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Ammonia and urea excretion in the tidepool sculpin (*Oligocottus maculosus*): sites of excretion, effects of reduced salinity and mechanisms of urea transport

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Abstract

Tidepool sculpins live in a variable environment where water temperature, salinity, gas tensions, and pH can change considerably with the daily tide cycle. Tidepool sculpins are primarily ammoniotelic, with 8–17% of nitrogen wastes excreted as urea. The majority of net ammonia (J^{net}_{amm}; 85%) and urea (J^{net}_{urea}; 74%) excretion occurred across the gill, with the remainder excreted across the skin, the kidney, and/or gut. Acute (2h) exposure to 50% seawater significantly increased J^{net}_{urea} (2.8-fold), but reduced J^{net}_{amm} (3.5-fold). In fish exposed to 50% seawater for 1 week, J^{net}_{urea} returned to control values, but J^{net}_{amm} remained slightly depressed. Unidirectional urea influx (J^{in}_{urea}) and efflux (J^{out}_{urea}) were measured using ^{14}C-urea to determine if urea was excreted across the gills by simple diffusion or by a carrier-mediated mechanism. J^{in}_{urea} increased in a linear manner with increasing urea water levels (0–11 mmol N l^{-1}), while J^{out}_{urea} was independent of external urea concentrations. As well, J^{net}_{urea} and J^{out}_{urea} were not significantly different from one another, indicating the absence of "back transport". Urea analogs and transport inhibitors added to the water did not have any consistent effect on unidirectional urea flux. These results demonstrate that ammonia and urea excretion rates and sites of excretion in tidepool sculpins are very similar to those found in other marine and freshwater teleosts. Urea and ammonia may play a role in osmoregulation as excretion rates and tissue levels were influenced by changes in water salinity. Finally, we found no evidence for a specific urea carrier; branchial urea excretion is likely dependent on simple diffusion.

Introduction

The intertidal zone is an environment of great variability; at low tide (approximately twice a day) tidepools are cut off from the open ocean. Organisms which live in these habitats must tolerate substantial changes in water temperature, gas tensions, pH, and salinity on a daily basis (Dejours 1981) and even air exposure lasting 3–4h every day, in some instances (Davenport and Sayer 1986; Kormanik and Evans 1991). Many tidepool fish have special mechanisms to cope with their unstable environment (Laming *et al.* 1982; Bridges 1988; Horn and Gibson 1988; Pelster *et al.* 1988).

In the present study, we focused on the characteristics of nitrogenous waste excretion in the tidepool sculpin, *Oligocottus maculosus*, a fish found almost exclusively in small rock pools in the intertidal zone of the Pacific northwest coast, and one that is not known to survive out of water for extended periods. The population density of these fish was often quite high, especially in smaller tidepools. Some fish were found in tidepools near the highest tide level, and therefore, at certain times

of the lunar cycle, the seawater was not renewed for several days. Due to the variable environmental conditions, we proposed that *O. maculosus* would have special mechanisms to cope with nitrogenous waste excretion.

Tidepool fish may be exposed to variations in water salinity due to evaporation or heavy rainfall. Very few studies have addressed the influence of water salinity on nitrogen excretion in teleosts and the information that is available is contradictory. For instance, ammonia excretion was unaffected by a decrease in salinity in two intertidal fish, *Blennius pholis* and *Periophthalamus sobrinus*, but urea excretion was greatly increased in the former (Sayer and Davenport 1987a), and greatly decreased in the latter (Gordon *et al.* 1965). In elasmobranchs, where urea is an important osmolyte, the situation is more clearcut. Euryhaline skate markedly increase renal urea clearance, decrease branchial urea excretion, and decrease urea tissue levels when acclimated to 50% seawater (Goldstein and Forster 1971; Payan *et al.* 1973; Forster and Goldstein 1976). Internal urea levels in most teleosts are only 1–10% of those in marine elasmobranchs, and urea is generally not considered to be an important osmolyte. Nevertheless, blood-to-water urea gradients are 5–50 fold greater than the corresponding ammonia gradients; Wood (1993) suggested that urea retention in teleosts might also serve an adaptive role. If urea is involved in osmoregulation in intertidal teleosts, then one would expect an initial increase in urea excretion, resulting in a decrease in tissue urea levels, in response to dilute seawater exposure. We tested this hypothesis in sculpin exposed to 50% seawater for 2h (acute) and 1 week (chronic).

Another aspect of nitrogen excretion that may be affected by environmental conditions, is the partitioning of excretion between the gills, kidney, gut and/or skin. Homer Smith (1929) first reported that the major site of nitrogen excretion in freshwater fish was across the gills (Smith 1929), but the contribution of other routes may become greater in seawater fish (*e.g.*, Read 1968; Morii *et al.* 1978; Sayer and Davenport 1987b; for review see Wood 1993). In the intertidal blenny, only about 50% of nitrogen was excreted by the head region (gills), while the remaining half was excreted by the "back end" (skin, kidneys, and/or gut) of the fish (Sayer and Davenport 1987b). We performed a partition experiment in which the front end of the sculpin was separated from the back end to determine the major sites of ammonia and urea excretion.

