J. exp. Biol. **113**, 133–141 (1984)
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THE PATTERN OF OSMOTIC REGULATION IN LARVAE OF THE MOSQUITO *CULISETA INORNATA*

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Accepted 29 March 1984

SUMMARY

1. Larvae of *Culiseta inornata* (Williston) can survive and complete development in dilutions of sea water ranging from 50–700 mosmol kg^{-1}. The larvae hyperregulate with regard to haemolymph osmotic concentration in dilute media (50–400 mosmol kg^{-1}) and osmoconform when external salinities exceed 400 mosmol kg^{-1}. This pattern of osmoregulation is distinct from that observed in freshwater and saline-water mosquito species. We propose that mosquitoes exhibiting this osmoregulatory pattern should be described as 'brackish-water' species.

2. Larvae of *Culiseta inornata* are able closely to regulate both sodium and chloride ion concentrations in the haemolymph over the full range of salinities tested (50–750 mosmol kg^{-1}).

3. The Malpighian tubules produce an isosmotic, potassium-rich fluid. *In vitro* and *in vivo* sampling of rectal fluids demonstrates that rectal secretions are isosmotic or only slightly hyperosmotic to the haemolymph and the surrounding saline media, and that they are isotonic with regard to sodium.

INTRODUCTION

The larvae of most species of mosquitoes are limited to a freshwater environment (O'Meara, 1976). The mechanism of osmoregulation in fresh water has been most extensively examined in *Aëdes aegypti*. Larvae of this species, which are unable to survive in environments more concentrated than 1·6 % NaCl (equivalent to 45 % sea water), are obligate hyperregulators (Wigglesworth, 1938). Osmotic homoeostasis is maintained by the elimination of excess water in the form of a dilute urine and uptake of ions by the midgut, rectum and the anal papillae (Wigglesworth, 1933*a*,*b*,*c*, 1938; Koch, 1938; reviewed by Stobbart & Shaw, 1974).

Larvae of some mosquito species are capable of surviving in both hypo- and hyperosmotic environments. Saline-water species of *Aëdes* and the closely related genus *Opifex* have now been extensively studied with regard to their mechanisms of osmoregulation (*Aëdes detritus*, Beadle, 1939; Ramsay, 1950; *Aëdes campestris*, Phillips & Meredith, 1969; *Aëdes taeniorhynchus*, Nayar & Sauerman, 1975; Bradley & Phillips, 1975, 1977*a*,*b*,*c*; *Aëdes togoi*, Asakura, 1980; *Opifex fuscus*, Nicolson, 1972). These species are hyperregulators in dilute media and hyporegulators in saline waters. Ultrastructural studies by Meredith & Phillips (1973) established that the

Key words: Osmoregulation, osmoconformer, mosquito larvae.

rectum of *A. campestris* contains two distinct regions. Ultrastructurally, the anterior region of the rectum resembles that of the rectum of the freshwater mosquito, *A. aegypti*, and is thought to serve a similar function in ion resorption and exchange. Meredith & Phillips (1973) noted that the cells of the posterior rectum were unlike those found in freshwater species of *Aëdes*. They proposed that the posterior rectum was the site of ion secretion. Bradley & Phillips (1975, 1977*a*,*b*,*c*) confirmed this hypothesis by demonstrating that the rectum secretes a hyperosmotic fluid rich in K^+, Na^+, Mg^{2+}, and Cl.

Several other genera of mosquitoes, e.g. *Anopheles, Aëdeomyia, Culex, Culiseta, Deinocerites* and *Psorophora*, possess larvae which can survive in saline water (O'Meara, 1976). No species in these genera have been examined with regard to their osmoregulatory mechanisms or capabilities in hyperosmotic media. We report here studies on larvae of the mosquito, *Culiseta inornata*, a species inhabiting brackish waters. The results demonstrate a pattern of osmoregulation distinct from that observed in any other species of mosquito.

MATERIALS AND METHODS

The colony of *Culiseta inornata* was maintained by Dr J. K. Nayar at the Florida Medical Entomological Laboratory, Vero Beach. Wild adult females from the vicinity of Vero Beach were collected and maintained in an 24 × 24 cm cage at a relative humidity of 70–80 %, temperature 27 °C, and 12: 12 light: dark photoperiod (Nayar, 1968; Nayar & Sauerman, 1970). Egg rafts or larvae were sent to Irvine and placed in 20 % sea water at 24 °C. The salinity of the medium was raised at a rate of 10 % sea water per day until the appropriate concentration was reached. All larvae remained a minimum of 3 days in the final salinity prior to examination. The various salinities were produced by the appropriate dilution of sea water (Instant Ocean, Aquarium Systems) with double distilled water (100 % sea water was set at 1000 mosmol kg^{-1}). Fourth instar larvae were used in all experiments. Some of the experiments were carried out on larvae of *Culiseta inornata* collected in California by Dr William Reisen of the Arbovirus Research Laboratory, Bakersfield, California. These larvae were identical with regard to all the physiological parameters examined to those from Florida.

