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STUDIES ON SALT AND WATER BALANCE IN CADDIS LARVAE (TRICHOPTERA)

II. OSMOTIC AND IONIC REGULATION OF BODY FLUIDS IN *LIMNEPHILUS STIGMA* CURTIS AND *ANABOLIA NERVOSA* LEACH

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INTRODUCTION

Only two species of caddis larvae are known to occur in water with a salt concentration approaching that of normal sea water. Larvae of *Philanisus plebeius* Walker live in rock pools on the coasts of New Zealand and Australia (McLachlan, 1882; Hudson, 1904), and larvae of *Limnephilus affinis* Curtis live in salt marsh pools on the coast of Britain (Sutcliffe, 1960). All other records of caddis larvae in salt water concern brackish waters with a salt concentration less than that of 30% sea water. Osmoregulation of the body fluids in *L. affinis* larvae was described in the first paper of this series (Sutcliffe, 1961). The object of the work reported here was to investigate osmoregulation of the body fluids in caddis larvae which normally occur only in fresh water, in order to compare their regulatory mechanism with that of the euryhaline larvae of *L. affinis*.

Two species of freshwater caddis larvae were used; *L. stigma* Curtis, which occurs in ponds and lakes, and *Anabolia nervosa* Leach, common in slow-flowing reaches of rivers. Regulation of the haemolymph chloride level has already been studied in one other freshwater caddis larva, *L. flavicornis* Fabricius (Boné & Koch, 1942). However, these authors only studied the chloride regulation of larvae kept in salt solutions in which the chloride concentrations were roughly equivalent to the range of concentrations found in natural fresh waters.

The osmoregulatory mechanisms of two other freshwater insects kept in saline media have already been studied in some detail. Wigglesworth (1933, 1938) and Ramsay (1950, 1951, 1953) worked with larvae of *Aëdes aegypti*. Beadle & Shaw (1950) and Shaw (1955) investigated larvae of *Sialis lutaria*. Chloride regulation has also been investigated in larvae of *Helodes* (Treherne, 1954) and in larvae of *Aeschna* and *Libellula* (Schoffeniels, 1950).

MATERIAL AND METHODS

Limnephilus stigma larvae were obtained from a small pond in Gosforth Park, Northumberland. *Anabolia nervosa* larvae were obtained from the River Blyth. Imagines of both species were reared out in the laboratory to confirm the identifications of the larvae.

Body fluids were removed and analysed as described previously (Sutcliffe, 1961). Larvae were not fed during the course of experiments, and usually were not removed from their cases until body fluids were required for analysis. Larvae were kept individually in beakers containing about 100 ml. of the experimental medium. Most of the experiments were carried out at 14–17° C.

Media were Newcastle tap water and local sea water from Cullercoats diluted with tap water to the required concentration. As in the previous paper, sea-water media are referred to in terms of equivalent concentrations of sodium chloride (mM./l. NaCl).

SURVIVAL IN SEA-WATER MEDIA

The majority of larvae of both species did not survive for more than a few days at external salt concentrations greater than about 60 mM./l. NaCl. Mortality was particularly high when larvae were transferred directly from tap water into high salt concentrations. Thus in 120 mM./l. NaCl about 50 % of the larvae died within 3 days. In 170 mM./l. NaCl about 75 % died within 3 days and in 220 mM./l. NaCl only a few individuals survived for more than 2 days.

In order to obtain measurements on the body fluids of larvae kept in 170–220 mM./l. NaCl it was necessary to increase the salt concentration gradually over a period of about 7 days. Survival was also slightly increased by lowering the temperature to 10–12° C. Even so, more than 50 % of the larvae died during the process, and none survived in 220 mM./l. NaCl for more than 6–7 days. Body fluids of larvae kept in concentrations above 120 mM./l. NaCl were usually analysed after 2–3 days at the final experimental salt concentration. In considering the following results it should be remembered that they were obtained only from those few larvae which were relatively successful in surviving the higher salt concentrations.