Many studies in recent years have focused on the mechanisms of branchial ammonia excretion in freshwater and seawater teleosts (*e.g.*, Maetz 1972; Wright and Wood 1985; Cameron and Heisler 1983; Evans and Cameron 1986; Evans *et al.* 1989; Wright *et al.* 1993), but comparatively little work has been done on the mechanisms of urea transport. There are two fundamental ways in which urea crosses cell membranes, either through specialized membrane transporters or by diffusion through non-specific aqueous pores (for review see Marsh and Knepper 1992). The traditional view of urea transport is that urea crosses all cell membranes by unspecified pathways. Substantial evidence exists, however, for active or facilitated urea transport across a variety of tissues in elasmobranchs, amphibians, and mammals (*e.g.*, Schmidt-Nielsen and Rabinowitz 1964; Levine *et al.* 1973; Kaplan *et al.* 1974; Curci *et al.* 1976; Hays *et al.* 1977; Katz *et al.* 1981; Brahm 1983; Chou and Knepper 1989; Effros *et al.* 1992). In some amphibians, active urea uptake mechanisms in the skin aid retention of elevated internal urea levels (Katz *et al.* 1981; Rapoport *et al.* 1989; Lacoste *et al.* 1991). It is not known whether a specialized urea transport protein exists in gill epithelial membranes. Boylan (1967) concluded that dogfish gills were relatively impermeable to urea and that this impermeability, rather than retention of urea through active transport mechanisms, was responsible for the maintenance of high internal urea levels in marine elasmobranchs. To identify the mechanisms of urea transport in the tidepool sculpin, we employed ^{14}C – urea to measure unidirectional urea transport (*i.e.*, J^{in}_{urea} = influx from water to animal; J^{out}_{urea} = efflux from animal to water) in relation to external urea concentration, along with independent measurements of net urea excretion (J^{net}_{urea}). We also examined the influence of a range of competitive urea analogs (acetamide, thiourea, methylurea) and urea transport inhibitors (phloretin, 1-(3,4-dichlorophenyl)-2-thiourea) on urea fluxes in the tidepool sculpin.

Materials and methods

Animals

Tidepool sculpins (*Oligoclittus maculosus*) weighing 1.37 ± 0.12 g (mean ± SEM) were collected from tidepools near the Bamfield Marine Station, Bamfield, B.C. Fish were held in an outdoor, fibreglass tank supplied with flow-through seawater (pH approx. 7.8, salinity approx. 31‰, temperature 12°C) for at least 3 days before experimentation. Fish were fed fresh mussels on alternate days. In many nitrogen excretion studies, fish are starved for 1 week prior to experimentation to eliminate the influence of diet on excretion rates. In this study, however, we chose to study animals fed up to 24h before experimentation in order to evaluate more typical nitrogen excretion rates. Faeces were removed from the experimental chamber prior to the start of each experiment and newly excreted faecal matter was not observed over the course of the experiments.

Experimental protocol

Eight series of experiments were performed.

1) A control experiment was conducted, in which net ammonia (J^{net}_{amm}) and urea (J^{net}_{urea}) excretion rates were determined over an 8h period, as well as ammonia and urea tissue concentrations (N = 6). Fish were placed in individual plastic chambers (indoors) containing approximately 80 ml of continuously-aerated seawater 24h before the start of the experiment. To determine net excretion rates, the volume of the aerated chamber was reduced to approximately 35 ml, and water samples (5 ml) were collected (0, 2, 4, 6 and 8h; volumes not replaced), frozen, and later analysed for urea and ammonia concentrations. At the end of the experiment, the fish were weighed, killed by a blow to the head, and frozen in liquid N_2. Tissue was ground to a fine powder in a pre-cooled mortar and pestle containing liquid N_2. The tissue was processed for measurement of urea and ammonia concentrations (see below).

2) An antibiotic series (antibiotic antimycotic solution (Sigma): 10,000 U penicillin, 10 mg streptomycin, 25 μg amphotericin B ml^{-1}, diluted (100 ×) was performed to determine if urea excreted by the fish over the 8h period was subsequently degraded to ammonia through the action of microbial urease (microbes may have been released in faecal material) (N = 6). As in series 1, J^{net}_{amm} and J^{net}_{urea} were determined over an 8h period.

3) A partition series was conducted where the "front end" (gills) and "back end" (skin, gut and/or kidney) excretion pathways were determined for J^{net}_{amm} and J^{net}_{urea} and unidirectional urea efflux (J^{out}_{urea}; see below) (N = 9). Fish, injected 24h previously with ^{14}C-urea (see below), were placed in a divided chamber where the front end of the chamber (50 ml) was separated from the back end (22 ml) by a rubber membrane (dental dam) placed immediately behind the pectoral fins. The procedure was performed without anaesthesia and was completed within 5 min. The patency of the membrane was achieved by elastic tension (*i.e.*, no stitches or glue) and was checked at the end of the experiment. Fish were left for 10 min before the start of the experiment. Water samples (5 ml) were collected from the aerated front and back chambers at 0 and 2h, for later analysis of ^{14}C-urea radioactivity, total urea, and ammonia concentrations. At the end of 2h, the fish was killed by a blow to the head, frozen in liquid N_2, and later processed for measurements of internal ^{14}C-urea radioactivity and total urea concentration (see below).

4) An acute low salinity series was performed in which fish were exposed to 50% seawater and J^{net}_{amm} and J^{net}_{urea} were determined (N = 6). Net excretion rates were measured in an initial 2h control period, followed by a 2h exposure to 50% seawater, and finally, a 2h recovery period (100% seawater). Water samples were collected at 0 and 2h for the control, 50% seawater, and recovery periods for later analysis of urea and ammonia concentrations.

