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Exposure to air, but not seawater, increases the glutamine content and the glutamine synthetase activity in the marsh clam *Polymesoda expansa*

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Summary

Polymesoda expansa **spends a considerable portion of its life exposed to air in mangrove swamps where salinity fluctuates greatly. Thus, the aim of this study was to evaluate the effects of aerial exposure (transfer from 10‰ brackish water directly to air) or salinity changes (transfer from 10‰ brackish water directly to 30‰ seawater) on nitrogen metabolism in *P. expansa*. We concluded that *P. expansa* is non-ureogenic because carbamoyl phosphate (CPS) III activity was undetectable in the adductor muscle, foot muscle, hepatopancreas and mantle when exposed to brackish water (control), seawater or air for 17 days. It is ammonotelic as it excretes nitrogenous wastes mainly as ammonia in brackish water or seawater. After transfer to seawater for 17 days, the contents of total free amino acids (TFAA) in the adductor muscle, foot muscle, hepatopancreas and mantle increased significantly. This could be related to an increase in protein degradation because exposure to seawater led to a greater rate of ammonia excretion on days 15 and 17, despite unchanged tissue ammonia contents. Alanine was the major free amino acid (FAA) in *P. expansa*. The contribution of alanine to the TFAA pool in various tissues increased from 43–48% in brackish water to 62–73% in seawater. In contrast, in clams exposed to air for 17 days there were no changes in alanine content in any of the tissues studied. Thus, the functional role of alanine in *P. expansa* is mainly connected with intracellular osmoregulation. Although 8.5–16.1% of the TFAA pool of *P. expansa* was attributable to glutamine, the glutamine contents in the adductor muscle, foot muscle, hepatopancreas and mantle were unaffected by 17 days of exposure to seawater. However, after exposure to air for 17 days, there were significant increases in ammonia content in all these tissues in *P. expansa*, accompanied by significant increases in glutamine content (2.9-, 2.5-, 4.5- and 3.4-fold, respectively). Simultaneously, there were significant increases in glutamine synthetase activities in the adductor muscle (1.56-fold) and hepatopancreas (3.8-fold). This is the first report on the accumulation of glutamine associated with an upregulation of glutamine synthetase in a bivalve species in response to aerial exposure, and these results reveal that the evolution of glutamine synthesis as a means for detoxification of ammonia first occurred among invertebrates.**

Key words: evolution, alanine, glutamine, glutamine synthetase, clam, *Polymesoda expansa*.

Introduction

While bivalves are considered to be aquatic animals, marsh clams *Polymesoda* spp. spend a considerable portion of their lives exposed to air during low tides. For instance, *Polymesoda caroliniana* is found most abundantly in areas of marsh in north Florida that are covered in water for only 12% of the year (Duobinis-Gray and Hackney, 1982). In Singapore, a related species, *Polymesoda expansa*, is commonly found in channels and pools formed by small streams running between the roots of mangrove plants in the landward fringe of mangroves. During spring tides the salinity of the pool is around 20‰ and at neap tides between 11 and 20‰ (Morton, 1988). However, *P. expansa* may not always be exposed to brackish water, because it is rarely covered by the tides in its natural habitat. In the laboratory, it can withstand considerable periods (up to months) of aerial exposure.

When exposed to air, lower intertidal and subtidal bivalves usually close their shells and shift to anaerobic metabolic pathways (Widdows et al., 1979; de Zwaan, 1983). In contrast, *P. expansa* gapes and slightly exposes the mantle margins posteriorly during aerial exposure (Morton, 1988). Adduction of the valves immediately following emersion and at periodic intervals helps to ventilate the mantle cavity (McMahon, 1988), and gas exchange occurs largely across the surface of the mantle. Such an adaptation sustains aerobic metabolism in

the tissues of *Polymesoda* spp. during long periods of aerial exposure. Thus, *P. caroliniana* is capable of the same rate of O_2 uptake whether in air or submerged in seawater (Deaton, 1991). The air-breathing capacity of *P. caroliniana* is exceptional among bivalve species found in high intertidal habitats; consequently, there is no marked bradycardia and no evidence of accumulation of metabolites indicative of anaerobic metabolism in this marsh clam during emersion (Deaton, 1991).