RESULTS

(*a*) *Haemolymph osmotic pressure and conductivity*

The relationship between osmotic pressure of the haemolymph and that of the medium is shown in Fig. 1A (*L. stigma*) and Fig. 1B (*A. nervosa*). The normal haemolymph osmotic pressure in both species is relatively low. The mean value for six *L. stigma* larvae was 102 ± 5 mM./l. NaCl, and the mean value for six *A. nervosa* larvae was 113 ± 10 mM./l. NaCl.

In sea-water media the haemolymph osmotic pressure increased gradually and remained slightly hyper-osmotic to the medium. This increase was due entirely to an increase in the electrolyte fraction of the haemolymph, estimated by measurements of its conductivity (Table 1). In this respect both species differ from *L. affinis* larvae, in which the electrolyte fraction of the haemolymph did not increase to the same extent as the haemolymph osmotic pressure (Sutcliffe, 1961).

(*b*) *Haemolymph chloride*

In both species there was considerable individual variation in the extent to which the haemolymph chloride concentration was regulated in sea-water media (Fig. 1A, B). In some larvae the chloride concentration increased to contribute practically all of the anion fraction of the haemolymph. Nevertheless, in the majority of larvae a low

chloride level was maintained against fairly high external concentration gradients, and in all cases the haemolymph remained hypotonic with respect to chloride until just prior to death.

Table 1. *Mean values of the haemolymph osmotic pressure and conductivity in larvae of* Limnephilus stigma *and* Anabolia nervosa *at different external salt concentrations*

		mM./l. NaCl				
Species	*N*	Haemolymph O.P.	S.D.	Conductivity	S.D.	Medium O.P.
L. stigma	4	101	—	88	—	Tap water
	6	154	14	148	7	108
	4	199	—	204	—	190
	2	232	—	228	—	226
A. nervosa	2	125	—	108	—	Tap water
	6	189	9	176	8	178
	5	233	26	193	14·5	220

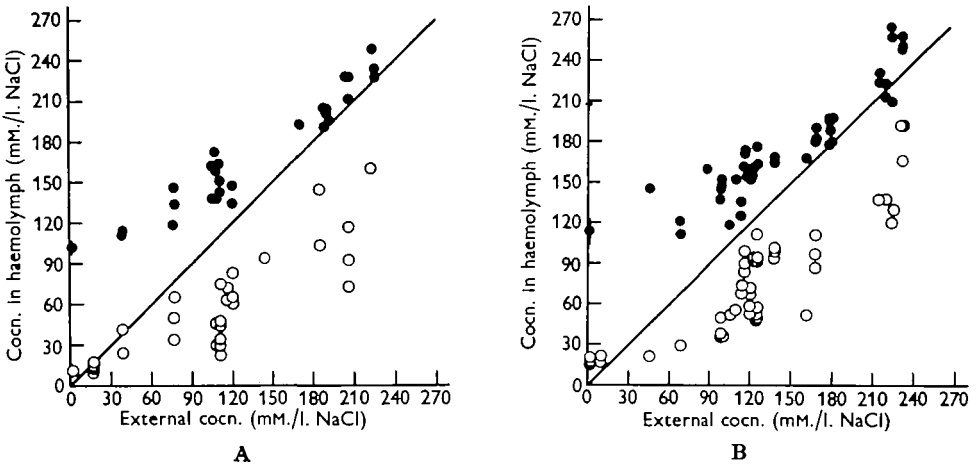

Fig. 1. The relation between the external concentration and the concentration in haemolymph of A, *L. stigma* and B, *A. nervosa* larvae. ●, Haemolymph osmotic pressure; ○, haemolymph chloride concentration. Vertical lines indicate extent of standard deviations from mean values for haemolymph osmotic pressure of six larvae kept in tap water (see text). In six *L. stigma* larvae kept in tap water the mean haemolymph chlorine concentration was 11 ± 4·5 mM./l.

(*c*) *Haemolymph sodium*

In both species the normal concentration of sodium in the haemolymph is very similar to that found in other aquatic insects. The mean value for six *A. nervosa* larvae was 101 ± 3 mM./l. sodium.

