Hydrobiologia vol. 36, 3-4, p. 489—503, 1970.

Ecology of the Sundays River
Part II. Osmoregulation in some Mayfly Nymphs (Ephemeroptera: Baetidae)

by

A. T. FORBES & B. R. ALLANSON

Institute for Freshwater Studies,
Zoology Department,
Rhodes University, Grahamstown, South Africa

INTRODUCTION

FORBES (1968) reported a number of apparent relationships between changing total dissolved solids (TDS) levels and the distribution and abundance of the fauna along the Sundays River. This was particularly noticeable in the case of one euryhaline species, *Cloeon crassi*, which had a wide distribution, including the estuary, but varied greatly in numbers at different stations. This species was characteristic of standing water, a habitat which occurred at all stations along the lower course, and hence the fluctuations appeared due to TDS variation.

Two other species, *Cloeon africanum* and *Centroptilum excisum*, were also found but their ranges were more restricted as they typically occurred only in flowing water. The range of *Cloeon africanum*, however, extended downstream to Station 4a (Part I, FORBES & ALLANSON 1970) while *Centroptilum excisum* was not found below Station 6 which suggested a greater TDS sensitivity in this species. This is supported by its presence in the Witrivier tributary which had much lower TDS levels than the main river.

In order to explain these distributional problems an investigation of the osmoregulatory abilities of these three species, and in particular *Cloeon crassi*, was undertaken.

Received February 5th, 1970.

Material and methods

All specimens of *Cloeon crassi* and *Cloeon africanum* were obtained from the Sundays River, usually at Station 5. *Centroptilum excisum* was obtained from the Palmiet River at Howiesons Poort near Grahamstown. Nymphs were transported in a minimum amount of water in plastic bags which were kept in a polystyrene insulated box if the weather was hot.

Only *Centroptilum excisum* and *Cloeon crassi* were used in these experiments which, unless otherwise stated, were performed in triplicate, using the method described by Allanson & Noble (1964). The containers used were 9 cm crystallizing dishes containing 100 ml of the experimental medium. Dishes were covered with a Petri dish to reduce evaporation and prevent escape of any sub-imagos that emerged. Control animals were kept in the river water in which they were caught under otherwise identical conditions.

Counts of the survivors were done at regular intervals depending on the TDS content of the experimental medium and the expected survival times. A record was kept of emergences and these were disregarded when the effects of the experimental medium were considered. No attempt was made to avoid the presence of late stage nymphs in the tolerance experiments since the tolerance of all stages were of interest.

All experiments were carried out at ambient temperatures which varied from 11.5 to 19°C. Temperatures measured during survey trips varied from 9 to 29°C along the stretch of the river inhabited by the above 3 species. Experimental animals were thus not exposed to abnormal temperatures.

During the chemical survey of the river (Forbes 1968) it was found that although the changes in ionic proportions along the lower course were negligible, there was a marked change in these proportions between Station 4, the last station on the lower course, and Station 3 at the head of the estuary. Since nymphs were found at both stations possible effects of these differences were investigated by exposing nymphs of *Cloeon crassi* to dilutions of seawater or to an artificial river water of equivalent TDS content.

The weights of the salts used in making up the artificial river water are shown in Table I.

In order to compare the effects of different media the time at which 50% of the experimental animals survived was obtained by inspection at regular intervals. This time will be referred to as the Median Time of Survival (MTS).

All results were expressed graphically following the probit transformation of Finney (1952). In this method the percentage of

TABLE I

Weights of salts used to produce solutions containing 7,000 and 10,500 parts per million (ppm) (approximately) when made up to 1 l.

TDS level reqd. (ppm)	Weight of each salt used (gm) $KHCO_3$	$NaHCO_3$	$NaCl$	Na_2CO_3	$MgSO_4.7H_2O$	$CaSO_4$	Total wt of solids
7,000 ppm	0.07	0.462	4.872	0.138	2.465	0.204	6.946
10,000 ppm	0.105	0.693	7.308	0.207	3.682	0.306	10.419

nymphs alive at any one time is converted to a probit value using probit tables. The probit values are then plotted against time on an arithmetic scale. A log time scale was tested but was not found to have any advantages over the arithmetic scale.