5) A chronic low salinity series was performed in which fish were exposed to 50% seawater for 8 days prior to determination of J^{net}_{amm}, J^{net}_{urea}, and unidirectional urea influx (J^{in}_{urea}; see below) and tissue ammonia and urea concentrations. Fish (N = 6) were held in a large aerated chamber (5l) in

50% seawater for 8 days and fed until day 7. A similar regime was repeated for a group of 6 control fish (100% seawater). One day before experimentation, sculpins were placed in individual aerated chambers (80 ml) and food was withheld. For J^{in}_{urea} measurements (see below), 6 small fish (body weight 0.57 ± 0.11 g) were held in a single aerated chamber (80 ml) for 24h before and during the experiment. J^{net}_{amm}, J^{net}_{urea}, and J^{in}_{urea} were measured over a single 2h period.

6) A unidirectional flux series was performed to characterize the dependence (or lack thereof) of unidirectional urea influx (J^{in}_{urae}) on external urea over the concentration range of 0.2 to 11.0 mmol N l^{-1}. J^{in}_{urea} was determined (see below) in 4 separate groups of fish (N = 6 per group) at external urea concentrations of 0.2, 1.25, 5.0 and 11.0 mmol N^{-1}.

7) Another unidirectional flux series was performed to characterize the dependence (or lack thereof) of unidirectional urea efflux (J^{our}_{urea}) on external urea over the same concentration range as in series 6. J^{out}_{urea} was determined (see below) in a group of fish (N = 8) injected 24h previously with ^{14}C-urea and transferred sequentially at 2h intervals to progressively higher external urea concentrations similar to those of series 6, but including also 0 mmol N l^{-1}.

8) A pharmacological series was conducted to investigate the mechanism(s) of urea transport. J^{in}_{urea}, J^{out}_{urea}, and J^{net}_{urea} were determined in the presence of urea analogs (acetamide, thiourea, methylurea; 5.0 mmol N l^{-1} to approximately match internal urea concentration) and specific urea transport inhibitors (phloretin, 1-3(3,4-dichlorophenyl)-2-thiourea (DCPTU); 0.25 mmol l^{-1}; N = 35) added to the external water. Phloretin (0.25 mmol l^{-1}) blocks facilitated urea transport in mammalian red blood cells and kidney tubules (*e.g.*, Brahm 1983; Chou and Knepper 1989). The inhibitor, DCPTU (0.5 mmol l^{-1}) has been shown to reversibly inhibit urea flux in frog urinary bladder epithelia (Martial *et al.* 1993). We chose a somewhat lower concentration of DCPTU (0.25 mmol l^{-1}) as the affinity of DCPTU for the urea transporter is known to be relatively high, for example, DCPTU has an inhibition constant (K_I) of 0.01 mmol l^{-1} in red cell membranes (Mayrand and Levitt 1983). Although urea analogs, phloretin and DCPTU have not been used in aquatic experiments in previous studies, their effects on urea transport in isolated tissues have been shown to be reversible (Chou and Knepper 1989; Martial *et al.* 1993). J^{net}_{urea}, J^{out}_{urea}, and J^{net}_{amm} were measured in an initial 2h control flux, a 2h experimental period (urea analogs (5.0 mmol N l^{-1}) *i.e.*, similar to internal urea levels) or inhibitors (0.25 mmol l^{-1})), and a final 2h recovery period. J^{in}_{urea} was measured in a separate group of fish over a single 2h period in the presence "cold" urea added to the water (5.0 mmol N l^{-1}) and either an urea analogue (5.0 mmol N l^{-1}) or inhibitor (0.25 mmol l^{-1}. Phloretin was dissolved in ethanol to a final water concentration of 0.4%. Control measurements were performed on fish exposed to 0.4% ethanol alone. DCPTU was dissolved in DMSO to a final water concentration of 0.5% (control experiment = 0.5% DMSO alone).

Unidirectional urea flux measurements

J^{in}_{urea} was determined by measuring the accumulation of ^{14}C-urea in the fish when the label was placed in the water. To determine J^{in}_{urea}, 0.013 μCi of ^{14}C – urea (ICN) per μmol l^{-1} of cold urea was added to 15 ml of aerated water containing the fish. After a 10 min mixing period, water samples (1.4 ml) were removed at 0 and 8h. Fish were placed in a 50 mmol l^{-1} urea solution for approx. 1 min at the end of the experiment to displace any ^{14}C-urea bound to the surface of the fish. Fish were killed by a blow to the head and weighed before digestion in NCS tissue solubilizer (50 mg tissue ml^{-1} NCS; Amersham). Tissue digests (2.1 ml) were mixed with acetic acid (60 μl) and OCS scintillation fluor (10 ml; Amersham) and radioactivity was measured with a Nuclear-Chicago (Unilux) scintillation counter. Water ^{14}C – urea was determined by mixing 0.1 ml of the experimental water with 5 ml of fresh seawater and 10 ml of scintillation fluor (ACS, Amersham). The efficiency of the 2 counting systems (65% for ACS versus 69.5% in OCS) was quantified using internal standardization and corrected appropriately (7% higher in OCS system).

To determine J^{out}_{urea}, ^{14}C-urea (4 μCi or 20 μl g^{-1} fish) in saline (160 mmol l^{-1} NaCl; 20–60 μl) was injected intraperitoneally into each fish 24h prior to the experiment to allow internal equilibration. Fish were left to recover in individual chambers during this period. The water in each chamber was replaced with 30–60 ml of fresh seawater containing the appropriate "cold" urea concentration and 0 and 2h samples (5 ml) were collected. At the end of the experiment, fish were placed in 50 mmol l^{-1} urea solution for approximately 1 min (see above), weighed, killed by a blow to the head, and frozen in liquid N_2. Tissue was ground to a fine powder in a pre-cooled mortar and pestle containing liquid N_2. A portion of the ground tissue was digested in NCS and counted, as above. The rest of the tissue was processed for measurement of total urea concentration (see below). Water ^{14}C-urea was determined by mixing 1 ml of the experimental water with 4 ml of fresh seawater and 10 ml of scintillation fluor.