In sea-water media the haemolymph sodium concentration increased and remained hypertonic to the medium (Fig. 2). Thus at an external concentration of 195 mM./l. sodium the concentration in the haemolymph of *L. stigma* larvae was twice the normal level. This is strikingly different from the regulation of haemolymph sodium in *L. affinis* larvae, in which the sodium was distinctly hypotonic at an external concentration of 195 mM./l., and was not increased to twice the normal level until the external sodium concentration reached 290 mM./l. (Sutcliffe, 1961).

(*d*) *Rectal fluid osmotic pressure and chloride*

The rectal fluid of tap-water larvae was considerably hypo-osmotic to the haemolymph, but when the concentration of the latter was raised by placing larvae in sea-water media the osmotic pressure of the rectal fluid increased rapidly to become

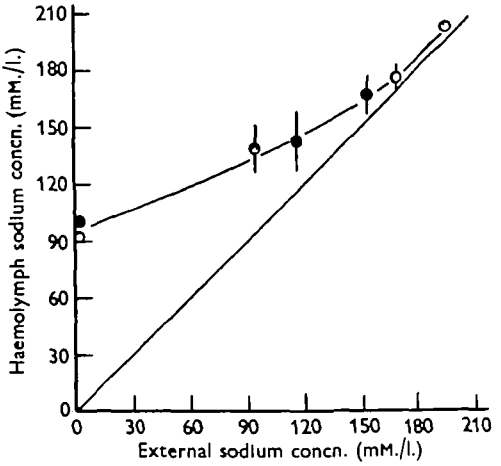

Fig. 2

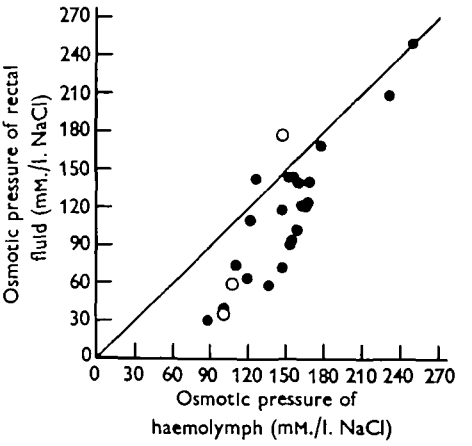

Fig. 3

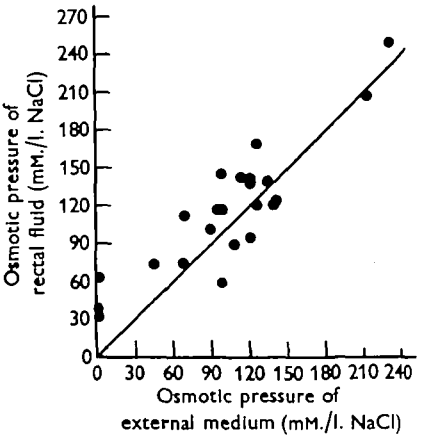

Fig. 4

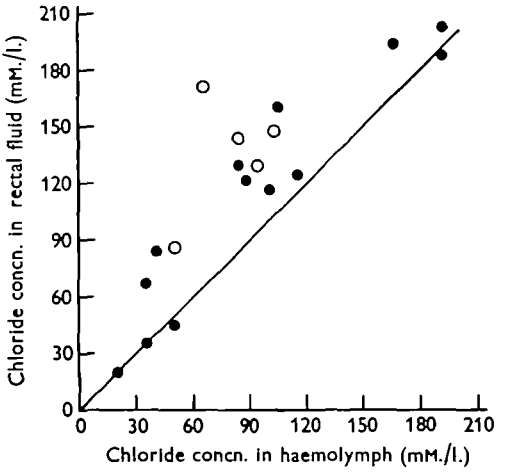

Fig. 5

Fig. 2. The relation between the external sodium concentration and the sodium concentration in haemolymph of *L. stigma* larvae (○) and *A. nervosa* larvae (●). Each point represents the mean value of haemolymph samples from five to six larvae (*A. nervosa*) and two to seven larvae (*L. stigma*). Vertical lines indicate extent of standard deviations of five or more haemolymph samples.

