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THE PHYSIOLOGY OF SALINITY TOLERANCE IN LARVAE OF TWO SPECIES OF *CULEX* MOSQUITOES: THE ROLE OF COMPATIBLE SOLUTES

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Summary

We investigated the physiological basis for differences in salinity tolerance ranges in mosquito larvae of the genus *Culex*. We examined the response of larvae of *C. quinquefasciatus*, a freshwater obligate, and *C. tarsalis*, a euryhaline osmoconformer, following transfer from fresh water to 34 % sea water. Hemolymph Na^+ and Cl^- levels increased similarly in both species, indicating that ion regulation does not differ under these conditions. *C. quinquefasciatus* responded to increased environmental salinity with increased hemolymph levels of serine, but suffered a significant reduction in levels of trehalose. *C. tarsalis* responded to increased environmental salinity with increased hemolymph levels of both proline and trehalose. When *C. tarsalis* larvae were held in 64 % sea water, which *C. quinquefasciatus* larvae cannot tolerate, hemolymph proline and trehalose were accumulated approximately 50-fold and twofold, respectively, relative to freshwater values. We found that proline serves as both an intra- and extracellular compatible solute in *C. tarsalis*, the first such circumstance documented in an animal in response to increased environmental salinity. Analyses of the acute responses of the two species to an increase in salinity (from 30 % to 50 % sea water) indicate that larvae of *C. tarsalis* are able to volume-regulate *via* drinking and to attenuate increases in hemolymph NaCl concentration using unknown mechanisms during large, rapid increases in salinity.

Key words: mosquito, larva, *Culex tarsalis*, *Culex quinquefasciatus*, proline, trehalose, compatible solute, osmolyte, ion, osmoconformer, volume regulation, drinking.

Introduction

Hochachka and Somero (1984) stated: ‘It should be noted that, in multicellular animals, organic osmolytes may play relatively little role in osmotic balance of the extracellular fluids’. The reasoning underlying this idea is that much of the metabolic and biosynthetic machinery of multicellular organisms resides within the intracellular compartment. During a hyperosmotic stress, organic osmolytes, such as free amino acids, polyols and methylamines, are accumulated within the cells. At high concentrations, these solutes do not perturb enzyme activity, as would high levels of inorganic salts, and they are therefore called compatible solutes (for a review, see Somero and Yancey, 1997). By accumulating these compatible solutes, the functioning of intracellular enzymes is preserved despite increasing cellular osmolality. This pattern is demonstrated by marine invertebrates (e.g. copepods, lobster, molluscs) that accumulate high levels of organic osmolytes within the tissues but allow the hemolymph to conform to the chemical composition of their environment (Gilles, 1987).

Garrett and Bradley (1987), however, demonstrated that the osmoconforming larvae of the mosquito *Culex tarsalis* accumulate high levels of proline and trehalose in the hemolymph in response to increased environmental salinity. This finding is contrary to the above statement by Hochachka and Somero (1984) and suggests that, in certain multicellular organisms, organic osmolytes do serve a role in the extracellular milieu. Indeed, other genera of mosquitoes (*Culiseta*, *Deinocerites*, Bradley, 1994) and insect orders that contain euryhaline species (caddis larvae, *Limnephilus affinis*, Sutcliffe, 1961; hemiptera *Cenocorixa* sp., Scudder et al., 1972; chironomids *Chironomus* sp., dragonfly nymphs *Enallagma clausam*, Stobbart and Shaw, 1974) osmoconform while inhabiting saline environments. It remains to be determined which osmolytes are accumulated in the extracellular compartment by these other insects. A certain level of overlap is to be expected because the types of compatible solute utilized by both prokaryotes and eukaryotes are few, indicating a strongly convergent pattern in osmolyte systems (Somero and Yancey, 1997).

Bradley (1994) pointed out that only 5 % of all extant species within the family Culicidae (mosquitoes) are capable of surviving in salt water. Salinity tolerance has evolved at least five times independently in the Culicidae, but only two physiological strategies have been used. One of the two

strategies, osmoregulation, is utilized by the salt-tolerant larvae of *Aedes*. These osmoregulating mosquito larvae have evolved an additional rectal segment, which is the site of active transport of ions from the hemolymph, thus producing concentrated urine and maintaining a stable hemolymph osmolality over a wide range of salinities. Those species of *Aedes* that are not salt-tolerant do not possess an additional segment of rectum and, therefore, cannot eliminate excess ions (Bradley, 1994). However, in the genera possessing osmoconformers, the distinction between species that are salt-tolerant and those that are restricted to fresh water has not been adequately addressed. It has yet to be determined whether the ability to accumulate organic osmolytes is essential to the osmoconforming strategy in insects. It is conceivable that other physiological traits (ion regulation, osmotic permeability of cuticle) are also determinants of euryhalinity. The ion-regulatory organs (Malpighian tubules, anal papillae and rectum) of the osmoconforming *Culex tarsalis* (Bradley, 1994) and *Culiseta inornata* (Garrett and Bradley, 1984b) do not differ morphologically from those of freshwater forms of *Culex* and *Aedes*. This suggests that there may be little difference in ion-regulatory abilities and that euryhalinity *via* osmoconformation is dependent upon the ability to accumulate organic osmolytes. We have addressed this question in the present study.

We examined the acute and chronic responses to increased environmental salinity in the larvae of two *Culex* mosquito species, *Culex tarsalis*, a euryhaline osmoconformer, and *Culex quinquefasciatus*, a freshwater obligate species. In this paper, we present results that reveal the physiological determinants that contribute to the salinity tolerance ranges in these two species. In addition, we are the first to report a compatible solute that functions in both the intra- and extracellular compartments of an animal in response to an increase in environmental salinity.

Materials and methods

Experimental animals and holding conditions

Colonies of *Culex tarsalis* and *Culex quinquefasciatus* were established in our laboratory from colonies provided by Dr M. S. Mulla, Department of Entomology, University of California, Riverside, CA, USA. Mosquito larvae used in the propagation of the laboratory colonies were hatched and held in Irvine tapwater (1 % sea water, 10 mosmol kg^{-1}) in large rectangular plastic trays (32 cm×18 cm×9 cm) with the water changed each week. Larvae were fed rabbit chow pellets and dry yeast. Room temperature was 19–23 °C and the light:dark photoperiod was set at 12 h:12 h. Experiments were conducted on fourth-instar larvae although, in some cases, large third-instar larvae may have been included.