If a purely arithmetic scale is used with percentage survival versus time a sigmoid curve will be obtained provided the response of the individuals in the sample follows a normal distribution. A probit transformation results in the conversion of this curve to a straight line. However, if the response is not random and modifying factors are present, use of the probit transformation will result in a series of points to which more than one straight line can be fitted. If such a "split" be obtained further investigation would be indicated to explain the differing response.

In all the graphs obtained a line was fitted to the points by eye. Only the randomness or otherwise of the response was of interest and hence, in accordance with FINNEY's recommendation, it was not considered necessary to resort to regression analysis in order to check the accuracy of the lines fitted.

RESULTS

Survival of Cloeon crassi

Survival in the controls is indicated in Fig. 1. The points on the graph are based on results obtained in four sets of control experiments. The results obtained were complicated by the emergence of mature nymphs and by one experiment which was truncated after 50% but before 100% mortality occurred. The set of points shown in Fig. 1 is thus incomplete. Although the emergences might have introduced some bias, an estimate of the MTS is possible. The MTS thus obtained was 276 hours while twice the standard error of the line fitted to the probit points gave a range from 230 to 320 hours.

The results of experiments on the survival times in dilute seawater and artificial river water are shown graphically in Figs. 2, 3, 4 and 5 while the median times of survival are summarised in Table II.

TABLE II

MTS of Cloeon crassi in dilutions of seawater and in artificial river water.

TDS content (ppm)	17,500	10,500	7,000	Control
MTS in:				
Seawater	45—60 mins	24 hours	259 hours	276 hours
Artificial River Water	not done	24 hours	230 hours	

From the above it is apparent that TDS levels of 17,000 and 10,500 ppm are above the limit of tolerance of most members of the population.

There is apparently no difference between survival times in dilute seawater and artificial river water at a TDS level of 10,500 ppm. Similarly the median times of survival obtained in a concentration of 7,000 ppm fall within the range mentioned during discussion of the mortalities in the control experiments. Finally, the above results show that TDS becomes limiting for some individuals in the population between 7,000 and 10,500 ppm.

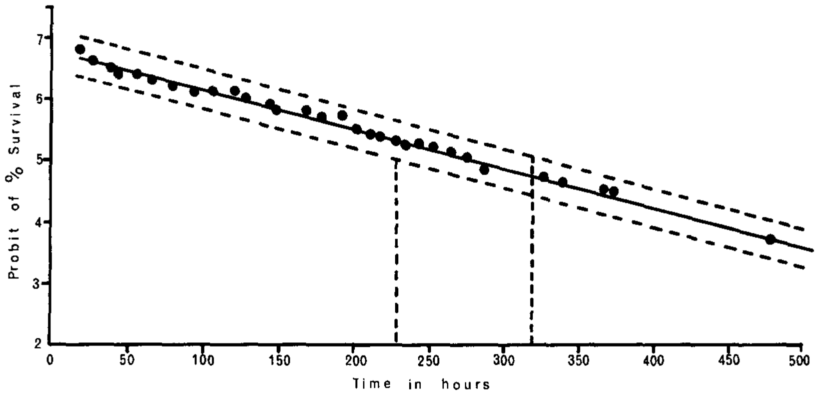

Fig. 1. Survival of *Cloeon crassi* in the control experiments in natural river water. MTS: 276 hours. Dashed lines parallel with the solid line indicate twice the standard error on either side.

Size effects on tolerance to different TDS levels

The "split probits" shown in Figs. 2, 3, 4 and 5 suggested the presence of some factors which modified the effect of the experimental media. MACAN (1961) states that tolerance to environmental factors may vary with age in freshwater animals. Since greater age is associated with greater size in most animals, the possibility that size affected TDS tolerance and was responsible for the "split probits" was considered.

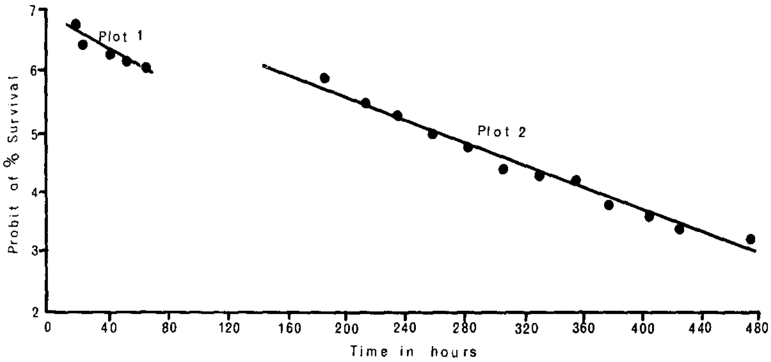

Fig. 2. Survival of *Cloeon crassi* in 20% seawater. TDS content $7^0/_{00}$. MTS: 259 hours. 100% mortality: 498 hours.

