SALT AND WATER REGULATION IN *MACROBDELLA DECORA* (HIRUDINEA: GNATHOBDELLIFORMES) UNDER OSMOTIC STRESS*

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SUMMARY

The anatomy, ultrastructure and innervation of the nephridia of the North American leech, *Macrobdella decora* (Say), are described. The osmotic concentrations of blood, crop fluid and final urine, as well as urine flow under normal conditions, were found to be similar to those of the well-studied European medicinal leech, *Hirudo medicinalis* L.

The capacity of the excretory system after changes in external salinity, and after salt and water loading with artificial blood meals, was investigated. In contrast to *H. medicinalis*, *M. decora* does not tolerate hypertonic environments and is less efficient in rapidly excreting excess salt and water. Three factors make salt and water regulation in *M. decora* different from that in *H. medicinalis*: a slower fluid resorption from the crop, a limited transport capacity of the primary urine-forming cells, and a lower rate of salt reabsorption in the central canal.

INTRODUCTION

Studies of the mechanisms of salt and water regulation in leeches have so far been limited to the European medicinal leech, *Hirudo medicinalis*. The main organs responsible for salt and water balance are the nephridia, and investigations now focus on their control (reviewed by Zerbst-Boroffka & Wenning, 1986; Wenning, 1986*b*). *H. medicinalis* is regarded as specialized for sucking vertebrate blood. The sanguivorous North American leech, *Macrobdella decora*, a close relative of *H. medicinalis*, is considered to be less specialized from an evolutionary point of view (Sawyer, 1986). As shown in a preliminary report (Wenning, 1986*a*), *H. medicinalis* and *M. decora* are similar with respect to nephridial structure and innervation. Physiological differences make *M. decora* ideal for evaluation of the factors that control salt and water regulation.

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* Dedicated to Professor Dr Ernst Florey on the occasion of his 60th birthday.

Salt and water regulation in *M. decora* were investigated and compared with earlier results from *H. medicinalis*. The present study deals with the following questions. How do the two species differ in their responses to salt and water loading? Has *H. medicinalis* evolved a more specialized functional organization to maintain salt and water balance than *M. decora*? Where is this specialization manifest? For ease of comparison, *M. decora* was subjected to the same osmotic stress used in studies on *H. medicinalis* (see Zerbst-Boroffka & Wenning, 1987).

MATERIALS AND METHODS

Specimens of *M. decora* were obtained from a commercial supplier and kept in tap water at 16°C. Experiments were carried out at room temperature (21–24°C), to which the animals had been adapted for 24 h.

For dissection, leeches were opened up dorsally and the excretory system was exposed. To study the flow of urine, stained fluid was injected into either the canaliculus system or the central canal of the nephridia (see also Boroffka, Altner & Haupt, 1970). Procedures for visualizing peripheral innervation of the excretory system and electron microscopy are described in Wenning & Cahill (1986).

Application of osmotic stress followed the procedure described for *H. medicinalis* (Boroffka, 1968; Zerbst-Boroffka, 1973; Wenning, Zerbst-Boroffka & Bazin, 1980). For these experiments, the usual methods of anaesthesia (alcohol, cooling) could not be applied without interfering drastically with the animal's response. Briefly, leeches were pinned through both suckers in an extended position in a dish with a change of tap water every 2 h. Initial muscle contractions ceased within a few minutes. Polyethylene catheters inserted into the urinary bladders allowed continuous monitoring of urine flow for 7–10 h in four midbody segments or collection of urine samples. A flame-polished glass capillary (diameter 0·5 mm) was inserted into the crop through the pharynx to collect samples or infuse salt solutions. Blood samples were taken from the dorsal vessel. The animals were weighed after being blotted with tissue paper.

In crop-loading experiments, 4 ml of the following salt solutions were used: 145 mmol l^{-1} NaCl + 5 mmol l^{-1} KCl (pH 7·4, 285 mosmol kg^{-1} H$_2$O) (hypertonic crop loading); 36 mmol l^{-1} NaCl (pH 7·4, 72 mosmol kg^{-1} H$_2$O) (hypotonic crop loading). To detect regurgitation, Lissamine Green (Chroma, Berlin, FRG) was added to the solutions. Urine flow measurements and determinations of the osmotic concentrations of body fluids (crop content, blood and final urine) were carried out on different animals. Osmotic concentrations were determined with a nanolitre osmometer (Clifton Technical Instruments, NY, USA). After the experiments, leeches were placed in fresh tap water and allowed to swim freely. The survival rate of animals used for physiological experiments did not differ from that of animals without any treatment.