Measurements

Water samples

Water ammonia levels were measured with the salicylate-hypochlorite assay (Verdouw *et al.* 1978) and urea levels by the diacetyl-monoxime method (Rahmatullah and Boyde 1980). Net urea-N (J^{net}_{urea}) and net ammonia-N (J^{net}_{urea}) excretion rates were calculated as follows:

$$J^{net} = \frac{(T_f - T_i)\ V}{Wt},$$

where T_i and T_f refer to initial and final concentrations of water ammonia or urea in nmol N l^{-1} (note 1 μmol urea = 2 μmol N), V is the volume of the system in liters, t is the elapsed time in h, and W is the weight of the fish in g.

Unidirectional urea influx (J^{in}_{urea} was calculated by monitoring the appearance of ^{14}C-urea radioactivity in the fish relative to its mean specific activity in the external water (SA_{ext} = cpm ml^{-1} water per μmol urea N ml^{-1} water) over the flux period. The average of the initial and final SA_{ext} was employed, but in practice the two figures were virtually identical.

$$J^{in}_{urea} = \frac{^{14}\text{C cpm in fish}}{Wt} \times \frac{1}{SA_{ext}}$$

Unidirectional urea efflux (J^{out}_{urea}) was calculated by monitoring the appearance of ^{14}C-urea radioactivity in the closed volume of external water over time relative to its mean specific activity in the fish (SA_{int} = cpm g^{-1} fish μmol urea-N g^{-1} fish). The SA_{int} could only be measured directly at the end of the experiment by terminal sampling. The measured value was then corrected to an integrated value for the entire flux period using the measured loss ^{14}C cpm to the water and the assumption of simple exponential turnover of the internal pool. A linear model gave virtually identical results.

$$J^{out}_{urea} = (\Delta \frac{^{14}\text{C cpm } l^{-1} \text{ in water})}{Wt} \times V \times \frac{1}{SA_{int}}$$

Tissue ammonia and urea levels

Tissue urea and ammonia concentrations were determined on whole animals. Fish were ground to a fine powder in liquid N_2 (see above). A portion of the tissue powder was immediately added to 5 volumes of ice-cold trichloroacetic acid (8%) and vortexed. The mixture was neutralized with saturated $KHCO_3$. The neutralized solution was centrifuged at 12,000 × g (2 min) at room temperature. Ammonia levels in the supernatant were determined with a commercial enzymatic kit (Sigma 171-c) and urea levels were measured as described above. Water content of the tissue was determined on a subsample of the ground fish tissue by drying to a constant weight. Final tissue concentrations were expressed as mmol N l^{-1} of tissue water.

Statistics

Data are presented as means ± SEM (N). Student's paired or unpaired t-test were employed as appro-

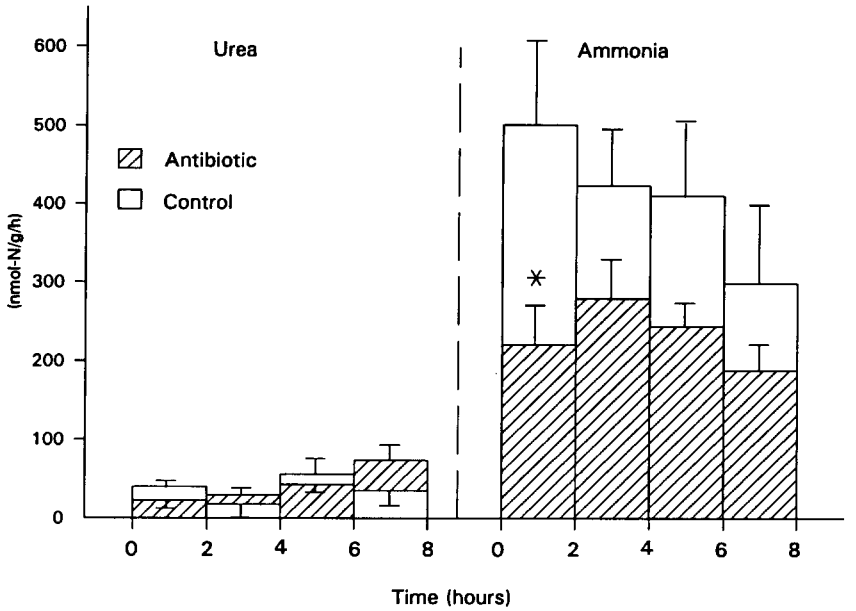

Fig. 1. Urea and ammonia excretion rates in control and antibiotic-treated sculpins (N = 6).

priate, to evaluate the significance of differences between mean values ($p < 0.05$). An ANOVA was used for comparisons of unidirectional fluxes at different external urea concentrations (Fig. 5) and the *t* value was adjusted by the Bonferroni procedure in the case of multiple comparisons.

