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OSMOREGULATION IN THE LARVA OF THE MARINE CADDIS FLY, *PHILANISUS PLEBEIUS* (WALK.) (TRICHOPTERA)

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INTRODUCTION

Few insects have successfully colonized the sea, and even fewer of these have been subjected to physiological analysis of their osmoregulatory capacity. Beadle (1939) demonstrated that the larva of *Aedes detritus* can tolerate external salt concentrations more than twice that of normal sea water, while Sutcliffe (1960) showed that the larvae of *Ephydra riparia*, *Cricotopus vitripennis* and *Coelopa frigida* were capable of withstanding a wide range of external salinities. The marine larva of *Chironomus salinarius* was investigated by Neumann (1961), while the regulation of the larva of *Ephydra cinerea* from the Great Salt Lake was studied by Nemenz (1960). Shaw & Stobbart (1963) distinguish certain common features in reports of the osmoregulation of these animals. Their haemolymph osmotic pressure and ionic concentration is not markedly different from that of related freshwater forms, but this can be maintained over a wide range of external salinities. The cuticle, however, is far less permeable to water and salts, and all are capable of elaborating an excretory fluid which is markedly hyperosmotic to the haemolymph. Regulation of the osmotic pressure of the haemolymph involves countering the small osmotic outflow of water across the cuticle, and this is achieved by drinking the medium and excreting a highly concentrated rectal fluid.

It is a necessary consequence of reduced permeability to water that the cuticle shall also be less permeable to oxygen, since no insect has evolved a membrane that is permeable to oxygen but impermeable to water (Hinton, 1953).

For those insects which possess spiracles and which are able to gain access to atmospheric air for their supply of oxygen, there appears to be no functional limit upon the degree of impermeability of the cuticle which can be tolerated. This is not the case for insects which depend upon diffusion of dissolved oxygen through the cuticle.

The euryhaline caddis larva *Limnephilus affinis*, which can tolerate salinities of up to 75% sea water, and which does rely on uptake of dissolved oxygen through tracheal gills, has been the subject of an intensive study by Sutcliffe (1961*a*, *b*). In marked contrast to the dipterous larvae quoted above, *Limnephilus* does not show a marked capacity to maintain the osmotic pressure of its haemolymph at a constant level. The cuticle is of the same order of permeability to water as in typical freshwater caddis larvae, and the ability of this larva to live in media of high salinity appears to be attributable to the capacity of the body tissues to withstand a high ionic concentration

Table 1. *Composition (mM) of the media used*

	Na	K	Mg	Ca	Li	Cho	Cl	SO_4	HCO_3	BeS
Artificial SW	468·5	10	54	10	—	—	548	28	2·5	—
Cho SW	234·25	10	54	10	—	234·25	548	28	2·5	—
BeS SW	468·5	10	54	10	—	—	274	28	2·5	274
Li SW	—	10	54	10	468·5	—	548	28	2·5	—
K SW	—	478·5	54	10	—	—	548	28	2·5	—

Abbreviations: SW, sea water, Cho, choline chloride; BeS, benzenesulphonate.

in the haemolymph. This tolerance breaks down, however, in salinities above 75% sea water, and the larva lives then only a short time.

In marked contrast to other caddis larvae, the larva of *Philanisus plebeius* occurs normally in littoral rock pools near low water, and also on subtidal coralline tufts, on the shores of New Zealand and Australia (MacLachlan, 1882; Hudson, 1904). It appeared likely that the osmoregulatory physiology might be qualitatively different from that of *Limnephilus*. The aim of the experiments reported in the present work was to investigate the mechanisms underlying the ability of this insect to exist in a saline environment.

METHODS

Larvae of *Philanisus plebeius* were collected at low tide from rock pools on Takapuna Beach, North Auckland, or from Leigh, 60 miles north of Auckland. They were kept in the laboratory in tanks of sea water, given fresh coralline regularly, and large larvae were selected for experimentation. Larvae were generally used within a week of collection. When used for experiments, larvae were placed, individually, in small glass tubes. They were not allowed access to food during the course of the experiment. In early experiments larvae were removed from their cases for experiments; but later, larvae were allowed to remain in their cases when possible, as it was found that without their cases larvae lost weight rapidly, although this did not affect their capacity for osmotic regulation. All experiments were carried out at room temperature, 20 ± 2 °C.

Media

In the first series of experiments, to test the osmoregulatory capacity of the animal in various salinities, the media were made up by diluting fresh locally obtained sea water with distilled water, or allowing it to become concentrated by evaporation. Later, artificial sea water was used (Smith, 1969*a*). For convenience the practice of Sutcliffe (1961*a*) has been followed throughout this paper in referring to sea-water concentrations in terms of equivalent concentrations of sodium chloride. For measuring the effect of changed external ionic concentrations upon the electrical potential difference across the body wall, the solutions whose composition is shown in Table 1 were made up. Choline chloride was washed with amyl alcohol and ether before use to remove impurities, while sodium benzenesulphonate was prepared by recrystallization from an alcohol/water solution.

Body weights of larvae were determined to the nearest 0·01 mg on a Sartorius balance (model 2405). The weight of the larvae used in the experiments was generally from 5–8 mg, although in summer larger larvae, weighing up to 10 mg, were occasionally found.

When it was necessary to prevent the larvae from either drinking or excreting, a ligature was applied round the terminal abdominal segment or behind the head. It was not found possible to seal the mouth with wax as the larvae have powerful jaws and can lever the wax off.

Analysis of body fluids was carried out on larvae which had remained in an experimental medium for at least 5 days. Preliminary experiments showed that larvae reached equilibrium within 48 h. Some analyses were carried out on the blood of larvae which had been in their experimental medium for less time than this; these were larvae which had been placed in extremes of salinity and which appeared moribund.

For removal of haemolymph the larva was removed from its tube, washed rapidly with distilled water, and blotted with soft tissue to remove surface moisture. It was then placed under liquid paraffin in a watch glass, and held with forceps while the body was pierced with a fine tungsten needle. Haemolymph generally flowed out and could be picked up in a Pyrex capillary. Generally it was possible to collect between 1 and 1·5 μl of haemolymph from each larva, although sometimes this quantity could only be obtained by gentle squeezing of the larva, particularly in experiments conducted at the environmental extremes. Occasionally larvae from high and low salinities were found to have a very fragile fat body, which disintegrated on manipulation of the larva, giving samples which could be seen to be full of fat globules. Such samples were discarded. Wherever possible samples were analysed immediately, on other occasions they were stored under liquid paraffin in a deep freeze at -40 °C.