Leeches were subjected to artificial sea water of different salinities: 10 % sea water = 100, 20 % = 200, 30 % = 300 mosmol kg^{-1} H$_2$O. Body mass (as an indicator

of body water) was monitored for 14 days. In other animals, urine flow was monitored in 30% sea water for 2 h.

<div align="center">RESULTS</div>

Anatomy and physiology of the excretory system of untreated leeches

M. decora has a thinner musculature, a thicker crop lining and blood vessels of smaller diameter than has *H. medicinalis*. The excretory system consists of smaller nephridia, larger urinary bladders and a longer inner lobe (Fig. 1; Wenning & Cahill, 1986). The canaliculus cells are interconnected. Their lumina, the canaliculi, form a continuous network throughout the entire nephridium. As seen by injection of stained fluid into the canaliculi, they empty into the central canal which begins in the apical lobe and is surrounded by canaliculus cells along its entire length. Ultrastructure and innervation of the nephridia are similar in both species (Fig. 2; Boroffka *et al.* 1970; Wenning & Cahill, 1986). The urine-forming cells show characteristics of transporting epithelia: the canaliculus cells have apical microvilli, basal infoldings associated with mitochondria (Fig. 2A) and septate junctions (Fig. 2B); central canal cells are more flattened with fewer microvilli or basal infoldings. The basal areas of canaliculus and central canal cells interdigitate. The capillary endothelium in the nephridia is fenestrated (Fig. 2C). Blood and extracellular fluid can therefore be regarded as one compartment for ions and water. Innervation by at least two different neurones was found in the nephridia. Branches of one neurone contain neurofilaments and neurotubules, and lie in the extracellular space between the urine-forming cells and capillaries (Fig. 2B). Endings of a different neurone contain dense core vesicles (mean diameter 97 nm) and small clear vesicles, and lie between the basal infoldings of the canaliculus cells. (Fig. 2A).

Mean urine flow, and the concentration and composition of body fluids, are not different from those described for *H. medicinalis* (Boroffka, 1968; Wenning *et al.* 1980), but Na^+ concentration in the blood is significantly lower in *M. decora* (Table 1).

Table 1. *Urine flow and composition of body fluids of* Macrobdella decora

	Mean ± S.D.	*N*
Final urine concentration (mosmol kg^{-1} H_2O)	24 ± 7	23
Final urine flow ($\mu l\,cm^{-2}\,h^{-1}$)	3 ± 0·7	9
Osmotic inflow of water in 24 h (calculated assuming a 34 cm^2 body surface) (ml)	2·5	
Crop content (mosmol kg^{-1} H_2O)	170 ± 18	5
Blood concentration (mosmol kg^{-1} H_2O)	196 ± 15	6
(mmol l^{-1} Na^+)	100 ± 12	6
(mmol l^{-1} K^+)	3·8 ± 0·9	6
(mmol l^{-1} Cl^-)	35 ± 2·5	6