Results

Urea and ammonia excretion rates and tissue concentrations

Net urea and ammonia excretion rates are shown in Figure 1. As in most teleosts, the majority of nitrogen wastes were excreted as ammonia, with approximately 8–17% of total nitrogen (ammonia + urea) excreted as urea. There was a step-wise decrease in ammonia excretion (but not urea excretion) over the 8h experiment, probably due to the accumulation of excreted ammonia in the external water (Wright and Wood 1985; Wright *et al.* 1993). Tissue urea concentration in control fish was 3.67 ± 0.50 mmol N l^{-1} tissue water (N = 25) and ammonia concentration was 1.17 ± 0.54 mmol N l^{-1} tissue water (N = 19).

To determine if urea excreted to the water during the 8h experiment was degraded by microbial urease (*e.g.*, released in faeces), we measured urea and ammonia excretion rates in the presence of antibiotic added to the water. There were no significant differences in urea excretion rates between control and antibiotic-treated fish over the course of the experiment (Fig. 1). Ammonia excretion, however, was significantly lower in antibiotic-treated fish between 0–2h compared to the control fish at the same time (Fig. 1).

Sites of urea and ammonia excretion

Water in contact with the front end of the fish was separated from that in the back end by placing the fish in a divided chamber. Front end values in Figure 2 represent excretion mostly across the gills and back end values indicate excretion *via* the kidneys, gut and/or skin. The majority of J^{net}_{amm} (85%), J^{net}_{urea} (75%), and J^{out}_{urea} (66%) occurred across the gills. Kidney, gut and/or skin J^{net}_{amm}, J^{net}_{urea}, and J^{out}_{urea} accounted for 15%, 26%, and 34% of total excretion, respectively.

J^{out}_{urea} and J^{net}_{urea} values were almost identical

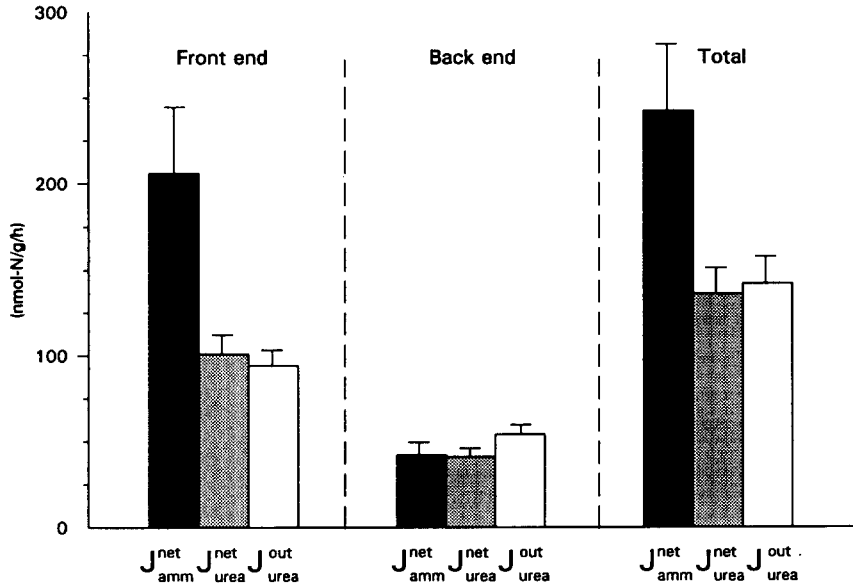

Fig. 2. Net ammonia (J_{amm}^{net}) and urea (J_{urea}^{net}) excretion rates and urea efflux (J_{urea}^{net}) across the gills (front end) and skin, kidney, and/or intestine (back end) in sculpin placed in a divided chamber. The third panel (Total) represents the sum of front end and back end excretion rates (N = 9). There were no significant differences between J^{net}_{urea} and J^{out}_{urea}.

(Fig. 2), indicating that the major direction for urea transport is from the fish to the water and little "back flux" occurred. The percentage of urea relative to total nitrogen (ammonia + urea) excretion was relatively high (36%) in this series compared to other experiments (control values in Fig. 1, 3, 4 were 8–17%).

Exposure to 50% seawater: acute and chronic effects

When fish were exposed to 50% seawater for 2h, there was a 2.8-fold increase in J^{net}_{urea} and a 3.5-fold decrease in J^{net}_{amm} (Fig. 3). Consequently, urea excretion accounted for 67% of total nitrogen excretion (ammonia + urea) during acute exposure to dilute seawater. Following return to 100% seawater, J^{net}_{urea} and J^{net}_{amm} recovered to control excretion rates (Fig. 3). In fish exposed to 50% seawater for 1 week, J^{net}_{urea} was similar to the control group (100% seawater) but there was a trend towards reduced J^{net}_{amm} in 50% seawater ($p < 0.06$; Fig. 4). Urea, therefore, accounted for about 20% of total nitrogen excretion under these conditions. J^{in}_{urea} (in the presence of 0.1 mmol N l^{-1} external urea) was approximately 5-fold higher in fish acclimated to 50% seawater (2.33 ± 0.47 nmol N g^{-1} h^{-1} (N = 5)) compared to control animals (0.50 ± 0.09 nmol N g^{-1} h^{-1} (N = 6)). Tissue urea levels were not significantly affected by chronic exposure to 50% seawater. Tissue ammonia levels were significantly lower in the 50% seawater group (1.38 ± 0.07 mmol N L^{-1} (N = 6) compared to the control group (2.28 ± 0.20 mmol N l^{-1} (N = 6)) for this experiment.