Fig. 3. Increase in the osmotic pressure of rectal fluid as the osmotic pressure of the haemolymph is raised. ●, Rectal fluid of *A. nervosa* larvae; ○, rectal fluid of *L. stigma* larvae.

Fig. 4 The relation between the osmotic pressure of sea-water media and the osmotic pressure of rectal fluid in *A. nervosa* larvae.

Fig. 5. Increase in the chloride concentration of rectal fluid as the concentration is raised in the haemolymph of *L. stigma* larvae (○) and *A. nervosa* larvae (●).

roughly iso-osmotic with the haemolymph (Fig. 3). In one instance in both *L. stigma* and *A. nervosa* the rectal fluid was hyper-osmotic to the haemolymph.

It is of some interest to note that in most instances the rectal fluid was slightly hyper-osmotic to the external medium (Fig. 4). Thus it is possible for the larva to drink salt water and excrete it as a slightly concentrated solution of salts, thereby gaining a small quantity of osmotically free water. In this respect it appears that the freshwater caddis larvae do not differ markedly from *L. affinis* larvae. Furthermore, as in *L. affinis*, the Malpighian tubule-rectal system in *L. stigma* and *A. nervosa* larvae can elaborate a fluid in which the chloride concentration exceeds that in the haemolymph (Fig. 5). This production of hypertonic rectal fluid is stimulated by the increase in haemolymph chloride which occurs when larvae are placed in sea-water media.

(*e*) *Osmotic pressure of the midgut fluid*

Samples of fluid were removed from the midgut and treated as described previously for *L. affinis* larvae. As in *L. affinis*, the midgut fluid in both *L. stigma* and *A. nervosa* contained a soluble dark brown pigment. The fluid was consistently hyper-osmotic

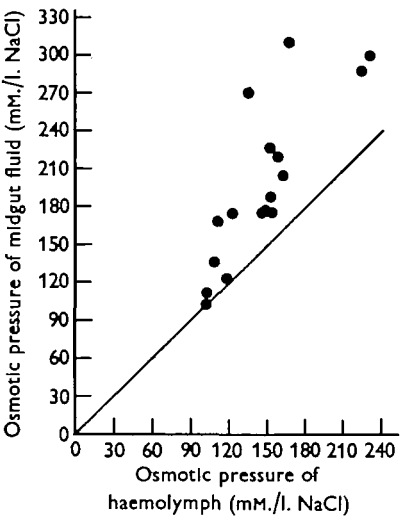

Fig. 6. The relation between the osmotic pressures of midgut fluid and haemolymph in *A. nervosa* larvae.

to the haemolymph, even when larvae were kept in tap water (Fig. 6). The significance of these findings cannot at present be explained, but it is worth noting that in mosquito larvae the midgut and caecal fluids are also hyper-osmotic to the haemolymph, associated with an internal circulation of water between the haemolymph and the gut (Ramsay, 1950).

(*f*) *Permeability of the body wall to sodium*

Using ^{24}Na as a tracer it was found that the body wall of *L. affinis* larvae is relatively impermeable to the inward diffusion of sodium, and that most of the sodium uptake occurs through the gut wall (Sutcliffe, 1961). Preliminary measurements of ^{24}Na influx in larvae of *L. stigma* and *A. nervosa* were also carried out as described for

L. affinis. Larvae were first adapted to tap water and to an external sodium concentration of 100 mM./l., and then some of the larvae were prevented from drinking the medium by sealing the mouth with a small blob of wax. After a further 2 days both media were replaced by a filtered sea-water medium containing 100 mM./l. sodium and a very small quantity of ^{24}Na. Now the influx of ^{24}Na should proceed exponentially to equilibrium with the outside concentration of labelled sodium, and the rate of influx will be proportional to the ratio C_i/C_o where C_i is the internal concentration and C_o is the external concentration of unlabelled sodium (Sutcliffe, 1961). In the case of larvae adapted to tap water before transference to the labelled solution containing 100 mM./l. sodium, the ratio C_i/C_o may be regarded as unity (see Fig. 2). For larvae previously adapted to an external concentration of 100 mM./l. sodium, C_i = 136 mM./l. sodium (Fig. 2).