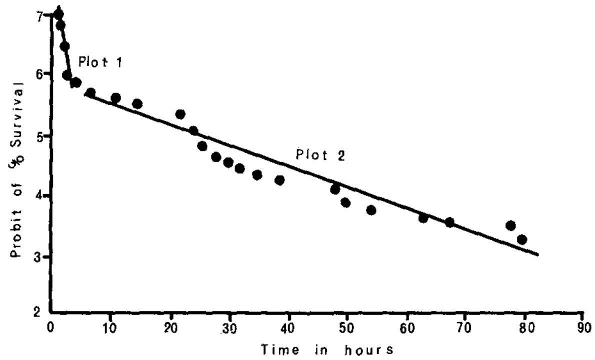

Fig. 3. Survival of *Cloeon crassi* in 30% seawater. TDS content $10.5^0/_{00}$. MTS: 24 hours. 100% mortality: 150 hours.

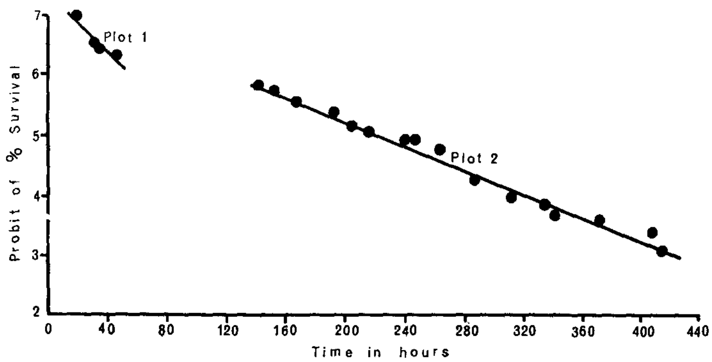

Fig. 4. Survival of *Cloeon crassi* in $7^0/_{00}$ artificial river water (equivalent to 20% seawater). MTS: 230 hours. 100% mortality: 563 hours.

All nymphs were measured immediately after death and placed in different size classes, viz. 1—1.9 mm, 2—2.9 mm etc. The mean sizes of the nymphs dying before and after the times at which the curves in figs. 2, 3, 4 and 5 showed marked changes in slope were calculated and then compared by means of a t-test. The results are summarised in Table III.

TABLE III

Comparison of the mean sizes of nymphs dying early and later in the experimental media used.

Experimental Medium	Mean Sizes (mm) of nymphs in each plot		$t_{0.99}$ d.f. = 60 is 2.62. Value of t obtained	
	Plot 1	Plot 2		
20% Seawater (Fig. 2)	3.8	2.8	2.26	possibly significant
30% Seawater (Fig. 3)	2.6	3.9	5.20	highly significant
7‰ Artificial River Water (Fig. 4)	4.3	3.8	1.31	not significant
10.5‰ Artificial River Water (Fig. 5)	3.4	4.3	4.71	highly significant

The differences in slope of the "split probits" obtained in Figs. 3 and 5 for the two higher TDS levels were far more marked than those obtained from the results for the lower TDS levels. These indicate a far more rapid initial death rate in the more concentrated media. Furthermore at these higher levels, in both artificial river water and dilute seawater, there was a highly significant size difference between those nymphs which died rapidly and those which survived for a longer period since smaller nymphs died first. It is thus possible to explain the "split probits" obtained in the more concentrated media.

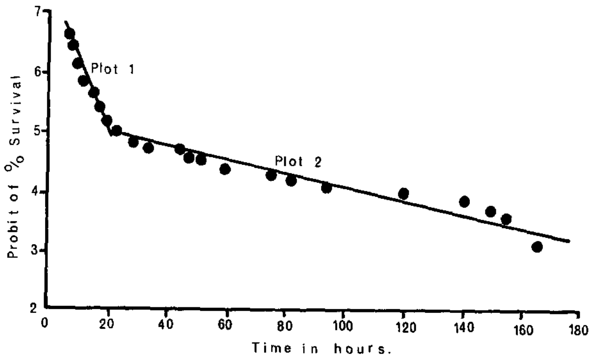

Fig. 5. Survival of *Cloeon crassi* in 10.5‰ artificial river water (equivalent to 30% seawater). MTS: 24 hours. 100% mortality: 175 hours.