Urea transport mechanisms

To determine if branchial urea transport was by passive diffusion or *via* a specialized transporter, we measured unidirectional urea transport in the presence of increasing concentrations of external urea. There was a linear relationship between the concentration of urea added to the water and J^{in}_{urea} (Fig. 5), in contrast to the saturation kinetics which might be expected with an inward transport system. It should be noted that J^{in}_{urea} does not only represent branchial uptake, but also uptake

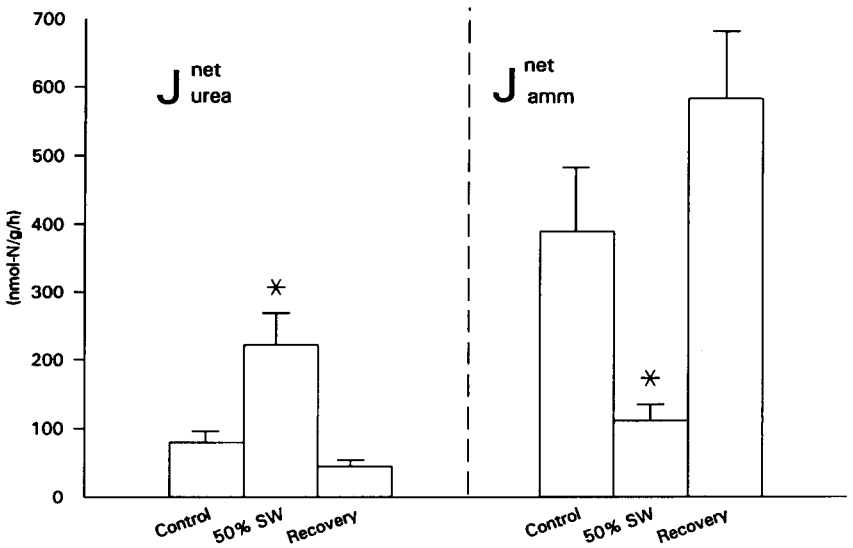

Fig. 3. Net urea (J_{urea}^{net}) and ammonia (J_{amm}^{net}) excretion rates in sculpins exposed to 50% seawater for 2h. Control and recovery measurements were made when fish were held in normal (100%) seawater (N = 6).

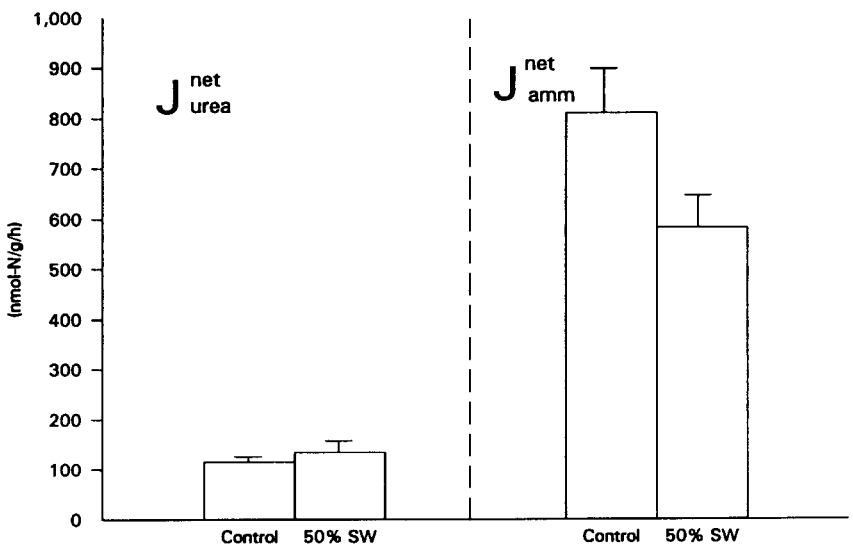

Fig. 4. Net urea (J_{urea}^{net}) and ammonia (J_{amm}^{net}) excretion rates in sculpins acclimated to 50% seawater for 1 week (N = 12).

due to drinking. Assuming that the drinking rate is similar to that in other marine teleosts (10 ml kg^{-1} h^{-1}; Evans, 1993), then up to 50% of uptake could be attributed to drinking. J^{out}_{urea} was independent of external urea concentration (Fig. 5), in contrast to the pattern of excretion with external concentration which would be expected if an exchange diffusion or back transport mechanism were present. Regardless of the absolute J^{in}_{urea} value, the results of these unidirectional flux experiments indicate that a specialized branchial urea transporter does not exist.

If urea transport is dependent on a specialized membrane transporter, then urea analogs in the water should competitively inhibit urea transport. Acetamide did not significantly alter J^{net}_{urea},

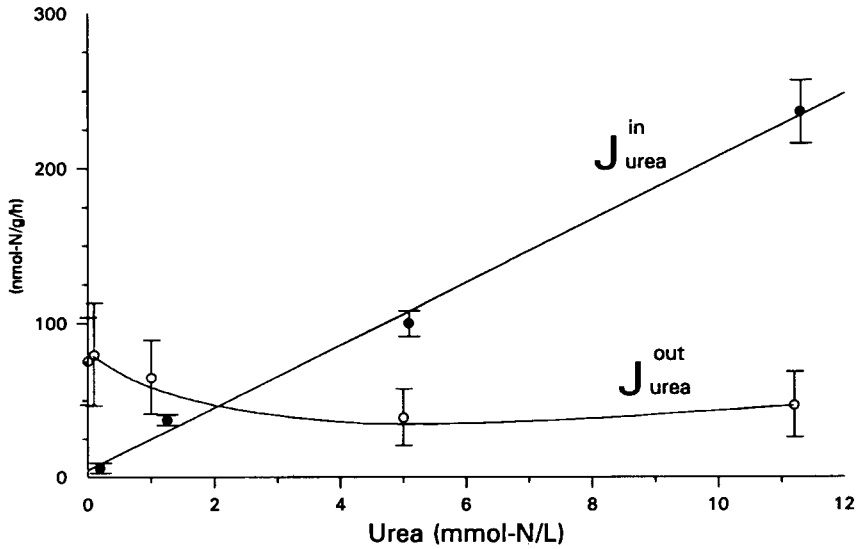

Fig. 5. Urea influx (J_{urea}^{in}; N = 6) and efflux (J_{urea}^{out}; N = 6) in sculpin exposed to increasing concentrations of urea in the external water.