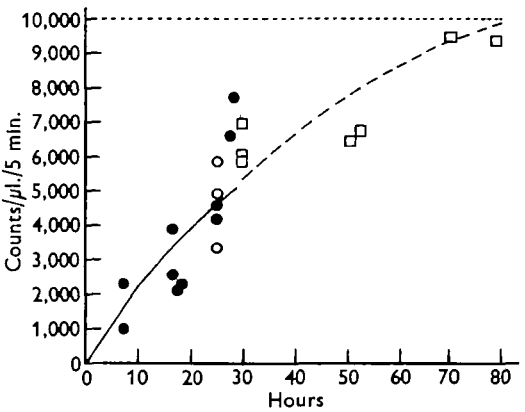

Fig. 7

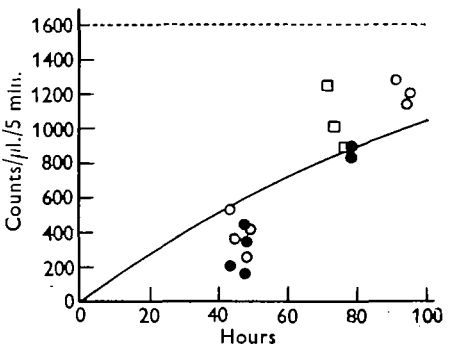

Fig. 8

Fig. 7. Influx of ^{24}Na into the haemolymph of *L. stigma* larvae in a sea-water medium containing 100 mM./l. sodium. □, Larvae previously adapted to the sea-water medium; ○, larvae transferred from tap water at the start of the experiment; ●, as above, but prevented from drinking the medium by sealing the mouths with wax. The two exponential curves describe theoretical increases in haemolymph radioactivity when T = 40 hr. and C_i/C_o = unity (solid curve), and when T = 60 hr. and C_i/C_o = 1·36 (broken curve). The horizontal broken line indicates the concentration of ^{24}Na in the sea-water medium.

Fig. 8. Influx of ^{24}Na into the haemolymph of *A. nervosa* larvae in a sea-water medium containing 100 mM./l. sodium. ○, Larvae previously adapted to the sea-water medium; ●, as above, but prevented from drinking the medium by sealing the mouths with wax; □, larvae transferred from tap water at the start of the experiment. The exponential curve describes the theoretical increase in haemolymph radioactivity when T = 150 hr. and C_i/C_o = 1·36. The horizontal broken line indicates the concentration of ^{24}Na in the sea-water medium.

The results of radioactivity counts on haemolymph samples from individual *L. stigma* larvae are shown in Fig. 7, together with two theoretical curves describing exponential increases in haemolymph radioactivity. The results obtained on larvae previously adapted to tap water conform approximately to a curve where T = 40 hr. and C_i/C_o = unity. Results obtained on larvae previously adapted to an external concentration of 100 mM./l. sodium conform to a curve where T = 60 hr. and C_i/C_o = 1·36. Two interesting points emerge clearly from a study of Fig. 7; (*a*) the

rate of ^{24}Na influx is very high, and (*b*) the rate of influx through the body wall is so fast that it obscures the influx through the mouth and gut wall, in spite of the fact that larvae not prevented from drinking were swallowing large quantities of the labelled sea-water medium (evidence of drinking will be given in a following paper).