At the lower TDS levels (7,000 ppm) Fig. 284, a suggestion of a split probit was obtained in both cases. However, the mean sizes of nymphs contributing to the first half of the split probit in each case were greater than those contributing to the second half. In addition the levels of significance of the differences were not as great as in the more concentrated media. It is possible that these early deaths were due to injury sustained during collection and handling.

It had been previously observed (FORBES 1968) that the numbers of *Cloeon crassi* nymphs decreased rapidly when the TDS levels in the river rose above about 9,000—10,000 ppm. In February 1968, in the course of a general survey, the following numbers of *C. crassi* were recorded at 3 stations above the estuary (see Table IV).

TABLE IV

Population density of Cloeon crassi in the lower course of the Sundays River in February 1968.

Station	5	4a	4
TDS (ppm)	4,482	9,310	14,896
Average number of *C. crassi*/metre sweep	170	19	3

Unfortunately the above specimens were not measured but in April 1968 more attention was paid to the sizes of the nymphs occurring at each station. The results are shown in Table V.

TABLE V

Size ranges of Cloeon crassi in the lower course of the Sundays River in April 1968

Station	5	4a	4
TDS (ppm)	4,950	7,728	9,934
Size Range	2—7 mm	2—7 mm	4—7 mm (20)

No detailed population density determinations were done in April but while nymphs were numerous at Stations 4a and 5 and a full range of sizes was collected, exhaustive collecting at Station 4 produced only 20 nymphs, none of which was under 4 mm in size.

Osmoregulatory Ability in Cloeon crassi

In order to determine the efficiency of the osmoregulatory mechanisms in *C. crassi* determinations of the freezing point depressions of the haemolymph under a variety of external concentrations were done. The method followed was that recommended by RAMSAY & BROWN (1955).

A number of haemolymph samples were taken from nymphs in natural river water. In addition nymphs were exposed to pond water having a TDS content of about 300 ppm. Nymphs were also exposed to artificial river water with a TDS content of about 12,000 ppm, and 10 were subsequently left in this medium which was allowed to evaporate for 10 days, after which further haemolymph samples were taken. Except in the latter experiment, all samples were taken after 48 hours exposure to any one dilution. Samples of haemolymph from nymphs in natural river water were taken 24—48 hours after arrival in the laboratory.

Except for one 2 mm specimen, all nymphs used in these determinations varied from 4 to 7 mm in length. Large nymphs were used as it was easier to obtain blood samples and because they survived better at high TDS levels. Unless otherwise stated all results are based on haemolymph samples taken from a minimum of 3 nymphs in any one medium. The results obtained are shown in Fig. 6.

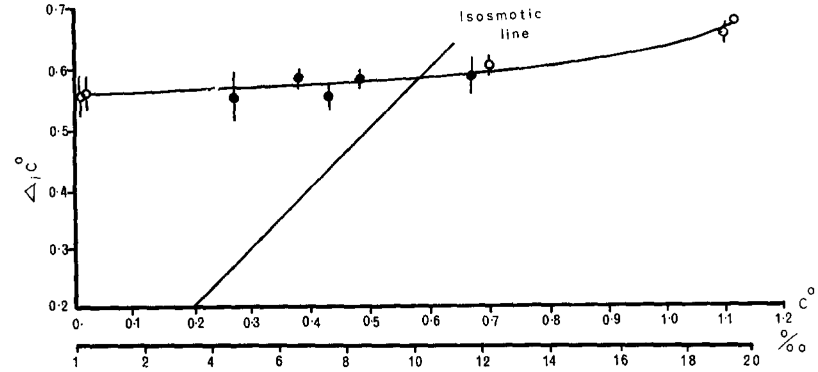

Fig. 6. Curve of freezing point depressions of the blood of nymphs of *Cloeon crassi* at a variety of external TDS levels. Solid circles indicate results obtained from nymphs in natural river water. Open circles indicate results obtained from nymphs in experimental dilutions. Vertical lines indicate range.