Table 1. Effects of the urea analogs, acetamide (5.0 mmol N l^{-1}) added to the water on unidirectional urea fluxes (influx = J^{in}_{urea}*, efflux = J^{out}_{urea}) and net urea (J^{net}_{urea}) and ammonia (J^{net}_{amm}) excretion in the presence of 5.0 mmol N l^{-1} "cold" urea in the external medium

	J^{in}_{urea} (N = 17)	J^{out}_{urea} (N = 13)	J^{net}_{urea} (N = 13)	J^{net}_{amm} (N = 13)
Control	88.1 ± 8.6	88.5 ± 24.7	96.0 ± 14.4	756.2 ± 127.9
Acetamide	75.9 ± 9.1	103.2 ± 30.5	133.5 ± 25.8	635.2 ± 77.5
Recovery	ND	63.6 ± 14.1	75.7 ± 10.7	558.1 ± 56.1

Data are shown as mean ± SEM (μmol N $kg^{-1} \cdot h^{-1}$); ND = not determined; *J^{in}_{urea} was measured in the presence of 5.0 mmol N l^{-1} of "cold" urea in the external medium.

Table 2. Effects of the urea transport inhibitor, phloretin (0.25 mmol l^{-1} in 0.4% ethanol), on unidirectional urea fluxes (influx = J^{in}_{urea}*, efflux = J^{out}_{urea}) and net urea (J^{net}_{urea}) and ammonia (J^{net}_{amm}) excretion

	J^{in}_{urea} (N = 12)	J^{out}_{urea} (N = 12)	J^{net}_{urea} (N = 12)	J^{net}_{amm} (N = 12)
Control	29.4 ± 8.5	43.6 ± 6.5	41.9 ± 7.0	501.4 ± 64.6
Phloretin	52.7 ± 9.1	39.1 ± 7.5	50.3 ± 7.5	389.9 ± 43.7
Recovery	ND	37.3 ± 5.6	32.3 ± 5.0	330.9 ± 59.0

Data are shown as mean ± SEM (μmol N $kg^{-1} \cdot h^{-1}$); ND = not determined; *J^{in}_{urea} was measured in the presence of 5.0 mmol N l^{-1} of "cold" urea in the external medium.

J^{out}_{urea}, J^{in}_{urea}, and J^{net}_{amm} (Table 1). As well, methylurea and thiourea did not significantly alter J^{net}_{urea}, J^{in}_{urea}, and J^{net}_{amm} (data not shown). Likewise, the urea transport inhibitor, phloretin, had no consistent effects on excretion flux rates (Table 2). Only J^{in}_{urea} was measured in the presence of the specific inhibitor. DCPTU and values were not significantly different from control values

(data not shown). Neither ethanol (0.4%) nor DMSO (0.5%) alone (control) influenced net excretion rates and unidirectional fluxes.

Discussion

The tidepool sculpin lives in an environmental in which water conditions can change markedly throughout the daily tide cycle. Despite this environmental variability, the pattern of nitrogen excretion was very similar to that of other teleost fish, *O. maculosus* were primarily ammoniotelic, with only 8–17% of nitrogen excreted as urea (Fig. 1). The major site of excretion was across the gills, but a significant amount of nitrogen, approximately 20%, was excreted by the "back end" of the fish (kidney, gut, and/or skin) (Fig. 2). Marine species, in general, appear to excrete a lower proportion of nitrogen wastes across the gills (~60%) compared to freshwater fish (~90%) (for review, see Wood 1993). In the immersed dab, *Limanda limanda* and emersed blenny, *B. pholis* and *Alticus kirki*, the skin is thought to play a major role in nitrogen excretion (Sayer and Davenport 1987b; Rozemeijer and Plaut 1993). The blenny has no scales (Horn and Gibson 1988) and this probably facilitates transfer of materials across the skin. Sculpins have scales and consequently, the skin may be less permeable, but it was not possible to differentiate between excretion across the skin, through the kidneys, or the gut in our experiments.

Ammonia and urea excretion rates were relatively variable, probably as a result of variations in individual feeding history. To ensure that gut microbes were not responsible for degradation of excreted urea, net urea and ammonia excretion were measured in the presence and absence of a broad spectrum antibiotic added to the water (Fig. 1). Urea excretion rates were unaffected by the antibiotic, indicating that microbial urease was not active in the experimental chamber. Ammonia excretion rates, on the other hand, were lower (significantly different 0–2h) in antibiotic-treated fish. The decrease in ammonia excretion may have resulted from a number of cell surface and/or metabolic effects typical of such a broad-based antibiotic treatment (see Materials and methods).