In order to collect haemolymph, larvae were rinsed briefly in double distilled water, blotted on filter paper and transferred to Parafilm where their cuticle was torn open. The haemolymph which flowed onto the Parafilm was collected using a Drummond 1 μl pipette. The volume of the haemolymph collected was estimated from the length of the fluid column (Kaufman & Phillips, 1973). Secretion studies on Malpighian tubules were conducted using isolated tubules stimulated with 5-hydroxytryptamine (50 μM) in mosquito artificial haemolymph (Bradley & Phillips, 1975), as described by Maddrell (1969). The volume of secreted drops was determined by measuring the diameter of drops under oil. For examining rectal function, experimental animals were ligated just anterior to the rectum and at a point between the rectum and the anal portion (Bradley & Phillips, 1975). In this preparation, the tracheae leading to the rectum remain intact and functional. The external cuticle was torn slightly and the isolated preparation was floated at the surface of the artificial haemolymph for a period of 2 h with its siphon open to the air. During the experimental period, the rectum was

bathed by a fluid of known composition. Rectal fluid was collected from these recta by puncture using an oil-filled micropipette (Bradley & Phillips, 1975). In some experiments examining rectal secretion, the osmotic concentrations of the artificial haemolymph was increased using sucrose.

Measurements of the osmotic concentration of haemolymph, Malpighian tubule secretions and rectal fluid were performed using a freezing point nanolitre osmometer (Clifton, Rochester, NY). Ionic concentrations of Na^+ or K^+ in these fluids were measured using a Varian AA275 flame spectrophotometer in the absorption mode. Samples of known volumes were placed in either 0·5 or 1 ml of double distilled water, for Na^+ measurements; or double distilled water plus 1·27 mg ml^{-1} caesium chloride, for K^+ measurements. To measure Cl^-, four larval haemolymph samples were pooled in a Drummond 5 μl pipette, the fluid column measured, and the sample placed in a vial with 4 ml of acid solution (1 part nitric to 4 parts acetic acid) containing gelatin (Buchler). Electrometric titrations were conducted on a Buchler Digital Chloridometer using the low range setting.

RESULTS

Larvae of *Culiseta inornata* are able to survive in 0–75 % sea water. Growth rates are reduced with increasing salinities. Survival to pupation is also lowered in media

