. The minor influence of pH on growth and development is rather surprising, especially considering that the effects of salinity on pupal mass and growth rates are much more dramatic (Clark et al., 2004). The minor influences of pH on pupal mass and growth suggest that either pH regulation is not metabolically expensive at extreme pH or that larvae can compensate by increasing dietary consumption and/or utilization of nutrients. One physiological attribute that may contribute to their pH tolerance is their method of gas exchange. Havens (1993) found that the three most acid-sensitive species of aquatic insects were those with the greatest surface area of external permeable structures. As air breathers, larval mosquitoes do not require intimate contact between highly permeable respiratory surfaces and the surrounding water.

Values obtained in the present study for hemolymph pH of *O. taeniorhynchus* are similar to those obtained by Giblin and Platzer (1984) in this species (7.62±0.14), and those obtained by Strange et al. (1982) for hemolymph of *Aedes dorsalis* reared at pH 10.5 under varying HCO_3^- concentrations. The data presented here extend these observations by demonstrating that both freshwater and euryhaline mosquitoes can maintain these hemolymph pH values across a wide range of ambient pH values (from 4 to 11 in these species). This remarkable pH regulatory ability is the key to the ability of mosquito larvae to tolerate such broad ranges of ambient pH. *Ochlerotatus taeniorhynchus* appears to be a somewhat more effective regulator than *A. aegypti*, as hemolymph pH of *O. taeniorhynchus* is constant across the entire pH range whereas that of *A. aegypti* changes by 0.1 pH unit as ambient pH increases from 7 to 11. *Ochlerotatus taeniorhynchus* thus has a hemolymph pH that is higher than that of *A. aegypti* at low and neutral pH. This change in hemolymph pH of *A. aegypti* with ambient pH is not associated with any noticeable difference in survival rates or changes in larval growth or development parameters, suggesting that it is not of great physiological significance.

In conclusion, this initial study into the physiology of pH tolerance in larval mosquitoes describes what must surely be one of the most pH-insensitive groups of animals in existence. Not only do larvae survive across seven orders of magnitude of ambient H^+ concentration, but instantaneous transfer from one extreme of the tolerable range to the other has little discernable effect on growth and development. Mosquito larvae are also unusual in that their acid tolerances in low Na^+ water are similar to those in 3.5 g l^{-1} NaCl.

The authors thank two anonymous reviewers for critical comments that improved the final version of this manuscript, and Dr C. R. Rutledge, Extension Medical Entomologist at the Florida Medical Entomology Laboratory, for efforts to obtain eggs. This work was supported by NRICGP no. 35302-8371 to T.M.C.

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