In the divided chamber experiments (Fig. 2), water in the front chamber was separated from the back chamber by a rubber dam which surrounded the fish. This confinement for several hours probably placed the fish under some stress. One indication of possible stress-related problems was the fact that the ratio of urea to total nitrogen (ammonia + urea) excretion rates was higher (~36%; Fig. 2) than for other free swimming control animals (8–17%; Fig. 1, 3, 4). An activation of ureagenesis in response to stress has been reported in the marine toadfish, *Opsanus beta*, subjected to confinement or crowding (Walsh *et al.* 1994). Unlike the majority of teleost fish, the toadfish has a functional ornithine-urea cycle (OUC) (Mommsen and Walsh, 1989). When confined to small volumes of water with conspecifics, glutamine synthetase activity was induced and urea excretion was stimulated, but how this response is regulated is not understood (Walsh *et al.* 1994). Clearly, the relationship between stress and ureagenesis needs to be further investigated.

Fish in small tidepools may experience marked reductions in water salinity during periods of heavy rainfall. The acute increase in urea excretion (Fig. 3) in response to 50% seawater may have been due to an increase in urea synthesis or an osmoregulatory strategy to reduce tissue urea levels, and/or a general increase in gill permeability. The first possibility seems unlikely, as the change in urea synthesis rates would have had to be relatively rapd (<2h). The second possibility, an osmoregulatory response, has not been studied in teleosts. In elasmobranchs, however, a reduction in external salinity increases renal urea clearance and results in lower tissue urea concentrations (Goldstein *et al.* 1968; Goldstein and Forster 1971; Payan *et al.* 1973; Forster and Goldstein 1976). Tissue urea levels in elasmobranchs, however, are two orders of magnitude greater than those in *O. maculosus* and therefore, urea probably contributes very little to osmoregulation.

Our results support the third possibility, a general increase in gill permeability. Isaia (1982) demonstrated that gill permeability to small nonelectrolytes, such as urea, was higher in freshwater-adapted *vs.* seawater-adapted rainbow trout. Similar findings were reported by Masoni and Payan

(1974) in the eel, *Anguilla anguilla*. In *O. maculosus*, urea influx ($^{in}_{urea}$) was more than 5-fold higher in fish acclimated to dilute seawater, also indicating an increase in gill urea permeability. Changes in gill permeability in acclimated fish may be due to changes in gill membrane phospholipid composition (Zwingelstein 1980; Daikoku *et al.* 1982). Incorporation of more sphingomyelin in gill epithelial membranes of saltwater-adapted fish, decreases the permeability of molecules that passively diffuse across the membrane (Zwingelstein 1980). Short term changes in gill permeability may be attributed to an increase in circulating catecholamines. Epinephrine greatly increases the permeability of the gill to small hydrophilic molecules, including water and urea (Isaia *et al.* 1978).

If there was a general increase in gill permeability to nonelectrolytes, then one would expect an increase in ammonia excretion rates as well, assuming that ammonia was excreted predominantly as a nonelectrolyte (NH_3). In contrast to urea excretion, there was a dramatic decrease in ammonia excretion in fish acutely exposed to 50% seawater (Fig. 3). The explanation for these results may relate to the mode of ammonia excretion. Evans *et al.* (1989) reported that *Opsanus beta*, excreted 43% of ammonia was NH_4^+, with the remainder as NH_3. NH_4^+ excretion was by both simple diffusion (presumably through paracellular channels) and by NH_4^+ $(H^+)/Na^+$ exchange. Moreover, when long-horn sculpin were exposed to reduced external Na^+ levels (20% seawater), there was a marked effect on the level of Na^+/H^+ exchanged (Claiborne and Perry 1991). A 50% reduction in external Na^+ levels in the present experiment, therefore, may explain, in part, the dramatic decrease in ammonia excretion. Furthermore, dilute seawater exposure would decrease the transepithelial potential (TEP) across the gills (Potts 1984). If the TEP was more negative in 50% seawater, then positively charged species would tend to be retained. After 1 week of acclimation to dilute seawater, ammonia excretion rates had almost returned to control values (Fig. 4), but tissue ammonia levels were significantly reduced. These results suggest that ammonia production was depressed in fish exposed to 50% seawater, but the physiological explanation for this effect is not clear.

Urea transport mechanisms

Considerable attention has been focused on the mechanisms of branchial ammonia transport in teleost fish (see Introduction), but very little work has been conducted on the mechanisms of urea transport in fish. Isaia (1982) reported that small nonelectrolytes, such as water and urea, were transported across the gill epithelium by a transcellular pathway. He suggested that these molecules were transported by passive diffusion because there was a linear correlation between their permeability and their oil/water partition coefficients. Marine elasmobranchs that retain urea have relatively impermeable gills with respect to urea (Boylan 1967) and transport is also thought to be by passive diffusion. In tissues with relatively high urea permeabilities, such as, mammalian renal inner medullary collecting ducts (*e.g.*, Chou and Knepper 1989) and red blood cells (*e.g.*, Brahm 1983), amphibian urinary bladders (*e.g.*, Levine *et al.* 1973), among others, specialized transporters facilitate urea passage across the membrane. In these tissues, urea analogs inhibit urea transport in a competitive fashion and phloretin and DCPTU partially or completely urea transport. To determine if branchial urea transport in *O. maculosus* was dependent on a specialized protein carrier, we measured unidirectional urea transport in the presence of urea, urea analogs, and urea transport inhibitors. The linear increase in J^{in}_{urea} (Fig. 5) and independence of J^{out}_{urea} (Fig. 6) with increasing external urea concentrations indicates that branchial urea transport in the sculpin is by passive diffusion, not by a facilitated transport process. The fact that the urea analogs, acetamide (Table 1), methylurea, and thiourea, and the inhibitors, phloretin (Table 2) and DCPTU, had no consistent effects on net and unidirectional urea flux supports this conclusion.

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