Samples of midgut fluid were obtained either by a similar technique, in which the larvae were induced to regurgitate midgut fluid by gentle squeezing of the body wall, or by dissecting out the gut, carefully blotting it, and opening it under paraffin. An initial group of animals was used to compare samples obtained by both methods from the same animal, and the results were found to be quite comparable.

Rectal fluid was obtained either by squeezing the animal in the anal region or by introducing a fine capillary into the rectum. It was generally possible to collect only a small sample by this method, about 0·1 μl, which was adequate for duplicate determinations of freezing point. Only clear samples were used for measurement.

Osmotic pressure was determined by the micro-cryoscopic method of Ramsay & Brown (1955). Temperatures were read to the nearest 0·005 °C. Duplicate measurements were made on each sample and the results expressed in terms of sodium chloride solutions with equivalent freezing-point depressions. A series of solutions of sodium chloride were tested and the relationship Δ/171 mM sodium chloride = 0·610 °C was found empirically, which is in close agreement with that given by Sutcliffe (1961*a*).

Sodium ion concentrations in the haemolymph and rectal fluid were estimated using a flame photometer (Evans Electroselenium Ltd). 1 μl samples were added to 2 ml distilled water in small glass beakers and compared with standards. It was not possible to prepare duplicate samples for body fluid analyses but duplicate samples of standards were accurate to ± 2 mM sodium chloride/l. Sodium content of the media was also determined using appropriate dilutions.

Chloride ion concentrations of the haemolymph were determined using a modification of the potentiometric method of Ramsay, Brown & Croghan (1955), in which the titration vessel was a conical depression in a perspex block. The sample was stirred by a transversely directed current of compressed air. Using 0·01 N silver nitrate, in

a Scholander micro-burette driven by an Agla micrometer syringe, as titrant, the results were accurate to ±3 m-equiv/l.

Drinking of the media was measured using amaranth (Treherne, 1957). Solutions fo 100% and 40% sea water were made up containing 0·01 M amaranth. Larvae acclimatized to these solutions were immersed in the medium for a known time, either 8 or 12 h. This was sufficient to fill the gut with a measurable amount of amaranth, but was too short to allow loss of amaranth through the rectum. At the end of the experimental period the larvae were removed, rapidly ligatured behind the head with fine silk to prevent regurgitation of gut fluid and the gut was then dissected out. It was immediately transferred to 2 ml distilled water in a glass homogenizer (Kontes) and ground up to liberate the amaranth. After light centrifugation to remove gross particulate matter, absorbance of the solution was measured in a Beckman DB spectrophotometer at 525 nm. A series of standards was made up using 1 to 10 μl of medium dissolved in 2 ml distilled water, and the volume drunk, accurate to 0·02 μl, was read off the standard curve.

The rate of exchange of body water in a sea-water medium was measured by two methods. In the first method a 20% solution of deuterium oxide was used as a tracer. Two groups of larvae, one ligatured at neck and anus to prevent drinking or excretion, were placed in the solution, and five from each group were removed at suitable intervals. Samples of blood were removed, distilled in fine capillaries and the elevation of freezing point of the distillate measured (Shaw, 1955; Staddon, 1966). In the second method tritiated water was used as a tracer. Larvae, ligatured and unligatured, were immersed in a sea-water solution containing tritiated water at an activity of 10 μc/ml. After removal and careful washing in distilled water, a sample of blood was removed and placed in 10 ml scintillant fluid (3% PPO, 0·3% dimethyl POPOP in toluene). Activity was determined in a Packard Tricarb Scintillation Counter programmed for tritium counting, with automatic background correction, and with automatic channels ratio printout to enable correction for differential quenching. Samples of the medium were also counted.

Exchange of sodium in sea water was determined using ^{22}Na. This isotope was obtained from Amersham as [^{22}Na]chloride at high specific activity and carrier free. A solution of artificial sea water was made up containing ^{22}Na at an activity of 20 μc/ml; 10 μc/mM. After a period of immersion in this medium, larvae were removed, washed, and a blood sample was added to 2 ml distilled water in a test tube. Activity in the samples was determined using a well-type Solid Scintillation Spectrometer (Baird Atomic 530). To measure the rate of washout of sodium from animals in sea water, larvae were loaded with ^{22}Na by placing them in the radioactive medium for 5–7 days. Some were then ligatured at mouth and anus, and each larva was placed in a small perspex cell within the well of the scintillation counter, and washed with an unlabelled sea-water solution which flowed through the cell at a rate of 1 ml/min. Activity was monitored continuously in 10 sec counts and recorded on a Heathkit pen-recorder. In this way the decay of radioactivity with time was recorded continuously. Counting efficiency of the cells was determined by counting the activity of a sample of medium under similar geometric conditions.

The potential difference across the body wall of the animal was measured by means of microelectrodes. An animal was ligatured at both ends and held with Plasticine in

a small perspex cell through which fluid could flow under gravity. Micro-electrodes were made from thin-walled Pyrex tubing of 2 mm bore, using a Palmer microelectrode puller, and filled with 3 M potassium chloride under vacuum. Suitable microelectrodes had a resistance of between 5 and 10 MΩ, and before use were tested both in sea water and 10% sea water. Those with ion selective tips were discarded. One microelectrode was inserted into the bath and the other was inserted into the haemocoel of the animal through an intersegmental membrane. The two microelectrodes were connected via agar/potassium chloride bridges to a pair of calomel half-cells, and the potential between them was measured on a Keithley Electrometer (602 C). The output from the electrometer was recorded on a Heathkit pen-recorder, while the animal was bathed in a series of media (Table 1). The potential generally reached a steady value within 2–3 min, and after each test solution the animal was bathed with sea water until the original potential was restored. The potential between the two electrodes was measured before and after each experiment and the mean value was subtracted from the measured difference.

RESULTS

Haemolymph osmotic pressure

Initial experiments showed that larvae were capable of living for at least 10 days in media of salinity from 90 to 900 NaCl/l. If given access to fresh food they could complete development in all salinities from 250–750 mM NaCl/l. Beyond these extremes, however, the larvae retreated into their cases for much of the time and eventually died.