The graph clearly indicates that *Cloeon crassi* possesses a marked osmoregulatory ability. The isosmotic point occurred at an external freezing point depression (Δ_e) of 0.58—0.60°C, which corresponds to a TDS level of just over 10,000 ppm; the internal freezing point depression (Δ_i) remained more or less constant over a Δ_e range from 0.01°C to at least 0.70°C and only showed a marked change when the Δ_e reached about 1.10°C. In terms of TDS levels this is a range from about 50 to 20,000 ppm.

Possible mechanisms involved in osmoregulation

Sutcliffe (1962) has investigated hyporegulation in the trichopteran *Limnephilus affinis* and he found that water is obtained by drinking the medium and excreting a hypertonic urine. The same basic mechanism has been reported in *Aedes detritus* by Beadle (1939), in *Ephydra cinerea* by Nemenz (1960) and is known to occur, with some modifications in the sites of production, in *Artemia salina* and in teleosts.

A suggestion that basically the same mechanism occurs in *Cloeon crassi* was obtained from experiments in which nymphs were exposed to an isosmotic solution of glucose and haemolymph samples taken from the survivors after 24 and 48 hours. Only late stage nymphs survived this treatment and freezing point determinations indicated erratic changes but a general trend towards a marked increase in $\triangle_i$ despite the constancy of $\triangle_e$. A maximum $\triangle_i$ of 1.18°C was recorded after 48 hours.

These results suggest that the nymphs normally osmoregulate by drinking the medium and excreting either the excess salts or excess water. Thus in the glucose solution the medium was taken up and the glucose taken in through the gut. However, once in the haemolymph there was apparently no mechansim for excreting it and this would have produced the high $\triangle_i$ levels recorded.

TDS tolerance in some other species of Baetidae

Following the experiments performed using *Cloeon crassi* some tolerance and regulatory tests were done using related species.

Centroptilum excisum

This species was not recorded below Station 6, which, although included in the lower course, always had a much lower TDS level (800—900 ppm) than the rest of the lower course. This species was also recorded from the Witrivier tributary in which the TDS levels varied between 200 and 300 ppm. Since similar habitats were present at places below Station 6 it seemed possible that TDS levels were limiting.

This possibility was investigated using the same methods as in the case of *Cloeon crassi* except that fewer animals were available and all results quoted are based on 20 animals in each dilution of seawater.

The results obtained are summarised graphically in Fig. 7 in which the MTS of *Cloeon crassi* under similar conditions have been included.

Survival times in 30% seawater (TDS 10,500 ppm) were similar, but *Cloeon crassi* survived three times as long as *Centroptilum excisum* in 20% seawater (7,000 ppm). In 10% seawater (3,500 ppm) the sur-

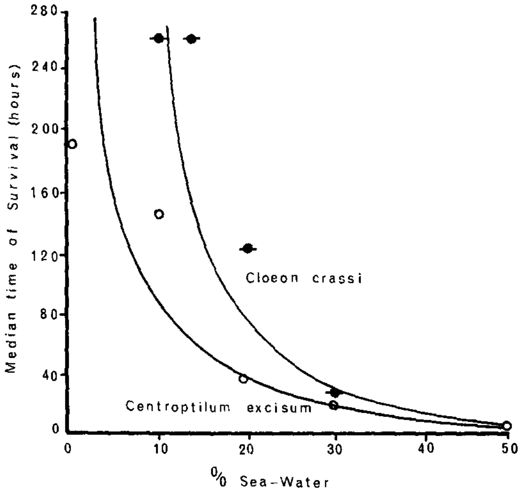

Fig. 7. Comparison of MTS of *Centroptilum excisum* (solid circles) and *Cloeon crassi* (barred circles) in dilutions of seawater.

vival times in both species were virtually the same as in the controls.

The available evidence thus shows that *C. crassi* can tolerate higher TDS levels than *Centroptilum excisum,* with the critical level for the latter species lying between 3,500 and 7,000 ppm.

Cloeon africanum

This species occurred at a number of stations along the lower Sundays River but appeared to be largely restricted to stones in currents. The possibility that increasing TDS levels downstream were an additional limiting factor was checked experimentally by determinations of freezing point depressions of the haemolymph as the limited numbers of this species precluded extensive tolerance tests.

Preliminary determinations of the normal $\triangle_i$ were done on nymphs from natural river water with a $\triangle_e$ of 0.30°C. These individuals had a $\triangle_i$ of 0.57°C which is very similar to that of *Cloeon crassi.* Of 30 nymphs transferred to artificial river water with a $\triangle_e$ of 0.70°C, only one survived for 24 hours.