The rate of influx through the body wall of *L. stigma* larvae (T = 40 hr.) may be compared with that in *L. affinis* larvae at an external concentration of 100 mM./l. sodium, where T = 400 hr. (Sutcliffe, 1961). Thus it appears that the influx rate in *L. stigma* larvae is ten times that in *L. affinis* larvae. Similar results were obtained with larvae of *A. nervosa* (Fig. 8). Here, the results may be compared with an exponential curve where T = 150 hr. and C_i/C_o = 1·36. Hence the rate of influx through the body wall is two to three times greater than in *L. affinis* larvae. Now if the rate of ^{24}Na exchange through the body wall is also a measure of the rate of diffusion of unlabelled sodium, it would follow that the body wall in the freshwater caddises is considerably more permeable to sodium than the body wall of the euryhaline larvae of *L. affinis*. In the case of *L. affinis* larvae the preceding assumption is not unreasonable, as the rate of ^{24}Na influx through the body wall is roughly proportional to the external concentration of unlabelled sodium (Sutcliffe, 1961). It is therefore possible that the rate of ^{24}Na influx in the freshwater caddises is also a measure of unlabelled sodium diffusion. However, the results here presented are also open to other interpretations. Thus the possibility is not excluded that either active uptake of sodium, or exchange diffusion, or a combination of both phenomena is responsible for the higher rate of ^{24}Na influx in the freshwater caddises. The interpretation of these differences in influx rates must therefore wait until the nature of the sodium exchange mechanism in these caddis larvae has been studied in more detail. Nevertheless, it appears that there is a distinct difference between the freshwater caddises and *L. affinis* with respect to the exchange of labelled sodium through the body wall, and it is suggested that this difference is an adaptive feature associated with the maintenance of a low sodium concentration in the haemolymph of *L. affinis* larvae.

DISCUSSION

The range of external salt concentrations tolerated by larvae of *L. stigma* and *A. nervosa* is strikingly different from that tolerated by larvae of *L. affinis*. The latter live for months at an external concentration of 410 mM./l. NaCl, and tolerate even higher salt concentrations for several days (Sutcliffe, 1960, 1961). Larvae of *L. stigma* and *A. nervosa*, however, begin to die at external concentrations greater than about 60 mM./l. NaCl, and only a few individuals survived for about one week at external concentrations of 170–220 mM./l. NaCl. This contrast in salt tolerance is accompanied by marked differences in regulation of the haemolymph composition. In the freshwater caddises the total concentration of the haemolymph is almost entirely accounted for by the cation sodium, and in salt water the haemolymph sodium level increases to remain hypertonic to the external medium. On the other hand, in *L. affinis* the haemolymph sodium concentration of larvae kept in fresh water accounts for only some 80% of the total haemolymph concentration, and the remaining 20% is due to non-electrolytes (Sutcliffe, 1961). Furthermore, in *L. affinis* the haemolymph sodium level is maintained strongly hypotonic to high external salt concentrations, and the

concentration of the non-electrolyte fraction in the haemolymph is greatly increased. The haemolymph chloride level in *L. affinis* is also maintained strongly hypotonic to high external salt concentrations. In the freshwater caddises, however, although haemolymph chloride is maintained hypotonic to salt water, the chloride concentration steadily increases as the external concentration is raised, and it eventually contributes most of the total haemolymph concentration on the anion side.

So far as chloride is concerned, it appears that the concentration in the haemolymph is to some extent actively regulated by the Malpighian tubule-rectal system. This system is very sensitive to changes in the haemolymph chloride level. An increase in the latter is rapidly followed by a considerable increase in the chloride concentration of the rectal fluid, which can be slightly hypertonic to the concentration in the haemolymph. In *L. affinis* larvae the ability to regulate the haemolymph chloride level is greatly increased, and the chloride concentration in the rectal fluid can exceed that in the haemolymph by a factor of three (Sutcliffe, 1961). This must be regarded as an adaptive feature for survival at high external salt concentrations.

The ability of caddis larvae to elaborate an excretory fluid in which the chloride concentration exceeds that in the haemolymph was first demonstrated in *Limnephilus flavicornis* by Boné & Koch (1942), and is now firmly established as a characteristic feature of the Limnephilidae. Boné & Koch also showed that a reduction in the haemolymph chloride concentration of *L. flavicornis* larvae resulted in re-absorption of chloride from the fluid in the rectum. Thus the Malpighian tubule-rectal system regulates the haemolymph chloride level over a fairly wide range of external concentrations. This is strikingly different from the behaviour of *Sialis* larvae, which are unable to produce an excretory fluid hypertonic with respect to the haemolymph chloride concentration (Shaw, 1955). In *Sialis* the excretory system is slow to respond to changes in the haemolymph chloride level, which remains hypertonic over a range of external concentrations up to at least 120 mM./l. NaCl. In view of this difference between caddis larvae and *Sialis*, it seems likely that the excretory system in freshwater mosquito larvae (Wigglesworth, 1938) and *Helodes* larvae (Treherne, 1954) can also concentrate chloride from the haemolymph, since these insects also maintain the haemolymph chloride hypotonic to salt water. It is certainly probable that the salt-water larvae of *Aedes detritus* are able to concentrate chloride, as these larvae drink salt water and produce a rectal fluid considerably hyper-osmotic to the haemolymph (Ramsay, 1950).