The mean osmotic pressure of the haemolymph of the larvae from sea water (606 mM-NaCl/l) is 202 ± 19 mM-NaCl/l. This is high for a trichopteran larva; Sutcliffe (1962*b*) gives values of 106–134 mM-NaCl/l for various larvae. It is, however, well within the range of published values for insect haemolymph (Florkin & Jeuniaux, 1964). Over a wide range of external salinities the value of the osmotic pressure of the blood changes only slightly (Fig. 1), rising from a mean of 160 mM-NaCl/l in a medium of 60 mM-NaCl/l to 220 mM-NaCl/l in a medium of 900 mM-NaCl/l. Samples of haemolymph taken from larvae kept in sea water of salinity beyond these extremes showed that the osmotic pressure of the blood changed rapidly and the animals died in a short time. Thus it appears likely that the larvae are not capable of tolerating large departures from the normal range of haemolymph osmotic pressures. In this respect it differs markedly from the larva of *Limnephilus affinis* (Sutcliffe, 1961*a*), which can tolerate a 3·5-fold change in blood osmotic pressure over its survival range.

Haemolymph sodium and chloride

In normal sea water (606 mM-NaCl/l) sodium ions (166 ± 23 m-equiv/l) account for most of the cation contribution to the haemolymph osmotic pressure, and the concentration of this ion is strongly regulated over the whole survival range (Fig. 2). In salinities above 900 mM-NaCl/l the ability to regulate sodium ion concentration breaks down and the concentration of this ion in the blood rises rapidly. The blood of three larvae which had been placed in a salinity equivalent to 1 M-NaCl/l, and which was analysed after 48 h, when the larvae appeared to be moribund, gave haemolymph

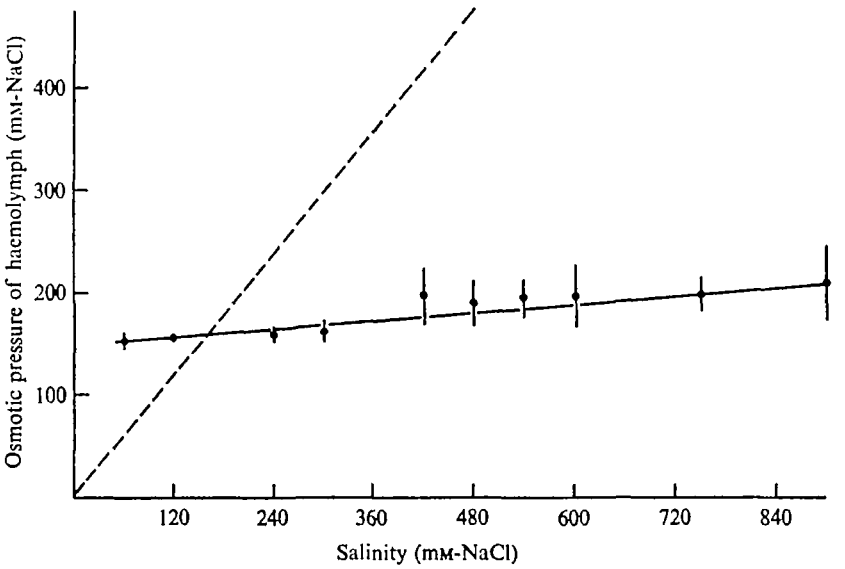

Fig. 1. The relation between the salinity of the external medium and the osmotic pressure of the haemolymph of the larvae of *Philanisus plebeius*. Each point represents the mean of haemolymph samples from 6 to 18 larvae.

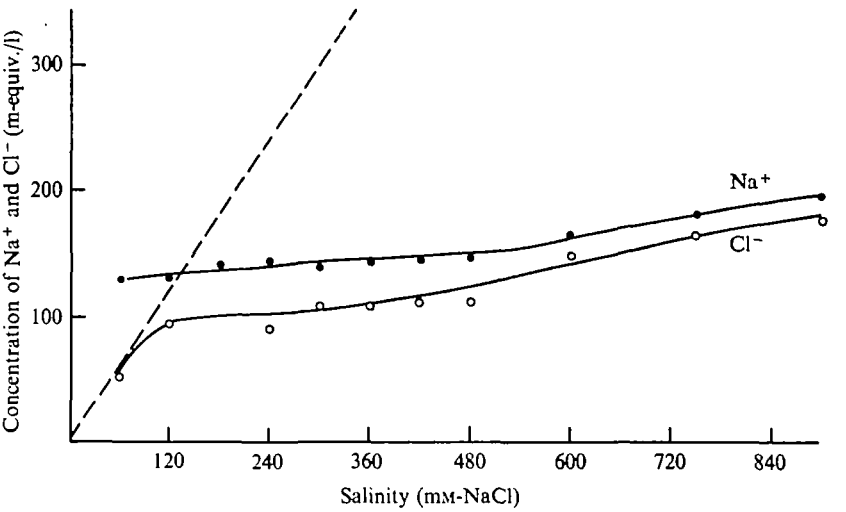

Fig. 2. The relation between the salinity of the external medium and the concentration of sodium and chloride ions in the haemolymph of the larvae of *Philanisus plebeius*. ●, Sodium concentration; ○, chloride concentration. Each point represents the mean of values of 5–16 larvae.

sodium concentrations of 650, 595 and 713 m-equiv/l respectively. Similarly, at low external concentrations the level of blood sodium falls rapidly.

The level of chloride ions in the blood is likewise regulated strongly over a wide range of external salinities. In normal sea water (606 mM-NaCl/l) the value of haemolymph chloride was 144 m-equiv/l (N = 15, S.D. ±16 m-equiv/l). In 50% sea water the chloride concentration falls to 110 m-equiv/l but further reduction of external salinities to 120 mM-NaCl/l led to a reduction of blood chloride level by only a further

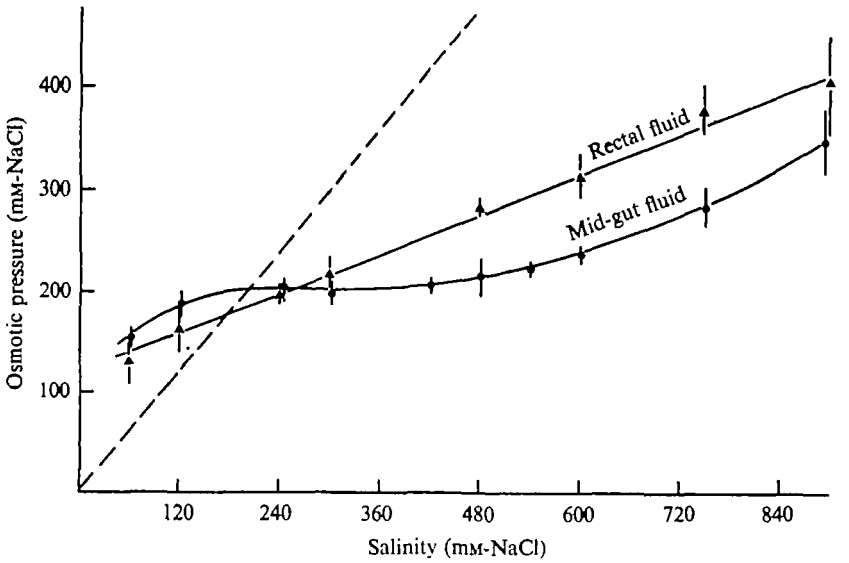

Fig. 3. The relation between the salinity of the external medium and the osmotic pressure of the midgut fluid and the rectal fluid of the larvae of *Philanisus plebeius*. ●, Midgut fluid; ▲, rectal fluid. Each point represents the mean of five or more determinations.