Effects of increased medium osmolality

As volume and salt flux change with increases in medium osmolality, the time course of adaptation reflects capacities of the excretory system. *M. decora* tolerates 10 % sea water (hypo-osmotic to its blood). In 20 % sea water (iso-osmotic), two out
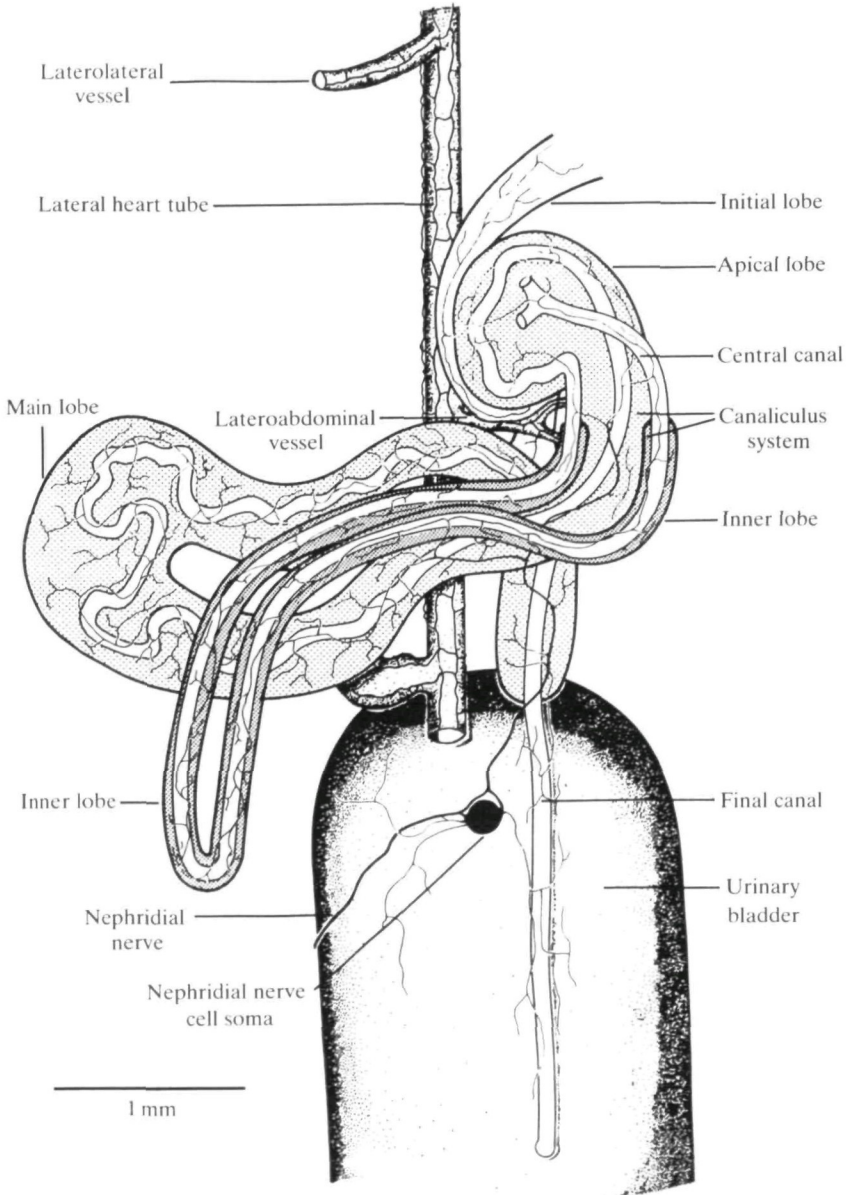

Fig. 1. Schematic drawing of the structure and innervation of a left midbody nephridium in *Macrobdella decora*. The canaliculus system (grey) is not shown in detail. Note the long inner lobe. The canaliculus system accompanies the final canal for a portion of its length. ×25.

of five leeches survived a 14-day experiment, the others died within 1 day (Fig. 3). In 30 % sea water (hyperosmotic), none of the four animals survived. In 30 % sea water, body mass decreased to 60 % and urine flow to zero in 2 h (Fig. 3). Some leeches showed a conspicuous mass increase before they died.

Volume and salt regulation after crop loading

The salt solution used for hypertonic crop loading resembles calf blood in its major ion content and osmolality (Zerbst-Boroffka, 1973). Hypotonic crop loading emphasizes volume loading. Under these conditions, changes in urine flow (Fig. 4) and osmolality of crop fluid, urine and blood (Fig. 5) were determined.

After hypertonic crop loading, urine flow decreased in 3 h to almost zero and then rose in the next 6 h to the control level. Blood osmolality increased, while crop osmolality decreased. As a result, blood and crop fluid became iso-osmotic. At 3 h, urine osmolality reached its maximum (176 ± 32 mosmol kg^{-1} H$_2$O) and remained elevated for at least 72 h. Crop osmolality returned to the control level within 24 h. At 8 h, most of the animal's mass loss (Table 2) could be due to expulsion of urine which was in the bladders prior to crop loading (total urine can account for 30 % of body mass). A discharge of urine shortly after crop loading has been observed. About 50 % of the infused volume is lost in 48 h.

After hypotonic crop loading, urine flow increased by up to three times (to $9 \pm 1 \cdot 7 \mu$l cm^{-2} h^{-1}). Urine concentration increased to 54 ± 14 mosmol kg^{-1} H$_2$O within the first hour and remained elevated for 72 h. Crop osmolality increased to the control level within 24 h. Blood osmolality, determined 3 h after crop loading, decreased. After 8 h, the volume lost through urine flow was 2·3 ml but, taking into account the urine produced during this time due to osmotic water inflow (approx. 1 ml), 'extra' loss was only 1·3 ml (33 % of the infused volume). Body mass indicated that 50 % (=2 ml) of the infused volume was lost in this time (Table 2). Again, this difference may be accounted for by the discharge of urine stored in the bladders prior to crop loading.