Determination of salinity tolerance

Ten larvae of each species were placed in individual plastic cups containing 40 ml of 10 % sea water (100 mosmol kg^{-1}) prepared using tapwater and Instant Ocean Salts (Aquarium Systems). A small amount of ground rabbit chow pellet was added to each cup. Eight replicate assays with *C. tarsalis* and six with *C. quinquefasciatus* were performed. After 2 days, dead larvae were counted and removed, and the salinity was increased by increments of 10 % sea water (100 mosmol kg^{-1}) using a concentrated seawater solution (Instant Ocean). The osmotic concentration of the medium was measured using a Wescor vapor pressure osmometer. This process was continued until all larvae were dead. Under our laboratory conditions, the salinity tolerance range (percentage mortality less than 20 %) was determined to be 0–40 % sea water for *C. quinquefasciatus* and 0–70 % sea water for *C. tarsalis*.

Chronic response to increased salinity

Batches of *C. tarsalis* and *C. quinquefasciatus* larvae were transferred to separate smaller trays (18 cm×13 cm×6 cm) containing 300 ml of 10 % sea water. The salinity of the experimental medium was increased by 10 % sea water every other day. For *C. quinquefasciatus*, the highest value used was 30 % sea water. *C. tarsalis* larvae were held in final salinities of 30 and 60 % sea water. For each species, separate control batches of larvae were maintained in tapwater, with water additions and hemolymph sampling occurring at the same time as for the experimental groups.

The osmolality of the two experimental media (30 % and 60 % sea water) was measured using a Wescor vapor pressure osmometer after the larvae had acclimated for 2 days and was found to be approximately 341±15 and 638±10 mosmol kg^{-1} (mean ± S.E.M., N=4) respectively. The approximate increase in osmolality of 40 mosmol kg^{-1} of the experimental media over the 2 days was attributed to evaporation. *C. tarsalis* larvae had therefore been acclimated to tapwater, 34 % or 64 % sea water, *C. quinquefasciatus* had been acclimated to tapwater or 34 % sea water. Larvae were removed from the acclimation trays, rinsed in distilled water and blotted dry on filterpaper disks. The larvae were exsanguinated on Parafilm, and the hemolymph was quickly collected using 0.5 µl microcapillary tubes (Drummond Microcaps). Hemolymph samples were then assayed for osmotic, ion, free amino acid and trehalose concentrations. To remove the Malpighian tubules, the larvae were immersed in mineral oil. Two incisions were made into the cuticle. The gut and Malpighian tubules were pulled away from the carcass. Pulled glass needles were then used to separate the Malpighian tubules from the gut and to transfer them to the appropriate sample tube. Intracellular fluid from the Malpighian tubules was assayed for free amino acid concentrations. Malpighian tubules were chosen because they can be easily dissected, they can be drained of the luminal contents and they lack extensive extracellular fluid space.

Analytical methods

Hemolymph osmolality was measured on freshly collected samples. Samples were quickly transferred from the microcapillary tube to the sample holder (filled with mineral oil) of a Clifton freezing point depression nanoliter osmometer.

The same osmometry standards (Wescor) were used to calibrate the Clifton and Wescor osmometers.

For cation analyses, 0.5 µl hemolymph samples were collected and diluted in 1 ml solutions of 2 g l^{-1} KCl for Na^+, 1.27 g l^{-1} CsCl for K^+, 10 g l^{-1} $LaCl_3$ for Ca^{2+}, and 10 g l^{-1} $LaCl_3$ plus 2 g l^{-1} NaCl for Mg^{2+} analysis. Sodium, potassium, magnesium and calcium hemolymph concentrations were then determined using atomic absorption spectrophotometry (Varian, AA275 series) (Garrett and Bradley, 1987). Hemolymph Cl^- concentrations were determined using a colorimetric assay. The assay is based on the liberation of thiocyanate ion from mercuric thiocyanate to form mercuric chloride. The thiocyanate then forms a quantity of highly colored ferric thiocyanate proportional to the chloride concentration (Gonzalez et al., 1998). Hemolymph samples (1 µl) were diluted with 1 ml of distilled water, and 100 µl of a 1:1 solution of mercuric thiocyanate:ferric nitrate was added. Samples and Cl^- standards were read spectrophotometrically at an absorbance of 480 nm.

Hemolymph trehalose concentrations were measured using a modification of the protocol of Parrou and François (1997) in which trehalose, a disaccharide, is hydrolyzed to its two glucose units whose concentration is assayed colorimetrically. Hemolymph samples (1 µl) were added to microcentrifuge tubes containing 50 µl of distilled water. The samples were then placed in boiling water for 1 min to destroy any substrates/enzymes that could interfere in the enzymatic hydrolysis of trehalose to two glucose molecules (next step). Once cooled, 50 µl of 0.8 mg ml^{-1} amyloglucosidase solution (*Rhizopus* mold; Sigma catalog no. A-7255) was added to each tube, mixed, and held at room temperature (approximately 23 °C) overnight. Trehalose standards were prepared and treated in the same manner as the hemolymph samples. Controls were prepared by omitting the addition of the amyloglucosidase and instead adding 50 µl of distilled water. The next day, the samples were assayed colorimetrically for glucose concentration (Sigma kit no. 510-A) at 450 nm.

Levels of free amino acids were assayed in both extracellular (hemolymph) and intracellular (lysed Malpighian tubules) fluid samples. A hemolymph sample of 0.5 µl was placed in a microcentrifuge tube containing 125 µl of 80 % ethanol. Isolated Malpighian tubules were lysed in 100 µl of 80 % ethanol. Both hemolymph and Malpighian tubule preparations were centrifuged for 5 min at 5000 ***g*** to remove hemocytes and cellular debris respectively. The supernatant was analyzed for free amino acids at the UCLA Protein Microsequencing Facility. A pre-column reaction with phenyl isothiocyanate (PITC) was performed on the samples. A Waters Pico-Tag system with a reverse-phase high-performance liquid chromatography (HPLC) column (Waters Novapak) and a sodium acetate/acetonitrile gradient were used to quantify levels of amino acids (Cohen and Strydom, 1988).

Acute response of drinking rates to increasing salinity

Drinking rates were measured by following the uptake of [^{14}C]inulin from the external medium (Bradley and Phillips, 1977b). In addition to measuring drinking rates following transfer to a higher salinity, rates were also determined in both species under non-transfer conditions in fresh water and 30 % sea water.