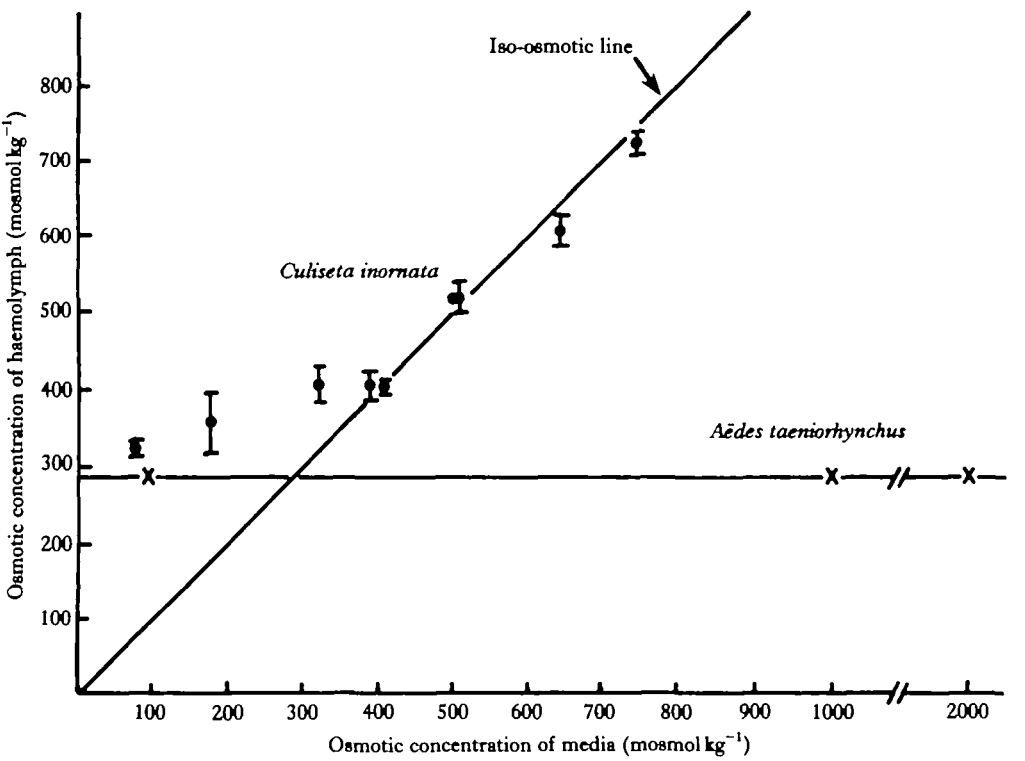

Fig. 1. The relationship between the haemolymph osmotic concentration and the osmotic concentration of the external medium (●) (mean ± S.D., $N > 8$). Measures of haemolymph concentrations for *Aëdes taeniorhynchus* under comparable conditions are also shown (×) (Phillips & Bradley, 1977).

above 60 % sea water. The LD_{50} for survival to pupae and, therefore, to adults, was approximately 65 % sea water. Larvae are able to survive for 24–48 h, in 80–100 % sea water but they cannot successfully pupate at these concentrations.

The relationship between the osmotic concentration of the haemolymph of *C. inornata* and the osmotic concentration of the medium is shown in Fig. 1. The larvae of *C. inornata* are hyperregulators in media less than or equal to 40 % sea water. In more concentrated media, the haemolymph is essentially isosmotic to the external medium, i.e. the larvae are osmoconformers. By comparison, the larvae of the saline water mosquito *Aëdes taeniorhynchus* are able to regulate their haemolymph osmotic concentration in both hypo- and hyperosmotic media (Fig. 1).

The relationships between the sodium and chloride concentrations of the haemolymph and of the medium are shown in Figs 2 and 3. The mean Na^+ concentration of the haemolymph lies between 90 and 160 mM over the entire range of salinities tested (Fig. 2). The mean chloride concentration in the haemolymph is approximately 60 mM in waters less concentrated than 40 % sea water, and approximately 100 mM in waters more concentrated than 40 % sea water (Fig. 3). Both ions are well regulated over a wide range of external concentrations.

Measurements were made of the concentration of K^+ in the haemolymph of larvae raised in 10 %, 18 %, 30 % and 42 % sea water. Within this range of salinities haemolymph K^+ concentrations were regulated, with a mean concentration of about 12 mM, well above the concentration in the external media.