Table 2. *Volume loss at various times after crop loading, determined by mass changes*

Time (h)	Hypertonic crop loading mean ± s.d. (*N*)	Hypotonic crop loading mean ± s.d. (*N*)
8–9	8·5 ± 7·6 (8)	49 ± 8 (11)
24	19 ± 14 (7)	72 ± 9 (10)
48	52 ± 16 (6)	81 ± 10 (10)
72	63 ± 14 (6)	85 ± 11 (9)
96	74 ± 13 (3)	85 ± 10 (5)
120	76, 101 (2)	91 ± 13 (5)

100 % = infused volume (4 ml).

lu

c

c

2A

a

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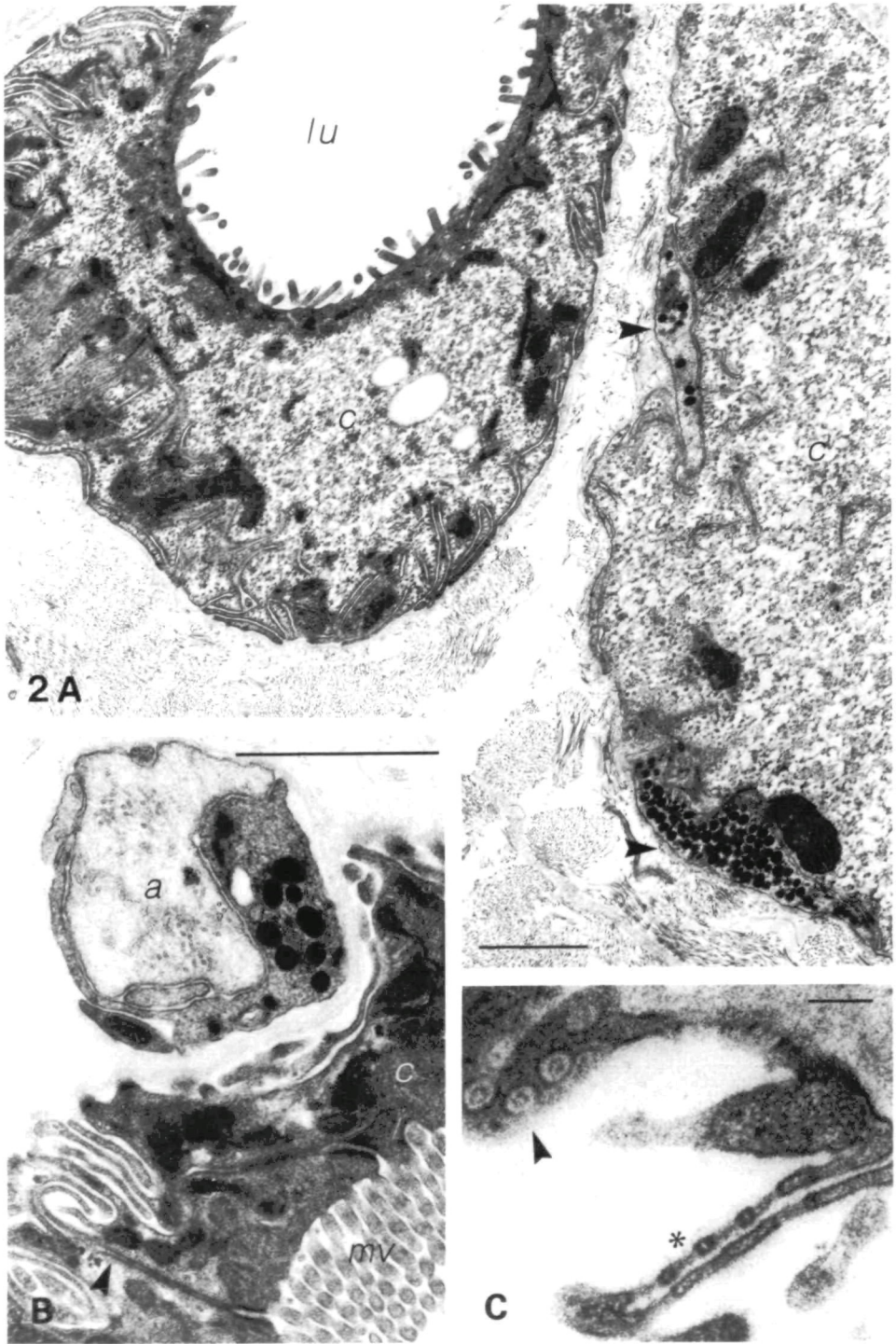
Feeding behaviour