Initial tests were performed to determine the period of linear increase of inulin levels in larvae (Bradley and Phillips, 1975). Uptake was linear for the first 3 h. A period of 30 min was used for all subsequent measurements. Batches of both species were acclimated to either fresh water or 30 % sea water for 2 days. On the day of the experiment, 4–7 larvae were placed in small, cylindrical glass vials (15 mm×45 mm) with 1 ml of tapwater or 30 % sea water (plus a small amount of ground rabbit chow pellet) and allowed a 2 h acclimation period. Four to six replicate vials of larvae were used to measure drinking rates in freshwater medium, 30 % sea water and following transfer from 30 % sea water to either 30 % (control) or 50 % sea water (experimental) at 0.5, 1 and 2 h. The salinity transfer was initiated by the removal of the medium from each vial and replacement with 1 ml of the appropriate medium (30 % sea water for the control larvae, 50 % sea water for the experimental larvae). To initiate the drinking rate measurement, 0.5 ml of the medium was removed from each vial, and 1 ml of medium (either 30 or 50 % sea water) containing 4.44×10^5 Bq (12 µCi) of [^{14}C]inulin was added. This gave a final medium activity of 20×10^3 cts min^{-1} μl^{-1}. After 30 min, larvae were removed, rinsed and blotted dry. Larvae were then transferred to a glass vial containing 200 µl of 10 % KOH, macerated and incubated in a water bath at 90 °C for 1 h. Vials were then cooled and samples diluted with 800 µl of distilled water. Samples (400 µl) were then added to 5 ml of liquid scintillation cocktail (Universol ES, ICN) and counted on a Beckman scintillation counter.

Acute response of whole body mass to increasing salinity

Acute changes in body volume were measured in both *C. quinquefasciatus* and *C. tarsalis* following acute salinity transfer. Batches of larvae of both species were held in 30 % sea water for 2 days. The day prior to the experiment, individual larvae were placed in containers with 40 ml of 30 % sea water (plus ground rabbit chow pellet). Eight larvae from each species were used in the control and experimental transfer treatments. To measure body mass, larvae were rinsed and blotted on filterpaper disks. Larvae were then weighed individually on a Cahn electronic microbalance (model 29) and quickly returned to their holding container. Two weighing measurements were made prior to transfer treatment to establish that there was a negligible effect of weighing and handling on larval body volume. Larvae were then either transferred to the same 30 % seawater solution (to control for the transfer effect) or to a 50 % seawater solution. Body mass was measured at 15 min and 1, 2, 4 and 20 h following transfer. *C. tarsalis* were also weighed at 28 h after transfer. Percentage change in larval body mass was calculated using the mass values of two adjacent time periods.

Acute response of hemolymph [Na^+] to increasing salinity

Hemolymph Na^+ concentrations were measured in both *C. quinquefasciatus* and *C. tarsalis*. Batches of larvae of both species were held in 30 % sea water for 2 days. Hemolymph samples were taken from both species held at 30 % sea water and following acute transfer to 50 % sea water. Hemolymph was sampled 1 h prior to (N=4 samples) and at 15 min and 1, 2, 4 and 6 h after transfer (N=5 samples at each time). The sampling process and sodium determinations using atomic absorption spectrophotometry were the same as stated above.

All experiments were replicated on separate batches of larvae and on different days.

Statistical analyses

All data are reported as means ± S.E.M. Comparisons between groups were performed using analyses of variance (ANOVAs) (overall $P \leqslant 0.05$) with multiple comparisons (Scheffe's test) if ANOVAs proved significant. Two-way ANOVAs were performed on the body mass and drinking rate measurements in the acute salinity transfer experiments.

Results

Chronic responses to increases in salinity

The levels of the two predominant hemolymph ions, Na^+ and Cl^-, increased significantly (40 and 100 % respectively) when *C. quinquefasciatus* larvae were transferred from fresh water to 34 % sea water (P<0.0001) (Fig. 1A). Levels of other cations, which occurred in much lower concentrations, exhibited a slight increase (K^+, P<0.0175), a decrease (Mg^{2+}, P<0.0017) or no change (Ca^{2+}). *C. tarsalis* larvae exhibited increases in Na^+ and Cl^- relative to freshwater values (26 and 53 % respectively, P<0.0001), whereas there was no change in K^+ and Ca^{2+} levels and a slight but significant (P<0.0117) decrease in Mg^{2+} levels upon transfer to 34 % sea water. Na^+ and Cl^- levels were elevated further when *C. tarsalis* larvae were held at 64 % sea water (increases of 67 and 180 % relative to freshwater values, P<0.0001), there was no change in K^+ levels and both Mg^{2+} (P<0.0001) and Ca^{2+} levels (P<0.0024) dropped compared with freshwater values (Fig. 1B).

Levels of hemolymph amino acids were measured in *C. quinquefasciatus* larvae acclimated to fresh water or to 34 % sea water (Fig. 2A). With increasing external salinity, serine concentration increased significantly (P<0.0001) and to the greatest concentration (6 mmol l^{-1}) of all amino acids measured; however, this increase was not osmotically significant. The concentrations of the remaining extracellular amino acids showed statistically significant increases (Thr, Ala, Pro, P<0.01) or no change (Glu, Gly, His, Arg) in response to the external salinity change. Malpighian tubules provided an intracellular sample (Fig. 2B). Glutamate was the predominant intracellular amino acid in both fresh water and 34 % sea water (significant increase, P<0.027). Serine did increase to the second highest concentration (P<0.001) (Fig. 2B), which approximated the extracellular serine level of 6 mmol l^{-1} (Fig. 2A), but again the increase was not osmotically significant. The concentrations of the remaining amino acids showed slight but statistically significant increases (Ala, Pro, P<0.01) or no significant change (Gly, His, Arg, Thr) (Fig. 2B).

In *C. tarsalis* larvae transferred from fresh water to 34 % sea water, proline was the amino acid showing the greatest increase in concentration in both the extracellular (P<0.0001) (Fig. 3A) and intracellular (P<0.001) fluids (Fig. 3B). The increase was statistically but not osmotically significant. The concentrations of proline in the extra- and intracellular compartments were not significantly different (P<0.27). Hemolymph serine (P<0.006) and alanine (P<0.006) levels increased slightly but significantly, whereas glutamate (P<0.038) and arginine (P<0.014) levels decreased slightly but significantly. The remaining amino acids measured did not respond significantly to the increase in external salinity