Discussion

The type locality of *Cloeon crassi* (Agnew, 1961) is the Vaal River where TDS levels, according to Chutter (1963) are low, seldom exceeding 500—600 ppm. Thus, until the discovery of this species in the Sundays River there was nothing to warrant it being attributed with any hyporegulatory ability.

The only previous indication of a possible hyporegulatory ability in ephemeropteran nymphs was provided by SCOTT et al. (1952) who recorded a *Cloeon* sp. in the Klein River estuary near Hermanus at a TDS level of 19.7‰. The laboratory experiments described in the present paper showed that all nymphal stages could survive TDS levels up to 7,000 ppm, but the smaller nymphs were adversely affected once the level reached about 9,000—10,000 ppm. However, the TDS tolerance increases markedly with age as shown by experiments in which late stage nymphs were left in artificial river water which was allowed to evaporate to a concentration of 20,000 ppm.

The similar tolerances of *Cloeon crassi* both to dilute seawater and to artificial river water are significant since it has been shown (FORBES 1968) that a distinct change in the ionic ratios in the water occurs between Station 4, the last station on the lower course, and Station 3 on the upper estuary. This change was permanent, regardless of the TDS levels. Nymphs in the section of the river between these two stations would thus be exposed to high or low TDS levels and ionic ratios typical of either seawater or of river water. The tolerance tests show that the ionic variation will be unimportant and that the important restricting factor will be the TDS level. This supports BEADLE's (1943) contention that overall TDS levels, and hence osmotic pressures, are of more importance to aquatic insects than the nature of the substances contributing to the osmotic pressure.

The "split probits" obtained when the results were expressed graphically have already been explained on the basis of size differences affecting the TDS tolerance. The greater effect on juvenile nymphs was apparently due to the greater surface to volume ratio of smaller animals and makes them more vulnerable to environmental stresses whose effects are proportional to the surface area of the animal.

As regards the osmoregulatory ability of *Cloeon crassi*, it is apparent from Fig. 6 that, in the size ranges studied, this species is an efficient hyper-regulator, a constant $\triangle_i$ of 0.54—0.58°C being maintained at $\triangle_e$ levels as low as 0.02°C. Hyporegulation appears less efficient and the $\triangle_i$ begins to rise once the $\triangle_e$ reaches 0.68—0.70°C. Although no determinations were done on small nymphs it appears probable that this increase is more rapid in their case and hence results in a more rapid death.

No experiments to test the effects of acclimation were done but there was a suggestion of acclimatory effects in the finding of 2 late stage nymphs in the estuary in July 1967 at a TDS level of 30,323 ppm. The only laboratory observation on the tolerance of nymphs to slowly increasing TDS levels was done when a batch of 10 late-stage nymphs was placed in artificial river water with TDS content of about 12,000 ppm and this was allowed to evaporate until the TDS content rose to almost 20,000 ppm. No deaths were recorded in this experiment.

The results of tolerance experiments indicate that *Centroptilum excisum* has a much lower level of tolerance.

Comparison of the osmoregulatory abilities of *Cloeon crassi* and *Cloeon africanum* indicated that the normal $\triangle i$ in both species is about 0.57°C. In addition, no sign of any hyporegulatory ability was found in *Cloeon africanum*. SUTCLIFFE's (1962) work on larvae of Trichoptera provides a parallel to this situation in that he also found species within the same genus with markedly different TDS tolerances. *Limnephilus affinis* can hyporegulate and survive in 50% seawater while *L. stigma* cannot survive more than a few days in more than 10% seawater.

Relation of the experimental results to the distribution of Ephemeroptera in the Sundays River

The absence of *Centroptilum excisum* below Station 6 despite its occurrence in the Witrivier indicated that although the adults are apparently capable of dispersing over at least the distance between these two stations, TDS levels in the main river below Station 6 were too high for the nymphs. The tolerance limits found by experiment indicated that TDS levels below Station 6 were favourable in winter. However, numbers of nymphs decreased at this time of year so that when conditions were favourable below Station 6 there were no nymphs to invade the new habitat.

Cloeon africanum is restricted by two factors. Firstly, it shows a preference for stones in currents. Secondly, it is restricted to TDS levels below about 9,000 ppm. These two factors would combine to restrict this species to that section of the river from Station 4a, where the last stony runs were found, upstream and occasionally to parts upstream from Station 5 when TDS levels rise to a limiting level at Station 4a (Part I, FORBES & ALLANSON 1970).