20 m-equiv/l, to 90 m-equiv/l. In salinities between this and 60 mM-NaCl/l the concentration of chlorides in the blood was the same as in the external medium. This suggests that the larva is incapable of concentrating chloride ions, a feature in which it is completely unlike any freshwater insect. However, in these low salinities it is capable of surviving for some time, and the blood sodium and the total osmotic pressure are maintained at a high level. It seems likely therefore that in low salinities the anionic deficit may be made up by the liberation of organic substances into the blood, a phenomenon reported for numerous freshwater insects at low external chloride concentrations – for example, *Aedes aegypti* (Wigglesworth, 1938) and *Sialis lutaria* (Beadle & Shaw, 1950).

At high salinities the rise of chloride concentration of the blood parallels that of sodium and of total osmotic pressure.

The osmotic pressure of the midgut and rectal fluids

The osmotic pressure of the rectal and midgut fluids from larvae adapted to a range of external salinities is shown in Fig. 3. The rectal fluid bears an approximately linear relationship to the external salinity, being markedly hyperosmotic to the blood at salinities above 120 mM-NaCl/l and hypo-osmotic to the blood in media more dilute than this. At all external salinities, however, the rectal fluid can play no more than a partial role in regulation, for though in high salinities it is considerably more concentrated than the haemolymph, it is still considerably hypo-osmotic to the medium.

The chloride concentration of the rectal fluid is shown in Fig. 4. Over most of the range studied the values found parallel those of the total osmotic pressure. In media below 100 mM-NaCl/l, however, the chloride values were found to fall away more steeply than the total osmotic pressure, and the mean value was slightly lower than the external chloride concentration. When the chloride concentration of the rectal

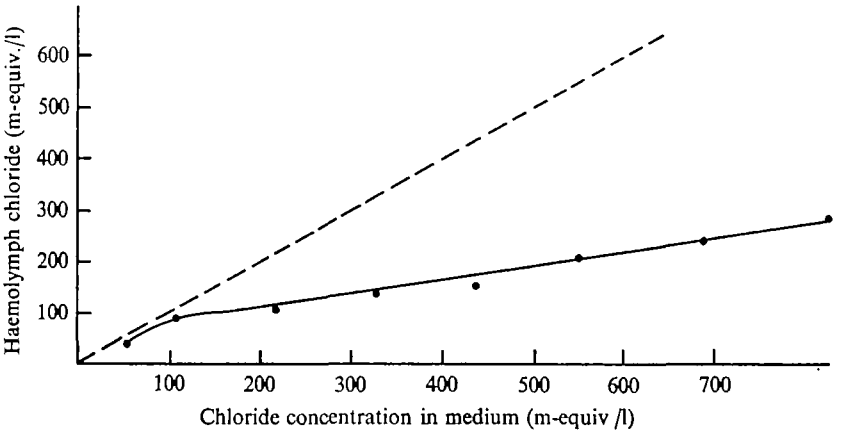

Fig. 4. The relation between the concentration of chloride ions in the medium and in the rectal fluid of the larvae of *Philanisus plebeius*. Each point is the mean of at least five determinations.

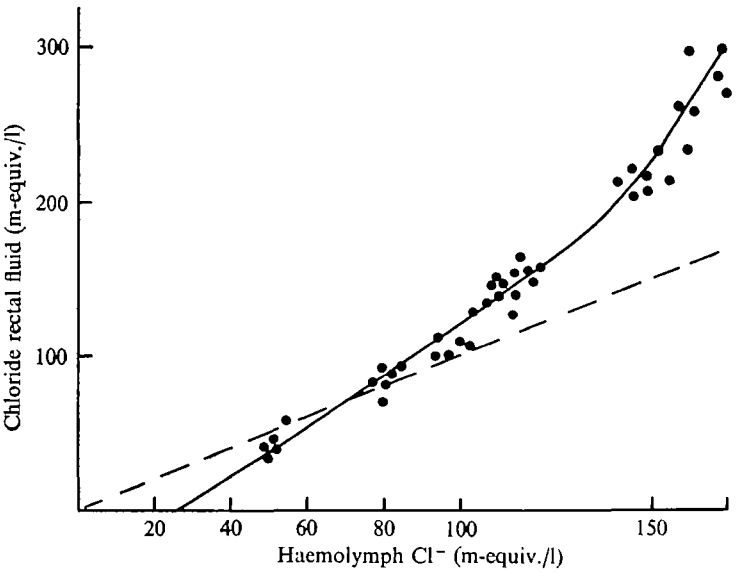

Fig. 5. The relation between the concentration of chloride ions in the haemolymph and in the rectal fluid of the larvae of *Philanisus plebeius*.

fluid is plotted against that of the haemolymph (Fig. 5) it can be seen that there is no doubt that the larva is capable of elaborating a rectal fluid containing a higher concentration of chloride ions than the haemolymph. In considerable dilutions of sea water (60 mM-NaCl/l), in which the haemolymph chloride fell to 50–60 m-equiv Cl/l, four of the five animals tested were found to have the chloride concentration in the rectal fluid lower than in the haemolymph, and slightly lower than in the medium.

The osmotic pressure of the midgut fluid is slightly higher than that of the haemolymph at normal environmental salinities but rises considerably in higher salinities and falls slightly in dilute media. Many authors have reported similar results for other animals but the significance of this in the overall regulation is obscure.