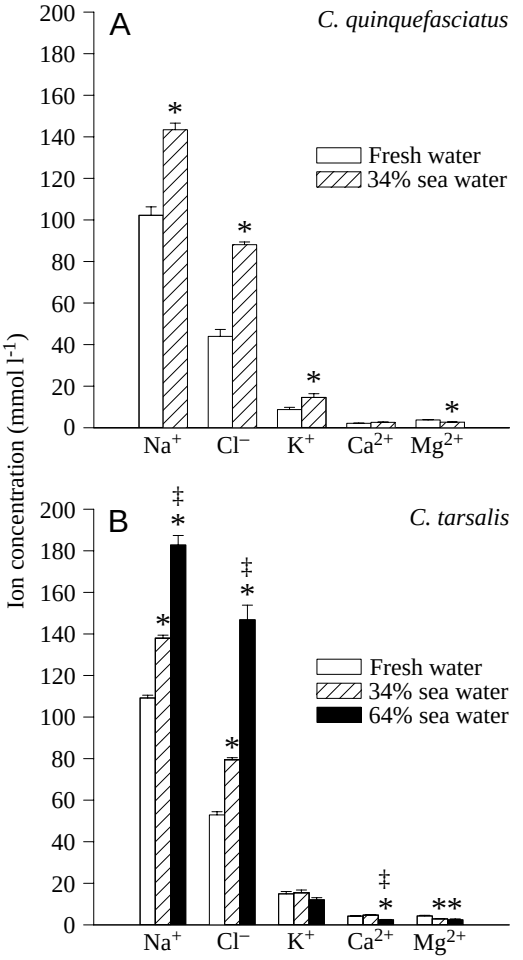

Fig. 1. Comparison of hemolymph ion concentrations (mmol l^{-1}) in larvae of (A) *Culex quinquefasciatus*, a freshwater obligate, and (B) *C. tarsalis*, a euryhaline osmoconformer. *C. tarsalis* were acclimated to fresh water and to 34 % and 64 % sea water (*C. quinquefasciatus* were acclimated to the first two media only). Values are means + S.E.M., N=5–14. An asterisk denotes a significant difference from the freshwater values ($P \leqslant 0.05$). ‡ denotes a significant difference between 34 % SW and 64 % SW values ($P \leqslant 0.05$).

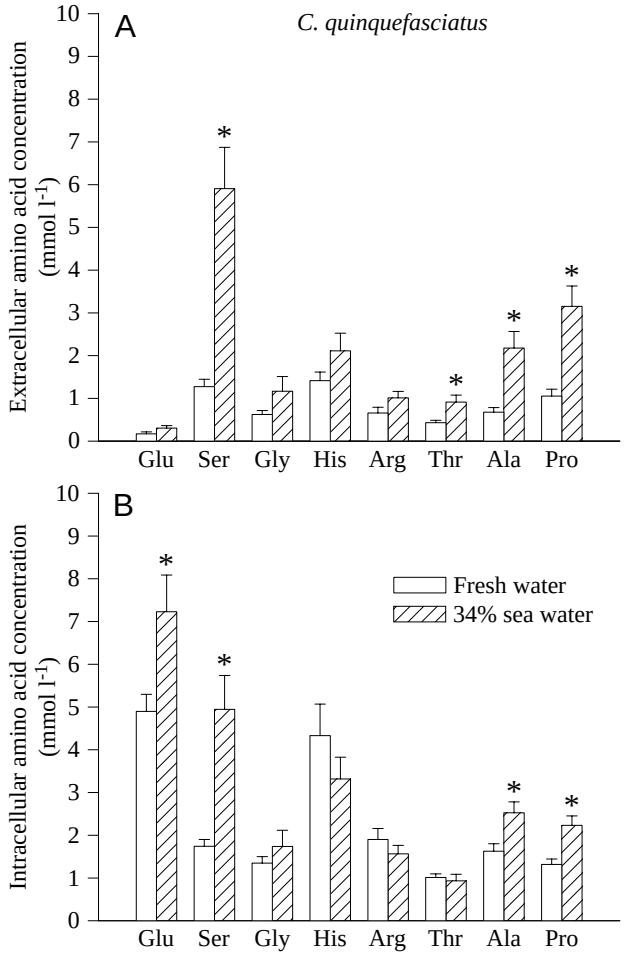

Fig. 2. Comparison of (A) extracellular and (B) intracellular amino acid concentrations (mmol l^{-1}) in larvae of *Culex quinquefasciatus*, a freshwater obligate, held in fresh water and 34 % sea water. Values are means + S.E.M., N=6–10. An asterisk denotes a significant difference from the freshwater values ($P \leqslant 0.05$).

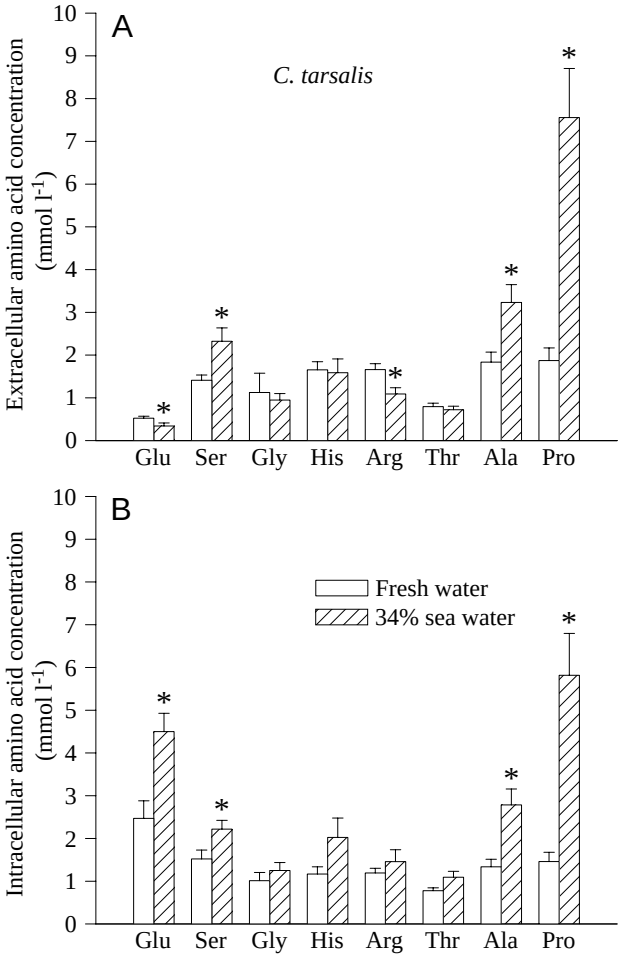

Fig. 3. Comparison of (A) extracellular and (B) intracellular amino acid concentrations (mmol l^{-1}) in larvae of *Culex tarsalis*, a euryhaline osmoconformer, held in fresh water and 34 % sea water. Values are means + S.E.M., N=6–10. An asterisk denotes a significant difference from the freshwater values ($P \leqslant 0.05$).