Thus both *Centroptilum excisum* and *Cloeon africanum* are restricted by their apparent lack of any hyporegulatory ability to waters in which the concentration of dissolved solids is less than the normal haemolymph concentration. This supports BEADLE's (1943) suggestion that upper limiting TDS levels are related to the normal haemolymph concentration.

When the effects of different TDS levels were being tested the parameter known as the Median Time of Survival was used. One of the advantages of this method is that it mitigates against the effect of outliers. However, these outliers, and especially the extremely resistant individuals, are of the utmost importance in the extension of the range into waters of a higher TDS level. Transport of stream dwellers by drifting with the current is well known. It is thus worthwhile noting that in 50% seawater, although the MTS was 45—60 minutes, 100% mortality required 45—173 hours in the different replicates. Similarly in 30% seawater the MTS was usually about 24 hours but 100% mortality varied from 63—336 hours.

These extended survival periods only occurred in more mature nymphs. This changing TDS tolerance with age is reflected in the changing composition of the population as the TDS level rises along the river. The decrease in numbers with increase in TDS content is initially due to the disappearance of the juvenile nymphs.

The greater tolerance of the larger, older nymphs could be of significance to the species since periods of high TDS in the river occurred in summer and early autumn when most of the population is close to emergence. Since tolerance is maximal at this time it will ensure that the maximum number of nymphs is able to complete their larval development. Unfortunately nothing appears to be known of the tolerance limits of the eggs to high TDS levels, nor of the hatching period in this species. Both of these factors would be of importance. The hatching period would be of particular significance if it was long enough to ensure that the nymph hatched in late autumn or winter. This would give the TDS sensitive early nymphal stage the maximum time to avoid the high TDS levels of summer.

Acknowledgements

This work was done in partial fulfilment of the requirements for the degree of Master of Science of Rhodes University by A. T. Forbes. The authors wish to acknowledge the support of the South African Council for Scientific and Industrial Research and the Sir Percy FitzPatrick Trust. Our thanks are also due to Dr B. J. Hill for reading and commenting upon this manuscript.

Summary

1. Nymphs of the mayfly *Cloeon crassi* can tolerate total dissolved solids concentration up to 10,500 ppm; these results agree with field observation.

2. Juvenile nymphs are more sensitive than adults to high concentration of dissolved solids.
3. No difference was detected in tolerance experiments on *C. crassi* between media made up with sea water or media of the same ionic composition as Sundays River water.
4. Measurements of haemolymph freezing point depression indicate that *C. crassi* can both hyper-regulate and hypo-regulate, depending upon the concentration of the media.
5. Haemolymph of nymphs of *C. africanum* in freshwater have the same osmotic concentration as that of *C. crassi* but no indication of any hyporegulatory ability was found in other media.
6. Nymphs of *Centroptilum excisum* cannot tolerate concentrations of total dissolved solids greater than 7,000 ppm.
7. The results are discussed in relation to the distribution of the three species in the Sundays River.

Zusammenfassung

1. Die Nymphen der Eintagsfliege *Cloeon crassi* sind imstande eine Lösung von einer 10.500 ppm hohen Konzentration fester Stoffe zu ertragen.
2. Kleine Nymphen sind empfindlicher gegen hohe Konzentrationen solcher Lösungen als große.
3. In Toleranzversuchen mit *C. crassi* wurde kein Unterschied zwischen mit Meerwasser zubereiteten Media und Media von gleicher ionischer Zusammensetzung, wie das Wasser vom Sundays River, festgestellt.
4. Messungen der Haemolymph-Gefrierpunkt-Depression zeigen daß *C. crassi* je nach der Konzentration des Mediums zu Hyper- und Hyporegulierungen fähig ist.
5. Haemolymph der Nymphen der Eintagsfliege *C. africanum* in Süßwasser ist von gleicher osmotischer Konzentration wie *C. crassi*; es wurde jedoch keine Andeutung irgendeiner Hyporegulationsfähigkeit in anderen Media gefunden.
6. Nymphen der Eintagsfliege *Centroptilum excisum* sind unfähig eine Lösung von einer Konzentration höher als 7.000 ppm fester Stoffe zu ertragen.
7. Diese Ergebnisse sind mit Bezug auf die Verbreitung der drei Arten im Gebiet des Sundays River diskutiert.

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