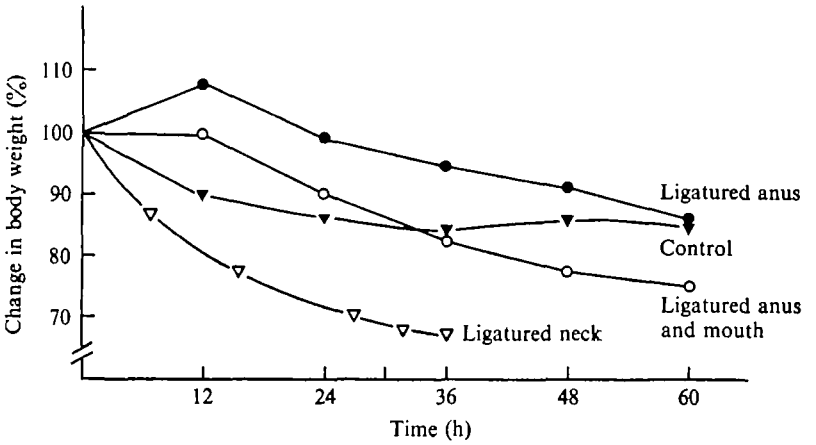

Fig. 6. The percentage change in body weight of larvae of *Philanisus plebeius* in sea water. ▽, Larvae with neck ligatured; ○, larvae with mouth and anus ligatured; ●, larvae with anus only ligatured; ▼, normal larvae.

The exchange of body water

It is clear from the results above that *Philanisus plebeius* is capable of maintaining its blood composition and osmotic pressure approximately constant over a wide range of external conditions. This is in contrast to the only other euryhaline caddis larva known, *Limnephilus affinis* (Sutcliffe, 1961*a*), which is able to live in high external salinities principally by tolerating a high internal ionic concentration. *Philanisus* resembles in this respect the larvae of marine Diptera in the capacity to regulate the blood concentration. It is therefore of interest to compare the permeability of the body wall of *Philanisus* with that of other marine insects.

The change in weight of ligatured larvae

To estimate the rate of osmotic loss of water through the body wall, the weight change of larvae in sea water was measured for larvae which had been prevented from drinking the medium by means of a ligature of fine silk around the neck and prevented from excreting by means of a second ligature around the anus. The change in weight of these larvae was compared with that in animals given an anterior ligature only, a posterior ligature only, or no ligature at all. Results of these experiments are shown in Fig. 6.

The interpretation of the results shown in Fig. 6 is not straightforward since the control animals removed from their cases and placed in small tubes in sea water lose weight at an appreciable rate during the experiment. To test the assumption that this loss of weight was attributable to removal of the larvae from their cases, rather than to handling involved in frequent weighing, a further experiment was set up in which five larvae were removed from their cases and kept in small tubes in sea water. Their change in weight was recorded and compared with members of a similar group which were carefully returned to their cases after each weighing. Results shown in Table 2 indicate that larvae returned to their cases maintain an approximately constant body weight even though not feeding, while those kept out of their cases lose weight at an appreciable rate under the same conditions. This loss of weight did not affect the animal's ability to regulate the osmotic pressure or ionic composition of the haemo-

Table 2. *Weight change of larvae of* Philanisus plebeius *in and out of their cases*

No. of larvae	Treatment	Mean % weight change in 12 h
5	100% sea water in cases	$-1 \pm 0{\cdot}9$
5	100% sea water out of cases	$-9 \pm 2{\cdot}3$
5	40% sea water in cases	$-4 \pm 2{\cdot}4$
5	40% sea water out of cases	$-12 \pm 3{\cdot}6$

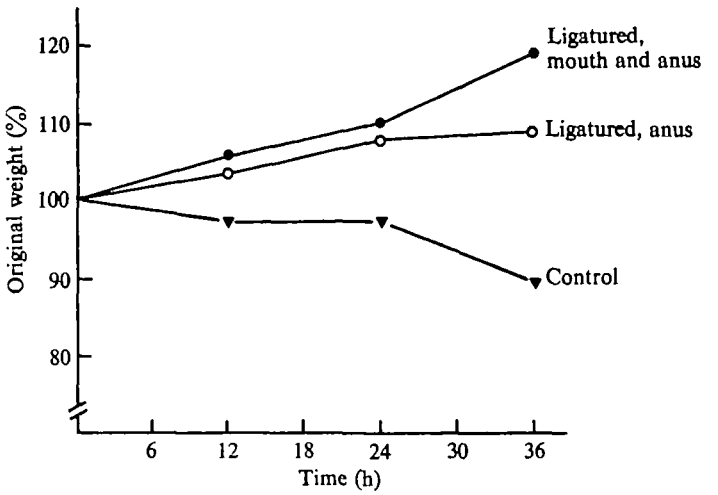

Fig. 7. The percentage change in weight of larvae of *Philanisus plebeius* in distilled water. ○, Larvae with anus ligatured; ●, larvae with mouth and anus ligatured; ▼, normal larvae.

lymph. Later experiments showed that this loss of weight was attributable to the great reduction of drinking in the latter group.

Examination of Fig. 6 shows that larvae prevented from drinking by an anterior ligature lose weight rapidly, the average weight of five larvae falling to 70% of its original value after 27 h. Much of this loss must be due to the production of rectal fluid, since in larvae with both mouth and anus sealed, and in those with only the anus sealed, the rate of loss is considerably reduced. Larvae with mouth and anus sealed lose water at a rate approximately equal to 10% of the body weight per day.

In distilled water (Fig. 7) larvae ligatured at both extremities gain weight at a rate of about 8% per day, which indicates that the larvae possess a body wall which is of the same order of permeability as that of *Limnephilus* and other typically freshwater insects. *Philanisus* is thus exposed in sea water to a constant loss of water through the cuticle which must be replaced, presumably by drinking.

The rate of drinking in sea water

The rate of drinking of the medium by the larvae with and without their cases is shown in Table 3. The mean rate of uptake of larvae in their cases was found to be 23·3% of the body weight per day, which is of the same order as that lost by larvae prevented from drinking the medium. These results also confirm the impression, gained in earlier experiments, that larvae removed from their cases lose weight as a result of a great reduction in the rate of drinking. At least in the short term it appears

Table 3. *Drinking rate of larvae of* Philanisus plebeius *in sea water*

No. of larvae	Duration of expt. (h)	Vol. sea water consumed/% original weight
In cases		
5	24	23·9 ± 6·2
5	16	15·6 ± 5·8
4	24	22·7 ± 9·6
Without cases		
5	24	12·2 ± 5·6

Table 4. *The weight change and chloride ion concentration in the haemolymph of larvae of* Philanisus plebeius *after ligation*

No. of larvae	Treatment	% weight change	Chloride in the haemolymph (m-equiv/l)
5	100% sea water; left 60 h; ligatured mouth and anus	−25	305
5	100% sea water; left 60 h; ligatured anus only	−15	300
5	100% sea water; left 60 h; control	−14	126

that a high rate of drinking is not essential to the regulatory process, since animals out of their cases were still able to maintain their osmotic pressure over 5 days. It seems likely that contact of the body wall with the case is important in maintaining the rate of water uptake and hence the total body volume. If larvae are prevented from drinking in a hyperosmotic medium, however, then the osmotic pressure and the chloride ion concentration of the blood rise. Table 4 gives results of an experiment in which larvae were prevented from drinking for 5 days in sea water. In the absence of any information on the volume of the blood or the distribution of chloride between blood and tissues it is not possible to estimate the extent to which this rise in chloride ion concentration is caused by loss of water from the body and how much is contributed by inward diffusion of chloride ions. It does serve to indicate, however, that a certain degree of drinking is essential if the animal is to maintain a normal osmotic balance, perhaps equivalent to the 10% of the body weight per day uptake shown by larvae out of their cases.