(Fig. 3A). With regard to the intracellular fluid of *C. tarsalis* larvae, glutamate showed the second highest increase in concentration (to approximately 4.5 mmol l^{-1}, P<0.005), followed by alanine (P<0.005) and serine (P<0.033). Concentrations of the remaining amino acids did not change significantly (Fig. 3B).

In *C. tarsalis* larvae acclimated to 64 % sea water, proline concentrations increased almost 50-fold in both the extra- (P<0.0001, Fig. 4A) and intracellular (P<0.002, Fig. 4B) fluids compared with values in freshwater-acclimated larvae. The proline values for both intra- and extracellular fluids were not significantly different. Relative to this osmotically significant increase, the concentrations of the remaining amino acids showed only slight increases (of less than 10 mmol l^{-1}) in both compartments (Fig. 4).

When held in fresh water, larvae of both species had hemolymph trehalose levels of approximately 20 mmol l^{-1} (Fig. 5). Upon transfer to 34 % sea water, *C. quinquefasciatus* experienced a statistically significant reduction (P<0.0001) in hemolymph trehalose concentration to 7 mmol l^{-1}, whereas *C. tarsalis* hemolymph concentrations of trehalose did not change. When *C. tarsalis* larvae were held for an additional day at 34 % sea water, hemolymph trehalose was accumulated to a significantly greater level (P<0.0006). In 64 % sea water, hemolymph trehalose concentration increased twofold from freshwater values (P<0.0001), and the increase was osmotically significant.

Hemolymph osmotic concentrations for *C. quinquefasciatus* in fresh water and 34 % seawater were 284±5.7 (N=9) and 345±13.4 mosmol kg^{-1} (N=5) respectively. Hemolymph osmotic concentration for *C. tarsalis* in fresh water, 34 % sea water and 64 % sea water were 310±1 mosmol kg^{-1} (N=22), 326±6.3 mosmol kg^{-1} (N=15) and 635±8.1 mosmol kg^{-1} (N=8) respectively. Hemolymph osmolality in *C. tarsalis* transferred from fresh water to 64 % sea water increased by 330 mosmol kg^{-1}. Increases in hemolymph ion concentrations (predominantly Na^+ and Cl^-) accounted for 49 % of this increase, whereas total free amino acid (predominantly proline)

and trehalose concentrations accounted for 25 % and 7 % respectively.

Acute response of drinking rate to increased salinity

Drinking rates in *C. tarsalis* and *C. quinquefasciatus* held in fresh water were not significantly different (Fig. 6). However, drinking rates for the two species became significantly different (P<0.047) when larvae were held in 30 % sea water. The drinking rate of *C. tarsalis* larvae increased by over 50 % from freshwater values (P<0.06), whereas *C. quinquefasciatus* experienced a slight decrease (25 %; P=0.046) in drinking rate. With regard to the control transfers (from 30 % to 30 % sea water), neither species showed a significant change over time; however, the drinking rates of *C. tarsalis* were almost twice the rates measured in *C. quinquefasciatus*. Upon transfer to 50 % sea water, both species exhibited significant yet contrasting departures from rates for the control transfer group

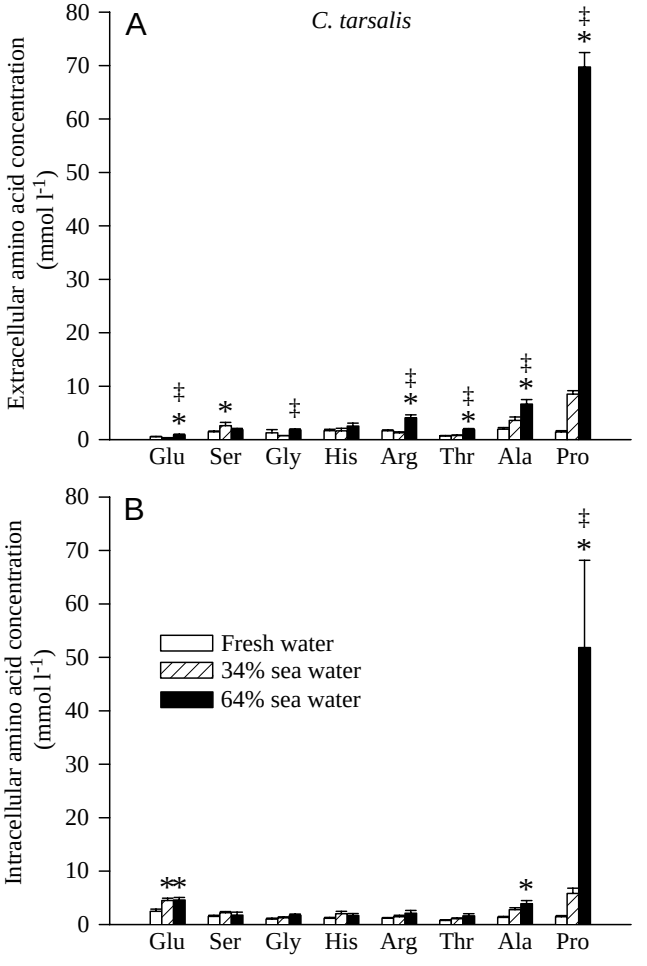

Fig. 4. Comparison of (A) extracellular and (B) intracellular amino acid concentrations (mmol l^{-1}) in larvae of *Culex tarsalis*, a euryhaline osmoconformer, held in fresh water and in 34 % and 64 % sea water. Values are means + S.E.M., N=6–12. An asterisk denotes a significant difference from freshwater values (P≤0.05). A double dagger (‡) denotes a significant difference from 34 % seawater values (P≤0.05).