In maintaining a haemolymph sodium level slightly hypertonic to high external concentrations the larvae of *L. stigma* and *A. nervosa* closely resemble the larvae of *Aëdes aegypti* (Ramsay, 1951, 1953) and *Sialis* (Shaw, 1955). Since neither of these latter insects can elaborate an excretory fluid hypertonic with respect to the haemolymph sodium concentration, it would be interesting to investigate sodium output in the rectal fluid of the caddis larvae. It seems *a priori* likely that the freshwater caddises are also unable to concentrate sodium. It is probable, however, that the euryhaline larvae of *L. affinis* can elaborate rectal fluid in which the sodium concentration exceeds that in the haemolymph. Indeed, if the Malpighian tubule-rectal system is the sole route for salt excretion, it may be argued that the ability to concentrate sodium is necessary in larvae which drink salt water with a sodium concentration greater than that in the haemolymph, and yet maintain the haemolymph sodium level

strongly hypotonic. Moreover, since the rectal fluid in *L. affinis* larvae consists of a highly concentrated solution of chloride, it is reasonable to suppose that this is balanced by a high concentration of sodium ions.

In the preceding paper it was suggested that maintenance of a low haemolymph salt concentration in *L. affinis* larvae is concerned with the regulation of a low salt concentration in the tissue cells. Shaw (1959) has suggested that the lethal effect of salt water on the freshwater crab *Potamon niloticus* is due to the action of the raised blood concentration on muscle fibres, and that part of this action is the increased penetration of sodium ions into the interior of the fibre. It is possible that a similar penetration of sodium into tissue cells occurs following an increase in the haemolymph sodium level in caddis larvae. If this is so, the active maintenance of a low haemolymph sodium level in *L. affinis* would be extremely important, and would form a major part of the adaptation for survival at high external salt concentrations. It is perhaps significant that *L. affinis* larvae do not survive for more than a few days when the external sodium concentration is raised to about 425 mM./l. At this concentration, regulation of a low sodium level in the haemolymph breaks down (Sutcliffe, 1961). In the freshwater caddises the haemolymph sodium level is not regulated, and larvae die rapidly when the sodium concentration in the haemolymph is raised to about twice the normal level.

The maintenance of a low haemolymph sodium level in *L. affinis* larvae may also be due to adaptive features situated outside the excretory system. One of these features may be a reduction in permeability of the body wall to sodium compared with that in the freshwater caddises. A reduction in permeability to sodium (and to chloride) would confer a considerable advantage, in that the salt load carried by the excretory system in order to eliminate excess salts gained by diffusion through the cuticle will be decreased. Consequently, the capacity of the excretory system to deal with excess salts gained by drinking salt water will be correspondingly increased. Combined with the ability to produce a concentrated excretory fluid, this feature alone could, to a certain extent, increase the tolerance of *L. affinis* larvae to salt water.

SUMMARY

1. Survival and regulation in sea-water media was studied in the freshwater caddises *Limnephilus stigma* and *Anabolia nervosa*.

2. The majority of larvae did not survive for more than a few days at external salt concentrations greater than about 60 mM./l. NaCl.

3. In sea-water media the haemolymph osmotic pressure increased to remain slightly hyper-osmotic to the medium. The haemolymph sodium level also increased to remain slightly hypertonic to the medium, but the chloride level was maintained hypotonic until just prior to death of the larvae.

4. When the haemolymph chloride concentration was raised above the normal level, the Malpighian tubule-rectal system elaborated fluid in which the chloride concentration was hypertonic to the haemolymph. The system is highly sensitive to changes in the haemolymph chloride level.

5. The regulation of body-fluid composition in the freshwater caddises is compared with that found previously in the euryhaline larvae of *Limnephilus affinis*. It is