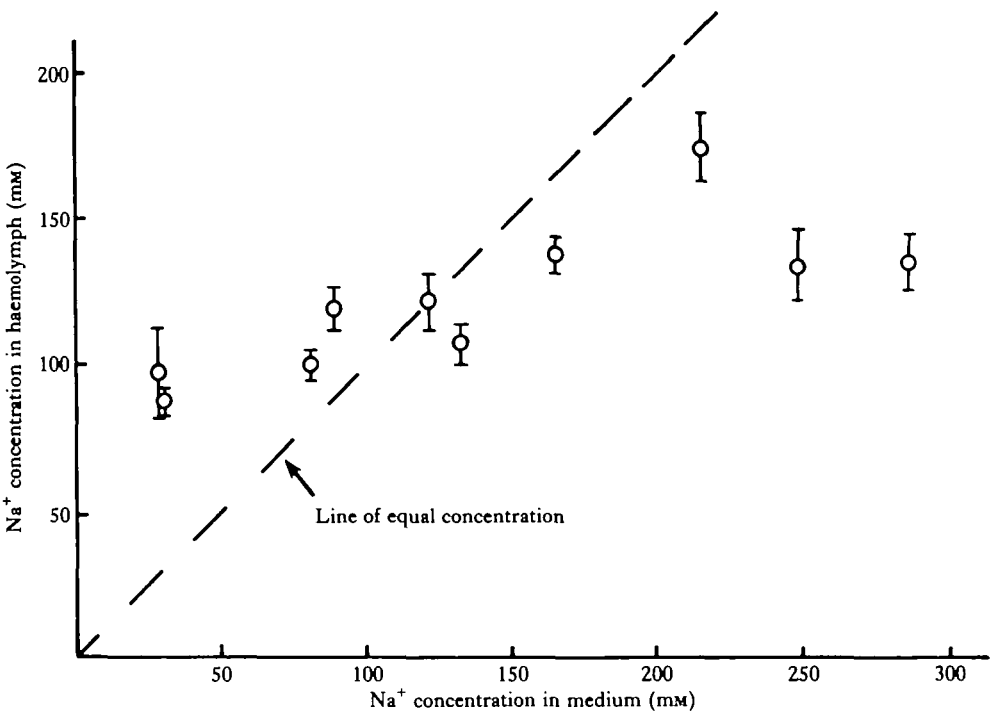

Fig. 2. Mean Na^+ concentration of the haemolymph of *Culiseta inornata versus* the Na^+ concentration of the medium (mM) (mean ± S.D., $N > 8$).

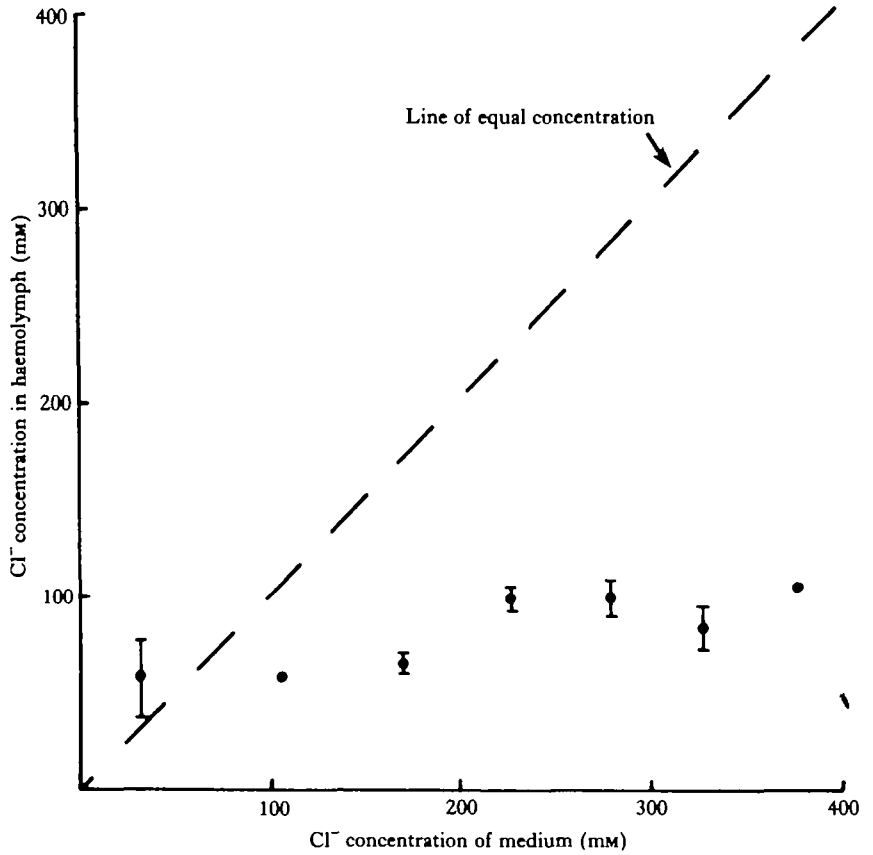

Fig. 3. The relationship between the mean Cl^- concentration of the haemolymph and the Cl^- of the medium (mM). Each point represents measurements of four samples each pooled from four larvae (mean ± S.D., $N = 4$).

Malpighian tubules

The osmotic concentration of fluid secreted by Malpighian tubules *in vitro* was examined. The secreted fluid was found to be richer in K^+ and lower in Na^+ than the bathing medium. Although the artificial haemolymph has a Na^+/K^+ ratio of approximately 11, the fluid secreted by the Malpighian tubules has a ratio of 1·1. Tubules were bathed in artificial haemolymph of varying osmotic concentrations (ranging from 350 to 550 mosmol kg^{-1}). Fluid secreted was always isosmotic to the bathing medium (±5 mosmol kg^{-1}).