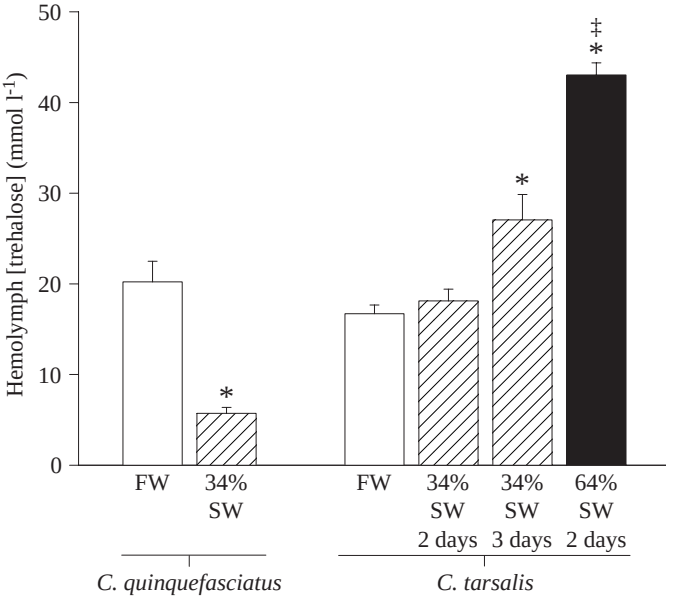

Fig. 5. Comparison of hemolymph trehalose concentrations (mmol l^{-1}) in the larvae of *Culex quinquefasciatus*, a freshwater obligate, and *C. tarsalis*, a euryhaline osmoconformer held in fresh water (FW) and in 34 % and 64 % sea water (SW) (*C. quinquefasciatus* was held in the first two media only). Note that an additional trehalose measurement was made on *C. tarsalis* larvae held for 3 days at 34 % sea water. Values are means + S.E.M., N=5–10. An asterisk denotes a significant difference from freshwater values (P≤0.05). A double dagger (‡) denotes a significant difference from all 34 % seawater values (P≤0.05).

(two-way ANOVA, *C. tarsalis* P<0.04, *C. quinquefasciatus* P<0.002). *C. tarsalis* larvae showed an elevation in drinking rate, which became significant (P<0.0018) at 1 h post-transfer. By 2 h post-transfer, drinking rate was not significantly different from control transfer values. *C. quinquefasciatus* larvae transferred to 50 % sea water exhibited a reduction in drinking rates that was significant by hour 2 (Fig. 6).

Acute response of whole body mass to increased salinity

The percentage change in larval body mass following an acute increase in salinity was used to indicate changes in body volume (Fig. 7). There was a significant difference in the pattern of body volume change between *C. tarsalis* larvae transferred from 30 % to 30 % sea water compared with those transferred from 30 % to 50 % sea water (two-way ANOVA, Scheffe's *post-hoc* P<0.008). Although both groups experienced slight increases in body volume by hour 28, only the 30 % to 30 % seawater group showed a significant increase from pre-transfer values (one-way ANOVA, Scheffe's *post-hoc* P<0.029). The two treatment groups of *C. quinquefasciatus* larvae exhibited a significant difference in the pattern of body volume change (two-way ANOVA, Scheffe's *post-hoc* P<0.004). *C. quinquefasciatus* larvae transferred to 50 % sea water experienced a 10 % reduction in body volume after 20 h in comparison with a 4 % increase in the control transfer group (Fig. 7).

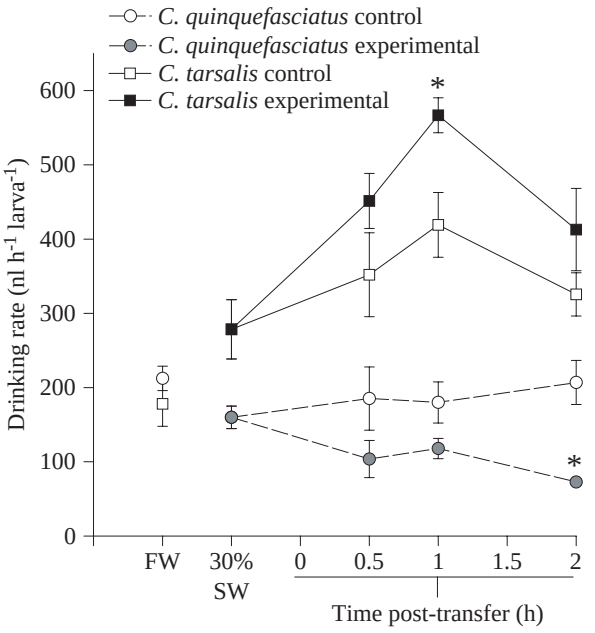

Fig. 6. Comparison of drinking rates (nl larva^{-1} h^{-1}) of larval *Culex quinquefasciatus*, a freshwater obligate (circles), and *C. tarsalis*, a euryhaline osmoconformer (squares), held in fresh water (FW) and in 30 % sea water (SW), and acutely transferred from 30 % sea water to either 30 % sea water (control) or 50 % sea water (experimental). Values are means ± S.E.M., N=4–7. An asterisk denotes a significant difference from pre-transfer, 30 % seawater values ($P \leqslant 0.05$).

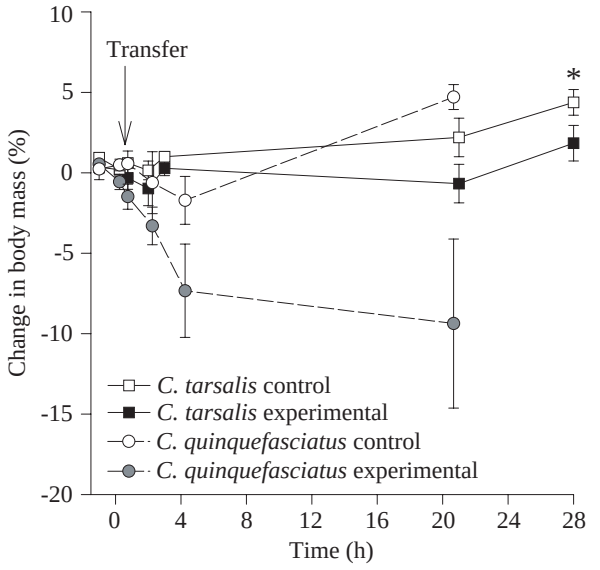

Fig. 7. Comparison of body volume changes (as measured by percentage change in body mass) of larval *Culex quinquefasciatus*, a freshwater obligate (circles), and *C. tarsalis*, a euryhaline osmoconformer (squares), held in 30 % sea water and acutely transferred to either 30 % sea water (control) or 50 % sea water (experimental). Values are means ± S.E.M., N=4–8. An asterisk denotes a significant difference from pre-transfer, 30 % seawater values ($P \leqslant 0.05$).

Acute response of hemolymph [Na^+] to increased salinity

C. quinquefasciatus hemolymph Na^+ levels prior to acute transfer from 30 % to 50 % sea water were slightly but significantly higher than those of *C. tarsalis* (P<0.006) (Fig. 8). Following transfer from 30 % to 50 % sea water, *C. tarsalis* hemolymph [Na^+] increased slightly, but this increase was not statistically significant until hour 4 (P<0.009). By 51 h, hemolymph Na^+ levels had decreased slightly and were not significantly different from pre-transfer values (P>0.1). *C. quinquefasciatus* larvae experienced an elevation in hemolymph [Na^+] that was significant by hour 2 (P<0.0001) and continued to increase thereafter. After 52 h post-transfer, only seven *C. quinquefasciatus* larvae were still alive, and hemolymph Na^+ concentrations were 250 mmol l^{-1} and significantly higher than pre-treatment values (P<0.0001, Fig. 8).