M. decora does not seem to be specialized for sucking vertebrate blood, as stated by Sawyer (1981) and its teeth seem to be too weak to penetrate intact skin. However, remnants of blood meals were occasionally found in the crop. In an attempt to determine the natural way of feeding, five *M. decora* were allowed to feed on fresh beef blood enclosed in beef gut lining. Two animals succeeded in penetrating the tissue and taking a meal. After some of the gut lining had been scraped away, the other leeches fed. They ingested approximately the equivalent of their own body
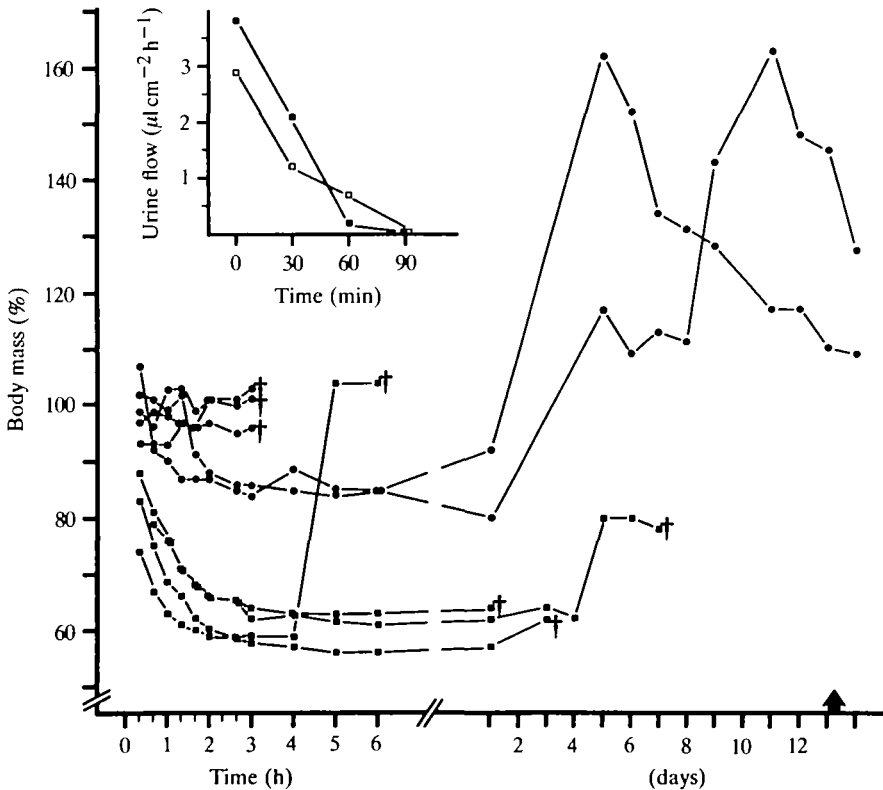
Fig. 3. Effects of increased medium concentration on body mass of individual animals (100 % = initial mass). ■, 30 % sea water; ●, 20 % sea water. In brackish water at time 0; return to tap water (arrow) at day 13; † = animal died. Inset: urine flow of two animals (■, □) subjected to 30 % sea water at time 0.

Fig. 2. (A) Section from the main lobe of a nephridium with two axon profiles containing dense-core vesicles (arrowheads). They lie in the basal region of the canaliculus cells. Scale bar, 1 μm. (B) Section from the inner lobe nephridium with an axon profile (*a*) in the extracellular space between the urine-forming cells. Note the septate junctions between adjacent canaliculus cells (arrowhead). Scale bar, 1 μm. (C) The fenestrated endothelium of a blood capillary in the nephridium sectioned in two different planes. Pores are visible in tangential (arrowhead) and cross section (asterisk). Scale bar, 0·1 μm. *c*, canaliculus cells; *mv*, microvilli; *lu*, lumen.
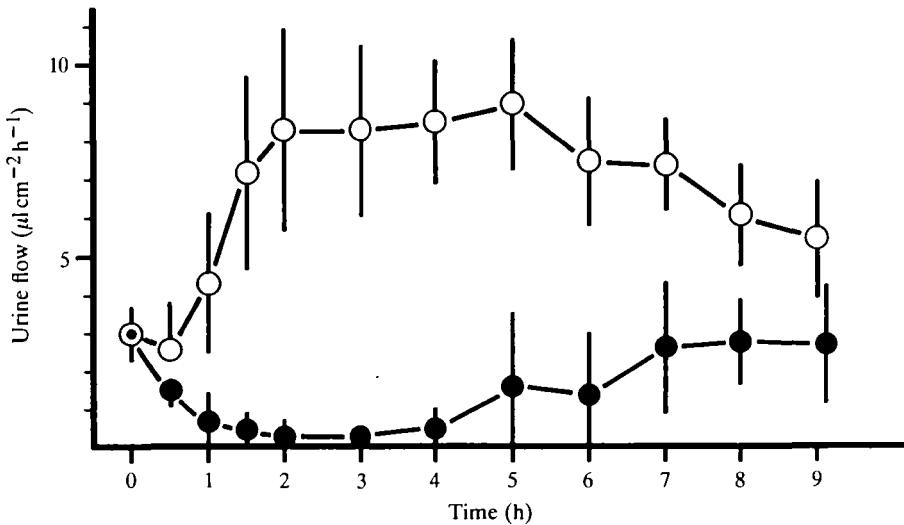
A. WENNING