Rectum

The osmotic concentration of rectal fluid in *C. inornata* was examined in order to elucidate the role of the rectum in ionic and osmotic balance in these larvae. Recta isolated by ligature were allowed to accumulate fluid in the lumen, while bathed in artificial haemolymphs with an osmotic concentration of either 350 or 635 mosmol kg^{-1}. The secreted fluid was not more than 50 mosmol kg^{-1} more concentrated than the artificial haemolymph (Table 1). The concentration of Na^+ in the rectal fluid was, however, less than the Na^+ concentration of the artificial haemolymph (150 mM). By

Table 1. *The mean osmotic and the mean Na^+ concentrations (N = 5) of the rectal secretion from ligated recta bathed in artificial haemolymph (*in vitro *preparation) or of rectal fluid sampled by micropipette through the anus of intact animals (*in vivo *preparation)*

Species	Bathing media (mosmol kg^{-1})	Rectal fluid (mosmol kg^{-1})	Rectal fluid $[Na^+]$ (mM)
Culiseta inornata			
in vitro	350	410 ± 24	—
	635	650 ± 10	133 ± 18
in vivo	387	386 ± 27	—
	650	649 ± 37	—
Aëdes taeniorhynchus			
in vitro	350	1000 ± 60	350 ± 45

Values for the osmotic and Na^+ concentrations of rectal fluid from larvae of *Aëdes taeniorhynchus*, under the same conditions, are also shown (Bradley & Phillips, 1977*b*, Fig. 7).
Rectal values are given as mean ± S.D.

comparison, the recta from larvae of *Aëdes taeniorhynchus* treated identically, secrete a fluid which is hyperosmotic to the bathing medium as well as hypertonic with regard to sodium concentration (Table 1). Cognizant of the limitations of the isolated rectal preparation (see Discussion), we also collected rectal fluid directly from the recta of intact larvae by inserting a micropipette through the anus. Samples collected in this manner were essentially isosmotic to the medium (Table 1).

DISCUSSION

The pattern of osmoregulation

Previous studies of osmoregulatory strategies in mosquito larvae have revealed two patterns of osmoregulation. Freshwater species are obligate hyperregulators, while saline-water species hyperregulate in dilute media and hyporegulate in hypersaline media.

In this study, we describe a new pattern of osmoregulation for larvae of the mosquito, *Culiseta inornata*. In dilute media, the larvae hyperregulate. In hypersaline media, the haemolymph is essentially isosmotic to the medium. Concentrations of Na^+ and Cl^- in the haemolymph are, however, regulated below those of the medium. Although the ability of larvae of *C. inornata* to tolerate saline water far exceeds that of freshwater mosquito species, they cannot survive for extended periods of time in waters more concentrated than 75 % sea water. In view of these differences observed between the osmoregulatory patterns of freshwater species, saline-water species, and that of *C. inornata* we propose the term 'brackish-water' species for mosquitoes possessing this pattern of regulation.

Although the osmoregulatory pattern of *C. inornata* is distinctly different from that described in any other mosquito species to date, similar patterns have been observed in aquatic larvae of the Tricopteran *Limnephilus affinus* (Sutcliffe, 1961) and the Hemipteran *Cenocorixida expleta* (Scudder, Jarial & Choy, 1972). Both of these insects, which can tolerate salinities up to 750 mosmol kg^{-1}, are osmoconformers and

can regulate their haemolymph Na^+, K^+ and Cl^- below that of the medium in hyperosmotic conditions. Interestingly, both these species belong to orders which have, in addition to strictly freshwater species, at least one saline-water species (Tricoptera: *Philanisus plebeius*, Leader, 1972, 1976; Hemiptera: *Trichocorixa verticalis interiores*, Tones & Hammer, 1975; reviewed by Scudder, 1976). The designations freshwater, brackish-water, and saline-water which we are proposing for mosquito larvae would seem to be applicable to other orders of insects as well.

In *C. inornata* the osmotic concentration of the haemolymph is essentially isosmotic to the environment at higher salinities, while sodium and chloride are regulated. As a result, the difference between the total osmotic pressure and the osmotic contribution of the major electrolytes gradually increases as the concentration of the medium is raised. In addition, a substantial anion deficit is observed in the haemolymph in all media. It is highly unlikely that magnesium, calcium or bicarbonate could account for this substantial increase in the osmotic activity of the haemolymph. Substantial portions of the osmotic activity are therefore presumably contributed by organic components.

Endopterygotes, including the Diptera, have high levels of organic acids and amino acids in the haemolymph (Florkin & Jeuniaux, 1974; Stobbart & Shaw, 1974). Florkin & Jeuniaux (1974) point out that the anion deficit observed in the haemolymph is associated with a high level of organic acids, particularly tricarboxylic acids, while the free amino acids have been proposed to make a net contribution to the cationic balance of the haemolymph. In *C. inornata*, therefore, it seems likely that organic substances, e.g. organic and amino acids, account for the increase in osmotic concentration (Yancy *et al.* 1982).