Discussion

This study is the first to document in insects, and to our knowledge in animals, a single compatible solute found in high concentration both intra- and extracellularly in response to increased environmental salinity. The only other case of intra- and extracellular solutes accumulated under high-salinity conditions is found in elasmobranchs. These organisms accumulate the nitrogenous waste product urea to raise the osmolality of their body fluids to equal that of their seawater environment. The protein-destabilizing effect of urea is counterbalanced by the accumulation of trimethylamine oxide (TMAO), a protein-stabilizing solute. This urea/TMAO counteracting system differs from the present finding because the accumulation of proline within the body fluids of *C. tarsalis* should not perturb protein function, as demonstrated in other organisms (Yancey and Somero, 1997).

When held at 34 % sea water, *C. tarsalis* and *C. quinquefasciatus* exhibited increases in levels of proline and serine, respectively, with no significant differences between the intra- and extracellular concentrations of these amino acids (Figs 2, 3). The accumulation of proline reached levels almost 50 times that of freshwater hemolymph values in both the hemolymph and tissues of *C. tarsalis* when the larvae were held in 64 % sea water (Fig. 4). In other organisms, the accumulation of free amino acids, in particular proline, has been reported to be only an intracellular event, a trait shared by a diversity of saline-tolerant organisms, including bacteria, algae, plants and marine invertebrates (Somero and Yancey, 1997). For example, during hyperosmotic shock, many euryhaline molluscs accumulate free amino acids such as proline, alanine and glycine within the tissues (Baginski and Pierce, 1977; Bishop et al., 1994), while the extracellular fluid is unregulated and conforms both in osmolality and ion composition to that of the marine environment (Burton, 1983). In contrast, *C. tarsalis* larvae hyporegulate hemolymph ion levels (Fig. 1B) while relying upon compatible solutes, one being proline, to raise the osmolality of both the tissues and the hemolymph (Fig. 4) to equal that of the saline environment.

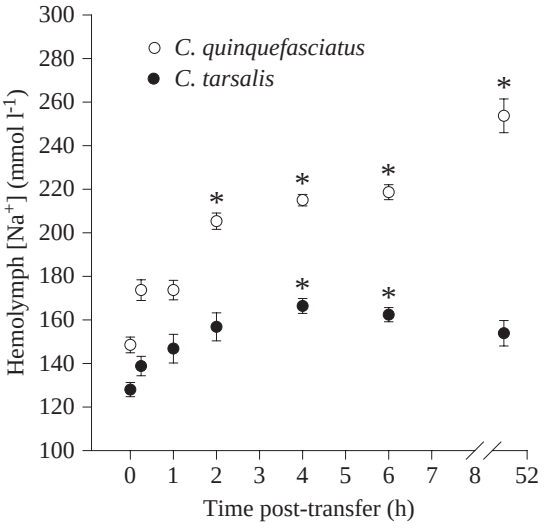

Fig. 8. Comparison of hemolymph Na^+ concentrations (mmol l^{-1}) of larval *Culex quinquefasciatus*, a freshwater obligate, and *C. tarsalis*, a euryhaline osmoconformer, held in 30 % sea water and acutely transferred to 50 % sea water. Values are means ± S.E.M., N=4–6. An asterisk denotes a significant difference from pre-transfer, 30 % seawater values (time 0 h) ($P \leqslant 0.05$).

Garrett and Bradley (1984a) point out that marine invertebrates often allow external ions to permeate the blood space and to change in concentration with the external medium, e.g. during tidally controlled salinity changes in estuaries. This strategy is not available to saline-tolerant organisms in inland environments where the ionic ratios, pH and total concentration of the waters can exhibit extreme changes. It would seem, therefore, that osmoconforming organisms inhabiting inland waters might employ both intra- and extracellular compatible osmolytes, a physiological strategy that has now been demonstrated for *C. tarsalis*.

The disparity in salinity tolerance ranges between the two species of mosquitoes is associated with differences in the manner in which the two organic osmolytes, proline (Figs 2–4) and trehalose (Fig. 5), are regulated in response to increased environmental salinity. The freshwater obligate *C. quinquefasciatus* is unable to accumulate either osmolyte at higher salinities, and this inability may contribute to its restricted tolerance range. Edwards (1982) reported that in the larva of *Aedes aegypti*, another mosquito restricted to water more dilute than its hemolymph (approximately 300 mosmol kg^{-1}), levels of hemolymph amino acids doubled but trehalose concentration remained unchanged when larvae were transferred from fresh water to 20 % sea water. It is not known whether this disparity can be attributed to phylogenetic or methodological differences. With regards to regulation of organic solute concentrations, it has been well documented that high levels of inorganic salts impair enzyme function in many biochemical pathways (Somero and Yancey, 1997). One example is high inorganic salt levels perturbing serine hydrolase function in the serine catabolic pathway (Burton, 1991a). It is plausible that the increased hemolymph NaCl (and potentially intracellular) concentrations (Fig. 1A) inhibited serine hydrolase in *C. quinquefasciatus*, thereby reducing serine degradation and increasing serine levels (Fig. 2). The significant decrease in hemolymph trehalose concentration experienced by the freshwater obligate held in 34 % sea water (Fig. 5) could also be a result of inorganic salts inhibiting trehalose synthesis or, more likely, causing stress, which leads to reduced trehalose accumulation.

In contrast, the osmoconforming *C. tarsalis* larvae are able to accumulate large amounts of proline (Fig. 4) and trehalose (Fig. 5) when held at a high salinity, indicating that the mechanism of regulation of these two osmolytes is different from that in the less tolerant species. Proline synthesis from glutamate has been found to be stimulated by salinity stress in the euryhaline copepod *Tigriopus californicu*s (Burton, 1991a) and in the osmotolerant bacterium *Bacillus subtilis* (Whatmore et al., 1990). This modulation by salinity has been explained by the ability of inorganic salts allosterically to block the negative feedback of proline on the first enzyme (γ-glutamyl kinase) in the synthesis pathway, thereby allowing proline synthesis to continue uninhibited (Kempf and Bremer, 1998). Proline accumulation is dependent upon protein synthesis, suggesting that this process involves the synthesis of new enzymes (Burton, 1991b; Whatmore et al., 1990). It is not known whether proline catabolic enzymes are downregulated under hyperosmotic conditions to avoid futile cycling. To understand fully how *C. tarsalis* accumulates proline in response to increased environmental salinity, these aspects of the regulation of proline synthesis and degradation will need to be examined. Some additional insights into this problem are presented in a companion paper (Patrick and Bradley, 2000).