Fig. 4. Urine flow (μl cm^{-2} h^{-1}; mean ± S.D., $N = 7$) after hypertonic (●) and hypotonic (○) crop loading. Control values are given for time 0, when crop loading started.

mass. When allowed to feed on frogs (*Pipa cortensis*), all leeches ($N = 5$) tried to attach themselves to the prey and probed the frog's skin, but none succeeded in taking a meal. It is concluded that *M. decora* might normally penetrate mucous membranes (e.g. nasal cavities) and ingest small blood meals.

DISCUSSION

Anatomy and physiology of the excretory system

The structure and innervation pattern of the excretory system are similar in the North American leech, *M. decora*, and the European medicinal leech, *H. medicinalis* (Fig. 1; Boroffka *et al.* 1970; Wenning, 1986*a*; Wenning & Cahill, 1986), as are urine flow and the concentration and composition of body fluid (Table 1; Zerbst-Boroffka & Wenning, 1986). Both species lack filtration structures in the nephridia (for *H. medicinalis* see Boroffka *et al.* 1970). This lack and the similar arrangement of urine-forming cells suggest that urine is formed in a similar way. Primary urine is secreted into the canaliculus system. From there it flows into the central canal where most of the salt is reabsorbed, resulting in a strongly hypotonic final urine (Table 1; Zerbst-Boroffka, 1975).

Since drinking is unlikely in a freshwater species and leeches feed only occasionally, final urine production in a given time equals the osmotic inflow of water through the body wall. When permitted to do so, *H. medicinalis* leaves the water, sometimes for long periods; *M. decora* does not. This difference in behaviour might explain the greater variability in final urine flow and concentration in *H. medicinalis* (Table 1; Zerbst-Boroffka, 1973; Wenning *et al.* 1980).

Regulatory capacity under osmotic stress

H. medicinalis tolerates salinities up to 40 % sea water (= 400 mosmol kg^{-1} H$_2$O) and invades brackish water (Boroffka, 1968). *M. decora* is less tolerant. The initial reaction to increased salinities is the same in both species. In the second phase of adaptation, volume regulation in *H. medicinalis* is characterized by salt gain in both iso- and hyperosmotic media. This, in turn, permits volume inflow and mass increase
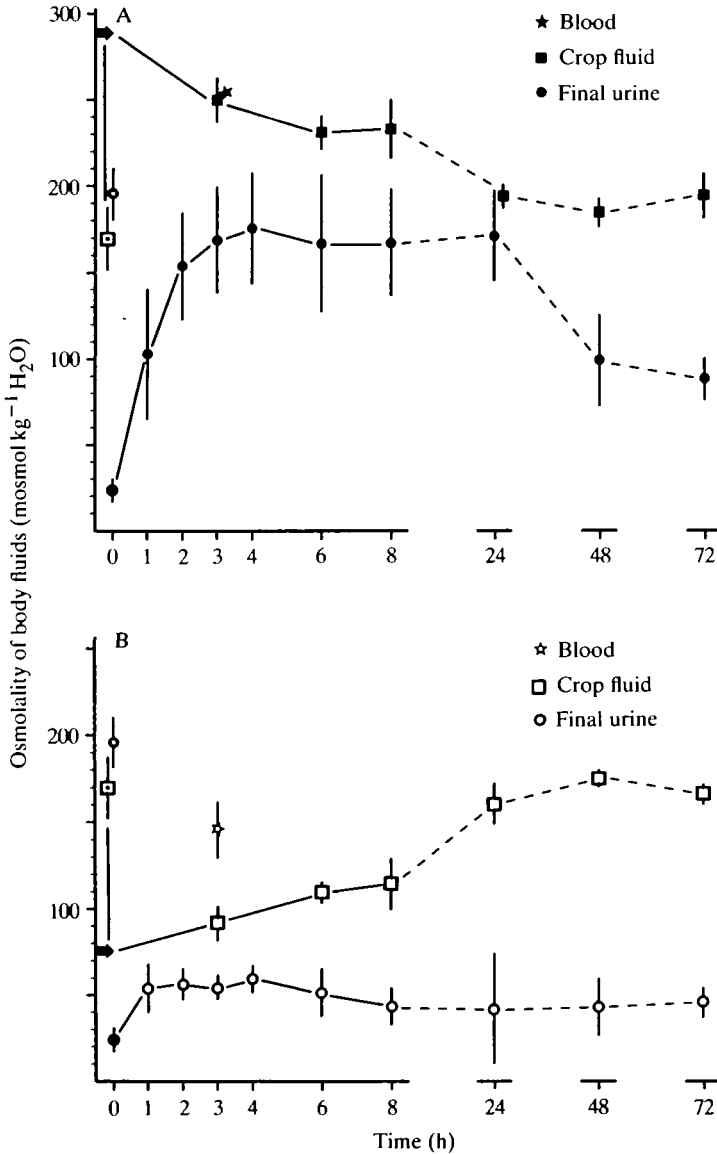