Physiological function of the osmoregulatory organs of Culiseta inornata

Ultrastructurally, the Malpighian tubules of *C. inornata* resemble those of other mosquito larvae (Bradley, Stuart & Satir, 1982; M. A. Garrett & T. J. Bradley, in preparation). Our physiological studies indicate that they are also physiologically quite similar in that they produce an isosmotic, K^+-rich fluid (Ramsay, 1951, 1953).

The rectum of *C. inornata* is composed of a homogeneous population of cells whose ultrastructure is consistent with a role in ion transport (M. A. Garrett & T. J. Bradley, in preparation). These rectal cells resemble those in the anterior portion of the rectum of *Aëdes campestris*. They are ultrastructurally quite distinct from the cells of the posterior region of the rectum of that species (Meredith & Phillips, 1973; Bradley & Phillips, 1975, 1977*a*,*b*,*c*). Based on these ultrastructural observations, it is suggested (M. A. Garrett & T. J. Bradley, in preparation) that the rectum of *C. inornata* does not function as a salt gland, such as is found in larvae of saline-water mosquitoes (Bradley & Phillips, 1975, 1977*c*).

In the present study, rectal secretions produced *in vitro* were only slightly hyperosmotic to the haemolymph. The Na^+ concentration of this rectal fluid is essentially isotonic to that of the haemolymph. Rectal fluid sampled *in vivo* yielded similar values for osmotic concentration. In studies with other brackish-water insects such as *L. affinus*, samples of rectal fluid taken under hyperosmotic conditions were also found to be relatively isosmotic or only slightly hyperosmotic to the haemolymph (Sutcliffe, 1961). Both rectal ultrastructure and *in vivo* and *in vitro* physiological results to date

suggest that the rectum in *C. inornata* is not the site of formation of a sodium-rich strongly hyperosmotic urine. A pattern of osmoconformity in hyperosmotic media is unavoidable if the larvae lack a site of hyperosmotic fluid secretion.

How then does *C. inornata* regulate its haemolymph Na^+ and Cl^- concentrations? We have shown that the Malpighian tubules produce an isosmotic secretion rich in K^+. These organs are therefore not a critical site of haemolymph Na^+ or Cl^- regulation. The rectum is also probably not the site of major ionic (certainly not Na^+) regulation for the reasons outlined above. Therefore, some additional site must be active in ion secretion. Studies on other mosquito species have implicated the anal papillae as sites of ion regulation. Ultrastructurally, these organs have the elaboration of apical and basal plasma membranes and numerous mitochondria which are characteristic of ion transport tissues (M. A. Garrett and T. J. Bradley, in preparation). The anal papillae of freshwater *Aëdes* and *Culex* species participate in ion uptake in dilute waters (Wigglesworth, 1933*a*,*b*,*c*, 1938; Koch, 1938). Phillips & Meredith (1969) have suggested that the anal papillae of the saline-water species, *Aëdes campestris*, may secrete ions against a concentration gradient. Studies by Asakura (1980) supported this suggestion, in that larvae of saline-water *Aëdes togoi* whose anal papillae had been removed showed elevated concentrations of ions in their haemolymph. It may be that the larvae of *Culiseta inornata* utilize their anal papillae for ion uptake in dilute media, and ion secretion in hypersaline media.

In summary, the larvae of *C. inornata* exhibit a pattern of osmoregulation previously undescribed in mosquitoes. The principal unique feature is that the larvae are osmoconformers in concentrated, brackish media. Unlike *Aëdes*, the rectum of *C. inornata* does not seem to be a major site of Na^+ regulation in concentrated media. A number of interesting questions remained unanswered. The osmotic concentration of the haemolymph rises substantially in concentrated media despite the fact that Na^+ and Cl^- are regulated. The osmotically active compounds responsible for this rise in haemolymph osmotic concentration deserve further study. The mechanism by which these high concentrations are maintained is also of interest, in view of the role of the Malpighian tubules in filtering the haemolymph. In addition, the sites of Na^+ and Cl^- regulation remain to be determined. It would be of particular interest to investigate the physiological function of the anal papillae.

We would like to thank Dr Jai Nayar for his consideration and the ready supply of *Culiseta inornata*. Dr William Reisen very kindly supplied us with *Culiseta inornata* from California. We are indebted to Juta Kiethe, Susan Fisher and Allison Oliva for their technical advice. This work was supported by California State Funds for Mosquito Research, Minigrant, NIH Grant AI 17736 and NSF PCM-8215420.

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