Similar issues arise regarding the regulation of accumulation of extracellular trehalose in *C. tarsalis* (Fig. 5). The osmotic regulation of trehalose accumulation has been thoroughly examined in osmotolerant prokaryotes. Trehalase, the enzyme that hydrolyzes the disaccharide trehalose to two glucose molecules, was characterized in the halo-alkaliphilic bacterium *Ectothiorhodospira halochloris* and was found to be inhibited by high inorganic salt levels (Herzog et al., 1990). Trehalose synthesis in *Escherichia coli* is stimulated by osmotic stress at both the enzyme and the gene expression levels (Kempf and Bremer, 1998). It is not known whether similar mechanisms are functioning in osmoconforming eukaryotes such as *C. tarsalis* larvae.

Under similar salinity conditions (34 % sea water), both *C. quinquefasciatus* and *C. tarsalis* exhibit similar and substantial increases in hemolymph Na^+ and Cl^- levels (Fig. 1A,B), indicating that similar ion-regulatory mechanisms are operating in both species. Detailed mechanistic investigations of ion regulation in the genus *Culex* have not been conducted to date. However, a study of the larvae of another osmoconforming mosquito, *Culiseta inornata*, indicated that the Malpighian tubules and rectum are not involved in the elimination of excess Na^+ when larvae are held in a brackish medium (65 % sea water) (Garrett and Bradley, 1984a). This

finding contrasts with the well-studied ion-regulating properties of saline-tolerant mosquito larvae in the genus *Aedes* (Bradley and Phillips, 1977a,b,c; for a review, see Bradley, 1994), in which the rectum is responsible for the production of a hyperosmotic urine *via* active transport of Na^+, Cl^- and Mg^{2+}. In freshwater mosquito larvae (*Aedes* and *Culex*), the anal papillae actively absorb Na^+ and Cl^- from the external medium (Wigglesworth, 1933a,b; Stobbart, 1971). It has been suggested that the anal papillae could function in the active excretion of ions when larvae are held in a hyperosmotic medium (Garrett and Bradley, 1984a,b; Phillips and Meredith, 1969). In larvae of the genus *Culex*, it is possible that the anal papillae are the primary site for Na^+ and Cl^- regulation both in fresh water and at higher salinities.

When larvae of *C. tarsalis* are transferred to a higher salinity, there is a period when a gradient for water loss across the cuticle exists before the osmoconforming response is complete. This water loss could be replaced by increased ingestion of the medium. It is clear that larvae of *C. tarsalis* respond to increased salinity by increasing their drinking rate (Fig. 6). This increased intake of fluid presumably allows the larvae to maintain body volume (Fig. 7) in the face of the osmotic loss of water to a more concentrated medium. Larvae of *C. tarsalis* must excrete the increased ion load (Fig. 8) that this rapid drinking entails, but the site and mechanism of excretion have not yet been determined. Larvae of *C. quinquefasciatus*, in contrast, do not show a similar elevation in drinking rate but instead a significant decrease (Fig. 6) when placed in 50 % sea water, and they are then unable to control hemolymph ion levels. As a result, the larvae lose volume (Fig. 7) and show increases in hemolymph ion levels (Fig. 8), a combination that may contribute to the death of *C. quinquefasciatus* larvae in this medium.

These differences in the acute response (i.e. at 1 h, Fig. 6) are distinct; however, the reason for the almost twofold greater chronic drinking rate in *C. tarsalis* compared with that of *C. quinquefasciatus* is unknown. Interestingly, drinking rates in *Aedes taeniorhynchus* were estimated to be approximately $350\,nl\,larva^{-1}\,h^{-1}$ and did not vary with salinity, but were instead correlated with the size (surface area) of the larvae. On the basis of these findings, Bradley and Phillips (1977b) proposed that drinking rates were not determined by osmotic water loss across the cuticle but may be regulated by metabolic rate and, hence, nutrient demand.

The present study has examined the physiological basis for differences in salinity tolerance ranges in the larvae of mosquitoes of the genus *Culex*. *C. tarsalis* has extended its upper salinity limit by utilizing an osmoconforming strategy involving the accumulation of two compatible solutes, proline and trehalose, within the body fluids, with the former serving in addition as an intracellular osmotic effector. The biosynthetic and catabolic pathways for these two solutes must be osmotically regulated in the osmoconformer in a manner that departs from that of the freshwater obligate species. In addition, the dynamic response of *C. tarsalis* suggests that volume regulation *via* drinking and the ability to attenuate hemolymph NaCl levels contribute to their tolerance of large, acute salinity increases.

The finding that proline functions as an osmolyte in both the intra- and extracellular environment raises many physiological questions. For instance, what is the source of proline? Organisms can extract amino acids from the environment, can produce them by degradation of proteins or can produce amino acids *de novo*. Previous work on plants, marine invertebrates, bacteria and fungi has indicated that the glutamate-to-proline synthesis pathway is responsible for most of the proline accumulated intracellularly (Mahan and Csonka, 1983; Rushlow et al., 1984; Tomenchok and Brandriss, 1987; Burton, 1991a,b; Hare et al., 1998). Second, what is the site of proline synthesis during the osmoconformatory response of *C. tarsalis* larvae? The fat body has been shown to be the primary site of proline synthesis in many insects, although other tissues are capable of low levels of proline production (Chapman, 1998). The cells of the fat body, which form thin sheets in the hemocoel, are in intimate contact and share a large surface area with the hemolymph. It is conceivable that proline synthesis could be elevated in the fat body, with proline being distributed to the hemolymph and tissues. The Malpighian tubules of locusts have been shown to secrete proline actively from the hemolymph into the tubule lumen (Chamberlin and Phillips, 1982). It is not known whether this mechanism is present in the *Culex* mosquito larvae. It is conceivable that, during exposure to high salinity, the transport of proline from the hemolymph into the tubule cell increases and/or the transport step from Malpighian tubule cell to the lumen is inhibited, thereby allowing accumulation of proline. Finally, upon return to an environment of lower osmolality, what is the fate of proline? Goolish and Burton (1989) determined that, when euryhaline copepods (*Tigriopus californicus*) were transferred from full-strength to 50 % sea water, proline and alanine, rather than being excreted, were retained and either oxidized or incorporated into proteins. To understand how proline functions as a ubiquitous compatible solute in *C. tarsalis* larvae, it will be necessary in our future studies to examine the regulation of proline accumulation, both intra- and extracellularly, and the coupling of proline to metabolism.

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