The rate of exchange of body water

If an isotopically labelled water is added to the environment of an aquatic animal, then the concentration of the isotope within the animal at time t will be given by the expression:

$$C_t = C_o(1 - e^{-kt}),$$

where k is the rate constant of exchange of body water. Rearranging this equation gives

$$-kt = \ln\left(1 - \frac{C_t}{C_o}\right)$$

and permits calculation of a rate constant for the penetration of deuterium oxide or tritiated water into the animal.

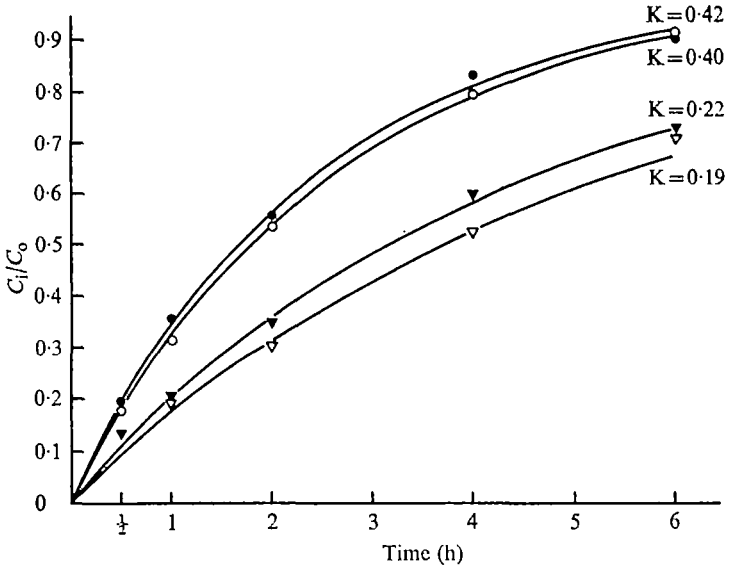

Fig. 8. The rate of exchange of body water of larvae of *Philanisus plebeius* in sea water, isotopically labelled with either deuterium or tritium. ▼, Larvae ligatured at the mouth immersed in tritiated water; ●, normal larvae in tritiated water; ▽, larvae ligatured at the mouth immersed in deuterium oxide in sea water; normal larvae in deuterium oxide in sea water.

Fig. 8. shows the change with time of the ratio of the internal and external concentrations of deuterium oxide and tritiated water, measured as elevation of freezing point in the case of deuterium oxide, and as radioactivity in the case of tritium. The points for deuterium and tritium, for the control animals in their cases, fit a curve where the value of k is $0{\cdot}40 \pm 0{\cdot}03$/h for deuterium oxide and $0{\cdot}42 \pm 0{\cdot}02$/h for triated water. For ligatured animals the value approximates to $0{\cdot}19 \pm 0{\cdot}06$/h and $0{\cdot}22 \pm 0{\cdot}03$/h for deuterium and tritium respectively. In another experiment, not shown in Fig. 8, for unligatured animals out of their cases, in tritiated water, the rate constant was found to be $0{\cdot}28 \pm 0{\cdot}05$/h, corresponding to the reduced rate of uptake of water by drinking. Owing to the branched nature of the gills it is not possible to determine a permeability constant for the cuticle, but figures for the rate constant confirm that the cuticle is at least of the same order of permeability as that of typical freshwater insects, and also that a large uptake of water occurs through the mouth under normal circumstances.

The exchange of sodium

The rate of exchange of sodium in normal animals in their cases, and in larvae with the mouth and anus sealed by ligatures, was measured by immersing a large number of larvae in a medium labelled with ^{22}Na as sodium chloride. At intervals five larvae of each group were removed and the radioactivity of the blood was measured.

The rise in activity of the blood of larvae with time is shown in Fig. 9. In unligatured larvae the rapid rise in the activity of the blood is undoubtedly associated with the high rate of uptake of the medium by drinking. If the values found are substituted in the equation

$$A_t = A\frac{C_t}{C_o}(1 - e^{-kt}),$$

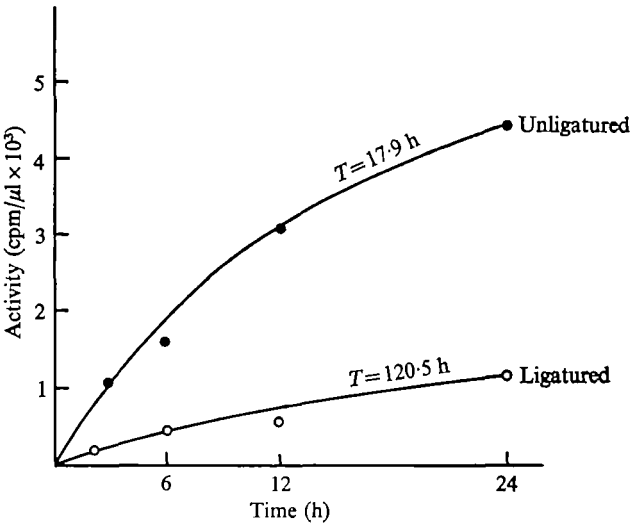

Fig. 9. The rate of exchange of sodium in larvae, in sea water isotopically labelled with ^{22}Na. ●, Normal animals in sea water labelled with ^{22}Na; ○, larvae ligatured at the mouth and anus in sea water labelled with ^{22}Na.

where A_t is the activity of the blood at time t, A is the activity of the medium, C_i and C_o the internal and external concentrations of sodium respectively, and k the rate constant for influx of sodium, then a value for the rate constant is found for normal larvae of 0·056/h. Comparison with Sutcliffe's (1961*a*) figures for sodium influx in *Limnephilus* may be made by calculating the time constant of sodium uptake, the reciprocal of the rate constant. The time constant for sodium exchange in normal *Philanisus* in sea water is 17·9 h, which is considerably smaller than that found for *Limnephilus* by Sutcliffe, even when consideration is given to the fact that his experiments were carried out at a much lower external sodium concentration. For ligatured larvae of *Philanisus*, however, the rate constant was found to be 0·083/h, corresponding to a time constant of 120·5 h. Assuming an internal sodium concentration C_i of 160 m-equiv/l, a time constant of 120 h, or 5 days, would correspond to an influx rate sufficient to raise the blood sodium by about 32 m-equiv/l/day if C_i was zero. This influx rate is about twice that calculated for *Limnephitus* in 300 m-equiv/l. Since the concentration of sodium in the external medium in the present experiment was 468 m-equiv/l, the results obtained were at least of the same order.