Fig. 5. Changes in the osmolality (mosmol kg^{-1} H$_2$O; mean ± S.D., $N = 4$–10) of crop fluid, urine and blood after hypertonic (●, A) and hypotonic (○, B) crop loading. Control values (▣ crop fluid, ✪ blood, ⊙ urine) are given for time 0, when crop loading started. Arrows indicate concentrations of the infused salt solution.

(Boroffka, 1968). *M. decora* apparently does not tolerate salt gain and the concomitant volume inflow to this extent: mass increase is maximal just before death (Fig. 3).

After hypertonic crop loading, volume and salt excretion is limited in two ways in *M. decora*: urine is less concentrated than in *H. medicinalis* (Fig. 5) (Zerbst-Boroffka, 1973; Wenning *et al.* 1980) and its flow even decreased (Fig. 4). In *M. decora*, salt excretion increases maximally by seven-fold (in *H. medicinalis* 60-fold) immediately after hypertonic crop loading. Consequently, *M. decora* requires more time to excrete excess salt and water present in an artificial meal. Mass loss is about $0.5 \, \text{g day}^{-1}$ (Table 2). This requires 20 % more urine $(= 0.6 \, \mu\text{l cm}^{-2}\text{h}^{-1})$ than normal. Extrarenal volume output (e.g. *via* the gut), as shown by the comparison of mass loss and urine volume, cannot be excluded, but is less important than nephridial activity (maximal discrepancy 15 %).

The qualitative and quantitative differences of the response to osmotic stress in the two species imply differences in fluid resorption from the crop, in the rate of primary urine formation by the canaliculus cells, and in salt reabsorption by the central canal cells.

After hypertonic crop loading, resorption of hyperosmotic fluid from the crop begins immediately in *H. medicinalis* (Zerbst-Boroffka, 1973; Wenning *et al.* 1980). *M. decora* is capable only of iso-osmotic volume resorption: the increase in urine flow at 3 h (Fig. 4) indicates that net volume resorption from the crop has begun. At that time, blood and crop fluid are iso-osmotic. These results indicate that salt resorption from the crop is less effective in *M. decora* and limits its ability to process large meals.

After hypotonic crop loading, fluid resorption from the crop occurs with the osmotic gradient and crop osmolality increases with time in both leech species (Fig. 5; Wenning *et al.* 1980), but at different rates. In *M. decora*, crop osmolality returns to the control level within 24 h; in *H. medicinalis*, within 4 h. Furthermore, while urine flow in *H. medicinalis* increases eight-fold within 30 min, it increases only three-fold and much more slowly in *M. decora* (Fig. 4). Is volume output in *M. decora* limited only by slow resorption or, additionally, by a limited transport capacity of the urine-forming cells? Initially, volume turnover can be expected to increase rapidly after crop loading. The slow increase in urine flow indicates that the transport capacity of the primary urine-forming cells is also limited. As similar mechanisms of urine formation are assumed for both species, urine volume depends mainly on the rate of primary urine formation in the canaliculus cells (Zerbst-Boroffka, 1975).

Final urine concentration depends on the rate of salt reabsorption in the central canal. *M. decora* does not concentrate the final urine to the same degree as does *H. medicinalis* (Fig. 5; Zerbst-Boroffka, 1973; Wenning *et al.* 1980), indicating a different capacity of the central canal cells.