The rate of efflux was measured in ten larvae kept in labelled sea water for a week and then counted individually and continuously while being bathed in flowing non-radioactive medium. Five of the larvae were ligatured at mouth and anus before measurement. The mean results of these experiments are shown in Fig. 10. They indicate that while the efflux rate of sodium (time constant of 19 h) is in good agreement with that found previously for whole larvae, the rate of loss of sodium through the cuticle is much higher than its rate of penetration, even though this is against the concentration gradient. The rate of efflux through the cuticle, however, is not as rapid as the rate of entry in the normal animal, indicating that at least some sodium must be lost through the rectal fluid.

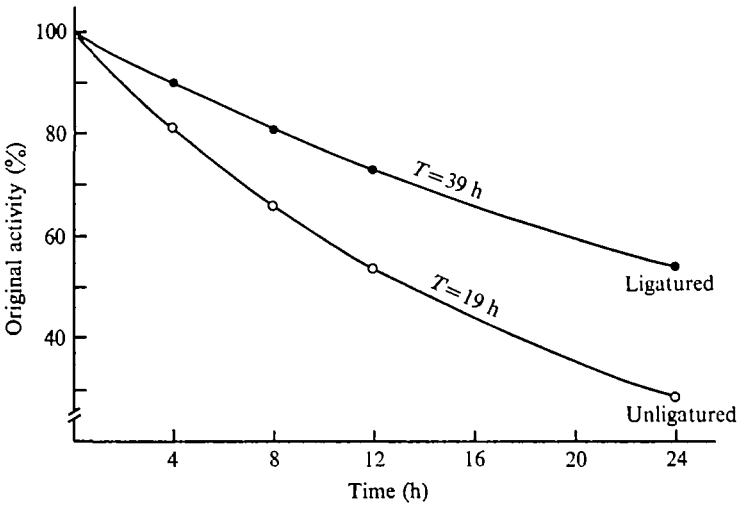

Fig. 10. The rate of efflux of ^{22}Na from larvae of *Philanisus plebeius* in sea water. ●, Normal larvae in sea water; ○, larvae ligatured at the mouth and anus in sea water.

Table 5. *Potential difference across the body wall of the larvae of* Philanisus plebeius *in various media*

Medium	No. of readings	Mean P.D. (mV)	Change relative to sea water (mV)
SW	10	−5·5	—
10% SW	6	−15·1	−9·6
BeS SW	8	+1·9	+7·4
Cho SW	8	−15·4	−9·9
K SW	5	−4·2	+0·7
Li SW	7	−9·3	−3·8

Abbreviations: SW, sea water, BeS, benzenesulphonate; Cho, choline chloride.

Potential measurements across the body wall

The results obtained from a study of the movement of labelled sodium across the body wall suggested strongly the possibility of active outward transport of sodium. For this reason studies of the electrical potential difference across the body wall were carried out to obtain further evidence for this possibility.

Table 5 summarizes the results of measurements of potential difference in various solutions. The potential difference between the blood and the external medium in artificial sea water was 5·5 ± 0·4 mV (10 measurements), the blood being negative with respect to the external medium. The equilibrium potential, calculated by the Nernst equation, for sodium ions, using a value for blood sodium concentration obtained previously, was 27·3 mV (blood positive) and for chloride ions 36·0 mV (blood negative). For each of these ions the value found was far from the equilibrium potential, indicating that both of these ions may be subject to active transport. The effect of reducing sodium in the medium to half its normal value by replacing it with choline and of reducing chloride concentration by replacement with benzenesulphonate is to cause a large change in potential difference in both cases, as does immersing the animal in 60 mM-NaCl/l (10% sea water). Smaller changes occurred in sea water made up with potassium or lithium.

DISCUSSION

It appears from the present work that the larva of *Philanisus plebeius* differs considerably from that of *Limnephilus affinis* in its mechanism for overcoming the physiological difficulties associated with life in a highly saline environment. This is perhaps not surprising since, although these two larvae are the only trichopterans found in salt water, *Limnephilus* normally lives in water of low salinity, and is only occasionally faced with a sudden and rapid rise in salt concentration when its environment is flooded from the sea (Sutcliffe, 1961*a*), whereas *Philanisus* is never found as a larva anywhere except in marine rock pools or subtidally. Thus although *Limnephilus* is capable of tolerating a high internal osmotic pressure, and can excrete a rectal fluid containing at least three times the haemolymph chloride concentration, this can only be a relatively limited resource, and the range of salinities which the larva can tolerate is fairly small. It can also easily live in fresh water. *Philanisus*, on the other hand, is adapted as a larva for life in the sea, and can maintain its blood osmotic pressure at a low level over a wide range of external concentrations, but is unable to complete its development in fresh water. It appears that the adaptations which have enabled this trichopteran of uncertain taxonomic affinities (Ulmer, 1953) to invade successfully a marine habitat are irreversible.

In general, it seems that marine insects, like terrestrial ones, must possess a cuticle relatively impermeable to water and salts. Thus Beadle (1939) regarded the cuticle of *Aedes detritus* as being totally impermeable to water and salts, a proposition apparently accepted by Ramsay (1950), while Nemenz (1960) regards the cuticle of *Ephydra cinerea* as being relatively, but not totally, impermeable to salts and water.

In *Opifex fuscus*, a New Zealand marine mosquito, Nicholson (1971, unpublished), using flux measurements of tritiated water, has calculated the permeability constant of the larval cuticle to water to be $0{\cdot}54 \times 10^{-2}$ cm/h, which accords favourably with the prediction of Shaw & Stobbart (1963) that the permeability of the cuticles of fully marine insects would be at least an order of magnitude less than that of freshwater forms. However, it is significant that those larvae studied have all been air-breathing dipterous larvae. The requirement of reasonable permeability of the cuticle to dissolved oxygen in those forms which rely on transcuticular diffusion as a source of respiratory gas would appear to limit the degree of impermeability to water which can be attained. Thus the larva of *Limnephilus* has a cuticle which is of the same order of permeability as that of other typical freshwater insects, and the present study has indicated that the cuticle of *Philanisus* is similar. It appears therefore that the generally accepted picture of osmoregulation of marine insects cannot hold for *Philanisus*.

The second aspect of the osmotic regulation of marine insects, the capacity to excrete a hyperosmotic rectal fluid, is also not sufficiently well proven to be regarded as a generality. Although it is a reasonable assumption, the only published data are Ramsay's (1950) figures for the rectal fluid of *Aedes detritus*. He found that in sea water the rectal fluid of seven larvae was in all cases markedly hyperosmotic to the haemolymph, but in only four cases was it also hyperosmotic to the medium. It is possible to argue that in these cases the rectal fluid was still being modified, but the recent work of Phillips & Meredith (1969) has drawn attention to the fact that the anal papillae may have a role to play in excreting chloride ions in hyperosmotic media.

The larva of *Limnephilus* is capable of excreting a rectal fluid hyperosmotic to the blood and to the medium (Sutcliffe, 1961*a*), but in this case the difference in osmotic pressure between the three fluids is small, and the rectal fluid, although somewhat different in composition from the haemolymph, is not greatly concentrated. Osmotic balance of the haemolymph is considered by Sutcliffe to be achieved by drinking the medium and excreting a hypertonic rectal fluid to balance the water lost by osmosis across the body surface. Clearly such a mechanism is not operative in the larva of *Philanisus*, since in order to 'gain' water from the medium, by drinking, followed by excretion of a hypertonic excretory fluid, the fluid excreted must be more concentrated than the medium. Although the rectal fluid of *Philanisus* is markedly more concentrated than the haemolymph in sea water, it is still considerably more dilute than the medium. In no case was a sample of the rectal fluid found to be hyperosmotic to the medium except in the lowest salinity tested. It appears, however, that the activity of the rectum is important to the functioning of the normal osmoregulatory mechanism, for if the larva is prevented from excreting by an anal ligature, the osmotic pressure of the blood rapidly rises.

There is also not doubt that active drinking of the medium is an important feature of the osmoregulatory mechanism of *Philanisus*. If the larva is prevented from drinking, the osmotic pressure and the chloride concentration of the blood rise rapidly in sea water. The animal under normal conditions drinks a volume of sea water equal to 25% of its own weight in a 24 h period. It seems that this quantity is not essential to osmoregulation since if the larvae are removed from their cases the rate of drinking is drastically reduced and the larvae lose weight, although the blood is still maintained at normal levels. The great flux of water under normal conditions appears to be associated with the maintenance of normal body volume.

It is clear that the larva is subject to considerable loss of water through the body surface. Direct measurements of weight change suggest that this may amount to about 10% of the body weight per day. The larva is incapable of supplying this loss by the mechanism found for *Limnephilus* by Sutcliffe (1961*a*, 1962*a*), that of drinking the medium and excreting a hypertonic rectal fluid. It follows that there must be an extra renal source of ion excretion which is capable of excreting ions against a concentration gradient at a sufficient rate to enable the larva to drink sea water and yet maintain its blood osmotic pressure at a low level. Some evidence is presented that this may be the case. In larvae loaded with labelled sodium the outward flux through the cuticle is considerably greater than the inward flux measured by a similar method. This net outward flux of sodium is in the opposite direction to the concentration gradient, and suggests either an asymmetrical permeability of the body wall to sodium ions, or the active transport of this ion out of the body. Measurement of the electrical potential difference across the body wall shows that the potential lies far from the equilibrium potential for either sodium or chloride ions, implying that the unequal concentrations of both ions across the membrane are maintained by active processes. This is further supported by the fact that a large change in potential occurs when either sodium or chloride in the bathing medium is reduced to half its normal value. Active transport of both sodium and chloride ions is unusual in epithelia; for *Artemia salina*, Smith (1969*b*) found that only chloride ions were actively transported out of the animal, sodium ions following passively, but Maetz (1971) finds that in the eel both sodium

and chloride ions are actively transported, as did Potts & Parry (1964) for the prawn *Palaemonetes varians* and Shaw (1960) for *Astacus*. Thus it appears that in *Philanisus* a large contribution to osmotic regulation is provided by the active transport outwards of both sodium and chloride ions. This is further confirmed by estimation of the transport numbers of chloride and sodium ions in the body wall by the method of Croghan, Curra & Lockwood (1965). They define the transport number T_i of an ion in a membrane by the relation

$$T_i = \delta V/\delta V_e,$$

where V is the membrane potential and V_e the equilibrium potential of the ion i, defined by the Nernst relation. A change ΔV_e in V_e is easily made by changing the ionic concentration on one side of the membrane; if the resultant change ΔV in V is measured, the transport number can be calculated using the approximation

$$T_i = \Delta V/\Delta V_e.$$

The transport number of sodium is found to be 0·545, and that for chloride to be 0·421. Bearing in mind the assumptions pointed out by Croghan, Curra & Lockwood (1965) it is apparent that the diffusional permeabilities of the cuticle to sodium and chloride are somewhat similar.

It is probable that many freshwater insect larvae possess mechanisms for the active uptake of ions through some part of the general body surface, even though this has been extensively studied only in the larvae of mosquitoes. It is not perhaps surprising that *Philanisus* should possess a mechanism for the active excretion of ions through the body surface. Furthermore it appears likely that this mechanism can be reversed, for the larva can maintain itself in balance in hypo-osmotic saline, although the rectal fluid is hypertonic to the medium.

SUMMARY

1. The larva of *Philanisus plebeius* is capable of surviving for at least 10 days in external salt concentrations from 90 mM/l sodium chloride (about 15% sea water) to 900 mM/l sodium chloride (about 150% sea water).

2. Over this range the osmotic pressure and the sodium and chloride ion concentrations of the haemolymph are strongly regulated. The osmotic pressure of the midgut fluid and rectal fluid is also strongly regulated.

3. The body surface of the larva is highly permeable to water and sodium ions.

4. In sea water the larva is exposed to a large osmotic flow of water outwards across the body surface. This loss is replaced by drinking the medium.

5. The rectal fluid of larvae in sea water, although hyperosmotic to the haemolymph, is hypo-osmotic to the medium, making it necessary to postulate an extra-renal site of salt excretion.

6. Measurements of electrical potential difference across the body wall of the larva suggest that in sea water this tissue actively transports sodium and chloride ions out of the body.

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