Control mechanisms of volume and salt excretion

In *H. medicinalis* (Wenning *et al.* 1980) and in *M. decora* (present investigation) the mechanisms controlling urine volume are independent of those controlling urine

osmolality (Figs 4, 5). Furthermore, the actual blood concentration does not determine final urine concentration (Fig. 5): urine osmolality increases after both hyper- and hypotonic crop loading. Final urine concentration depends on salt gain regardless of the volume in which the salt was contained.

In *H. medicinalis*, volume excretion depends on blood volume and is not correlated with blood osmolality (Zerbst-Boroffka, 1973, 1978; Wenning *et al.* 1980). This is also assumed to be true of *M. decora*. First, after hypotonic crop loading, volume turnover, blood volume and urine flow increase whereas blood osmolality decreases. Second, if *M. decora* is depleted of water osmotically (e.g. by transfer into hypertonic medium), urine flow decreases. The initial rapid mass loss will lead to a decrease in blood volume.

Nervous regulation of nephridial activity is assumed to be another means of control. The nephridia of *M. decora* are as densely innervated as those of *H. medicinalis* (Wenning & Cahill, 1986). Nerve branches in the extracellular space (Fig. 2B) presumably originate from the peripheral nephridial nerve cells, proposed as salt receptors in *H. medicinalis* (Wenning, 1986*b*). Branches of other neurones contain dense-core vesicles and contact the canaliculus cells (Fig. 2A). The equivalent neurones in *H. medicinalis* are suggested to mediate primary urine formation (Wenning, 1986*b*).

Salt and volume turnover

As blood volume has not been determined, salt and volume turnover are calculated by assuming the same relative blood (and extracellular) volumes in both leech species. In *H. medicinalis*, blood volume is about 10 % of the body mass and total extracellular water is 20 % (Hildebrandt, 1987). Blood volume increases maximally by 30 % after crop loading (Zerbst-Boroffka, 1978; Hildebrandt, 1987). For *M. decora*, blood volume is assumed to be 200 μl, containing 45 μosmol of salt.

The decrease in crop fluid osmolality in *M. decora* after hypertonic crop loading (Fig. 5) is mostly due to net water inflow and salt outflow rather than to resorption of large quantities of hypertonic fluid as in *H. medicinalis* (Wenning *et al.* 1980). This is shown by a rough calculation. In 3 h, 0·3 ml of water enters the leech passively due to the osmotic gradient, but only 0·07 ml of urine is excreted. If the remaining 0·23 ml of water were to remain in the blood or extracellular space, these compartments would increase in volume by 100 and 50 %, respectively. As blood vessels do not show any dilation during blood sampling, the 0·23 ml of water is assumed instead to be lost into the crop, leading to a decrease in crop osmolality to about 268 mosmol kg^{-1} H$_2$O. Since crop osmolality is even lower (250 mosmol kg^{-1} H$_2$O), salt (18 mosmol kg^{-1} H$_2$O, 4·23 ml = 76 μosmol) has left the crop with the osmotic gradient, but only a fraction (9 μosmol) has been excreted in this time. It is assumed that the remaining 67 μosmol are stored (e.g. intracellularly) and/or passively lost through the body wall. The increase in crop fluid osmolality (Fig. 5) in *M. decora* after hypotonic crop loading is mostly due to salt inflow into the crop rather than to resorption of large quantities of fluid less hypotonic than the one infused, as shown for *H. medicinalis* (Wenning *et al.* 1980). In 3 h, only 0·37 ml of

additional final urine is lost (0·67 ml minus 0·3 ml normal rate), but 0·957 ml of net water outflow is required to increase crop osmolality to 92 mosmol kg^{-1} H$_2$O and to excrete 35 μosmol. The increase in blood volume would then be 400 %. It is more likely that crop fluid volume decreases to 3·63 ml (4 ml minus 0·37 ml additional urine), containing 335 μosmol instead of the original 316 μosmol. Extra salt (35 + 19 = 54 μosmol) can be derived from intracellular storage, reduced integumental salt loss (due to decreased blood osmolality) or – less likely – from an increase in active salt uptake through the integument.

In conclusion, *M. decora* does not tolerate an environment hypertonic to its own blood and does not excrete extra salt and water as quickly as its close relative, *H. medicinalis*. Both species have more difficulty coping with salt than with volume stress, but *H. medicinalis* is capable of excreting larger amounts of salt (Wenning *et al.* 1980). The differences in response to salt and water loading imply the same evolutionary trend (i.e. higher specialization in *H. medicinalis*) considered by Sawyer (1986). As the structure and function of the excretory system are similar in both species, this study provides an excellent basis for evaluating the factors that might control nephridial activity.

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