Although *P. expansa* is not confronted with hypoxia during long-term aerial exposure, the mobilization of proteins and amino acids for energy supply would lead to the release of ammonia (Bishop et al., 1983). Indeed, ammonia accumulates in the haemolymph and mantle cavity fluid of *Mytilus edulis* exposed to air (Shick et al., 1988). Since ammonia is toxic, 'terrestrial' bivalves like *P. expansa* have to defend themselves against ammonia toxicity during long-term emersion. Therefore, this study was undertaken to determine the effects of aerial exposure (17 days) on nitrogen metabolism in *P. expansa*. In order to confirm that the effects and phenomena observed related specifically to aerial exposure, we also determined the effects of salinity changes (from 10‰ to 30‰) on this marsh clam for direct comparison.

Ammonia is detoxified to urea as an excretory product in many vertebrates, including a few fish species, the majority of amphibians, some reptiles and all mammals (Campbell, 1973). The synthesis of excretory urea in certain land planaria (Campbell, 1965), earthworms (Bishop and Campbell, 1963, 1965) and snails (Campbell and Bishop, 1970; Campbell and Speeg, 1968; Tramell and Campbell, 1972) *via* the arginine–ornithine–urea cycle (OUC) indicates that the modification of the basic nutritional pathway of arginine synthesis for ammonia detoxification first took place among invertebrate animals (Campbell, 1973). However, Andrews and Reid (1972) were unable to detect activities of carbamoyl phosphate synthetase III (CPS III), a crucial enzyme of the OUC, in the tissues of several bivalve species (*Mytilus californianus*, *Anodonta kennerlgi*, *Saxidomus giganteus* and *Compsomyax subdiaphana*). At present, no bivalve is known to possess a functional OUC (Bishop et al., 1983). Thus, the hypothesis tested in this study was that, like other bivalves, CPS III was absent from the tissues of *P. expansa*, and that this marsh clam was incapable of detoxifying ammonia to urea during aerial exposure despite the harsh environment conditions in its natural habitat.

In mammalian brains, ammonia toxicity can be ameliorated transiently through the action of glutamine synthetase (GS), resulting in the synthesis of glutamine. The accumulation of glutamine consequently leads to astrocyte swelling and brain damage (Brusilow, 2002). The capacity for detoxification of ammonia to glutamine in higher vertebrates can be traced back to fish. Several species of tropical air-breathing fishes can detoxify ammonia to glutamine not only in the brain, but also in liver, muscle, stomach and gut, during exposure to terrestrial conditions or environmental ammonia (Peng et al., 1998; Jow et al., 1999; Ip et al., 2001b, 2004a; Chew et al., 2001; Anderson et al., 2002; Tay et al., 2003; Lim et al., 2004). In contrast, glutamine is not known to be accumulated as a product of ammonia detoxification in tissues of invertebrates, although it functions as a minor osmolyte in certain species (Livingstone, 1985). Tracer studies using ^{14}C-glucose with clams, mussels, oysters, and pulmonate and prosobranch snails indicate that all these molluscs have a strong capacity for rapid biosynthesis of glutamate, alanine and aspartate, but the capacity for glutamine biosynthesis is weak or nonexistent (see review by Bishop et al., 1983). Glutamine synthesis is energy dependent; one mole of ATP is hydrolysed for each mole of amide-N formed. To date, no bivalve is known to accumulate glutamine during emersion, because bivalves usually shift to anaerobic energy metabolism and accumulate alanine, and sometimes alanopine, during aerial exposure (Bishop et al., 1983).

P. expansa is capable of withstanding aerial exposure without undergoing anaerobiosis or a reduction in metabolic rate, so it is an ideal specimen to study whether the capacity for the detoxification of ammonia to glutamine first evolved among invertebrates. Therefore, in this study, we aimed to verify that glutamine accumulation occurred in association with increased ammonia levels in clams exposed to terrestrial conditions but not in those exposed to seawater, when ammonia could be excreted freely. We also aimed to demonstrate that glutamine accumulation occurred in association with an upregulation of GS activities in tissues of clams exposed to air.

Materials and methods

Animals

Specimens of *Polymesoda expansa* Mousson 1849 (39.3–80.2 g) were collected from mudflats of the mangrove swamp at Kranji, Singapore. They were maintained in 15 l of brackish water (10‰) with aeration in glass aquaria (43 cm×28 cm×30 cm, L×W×H) and fed newly hatched *Artemia salina*. Water was changed every 3 days. The clams were acclimatized to laboratory conditions for at least 1 week before, and food was withdrawn 3 days prior to, experimentation.

Determination of wet mass of tissues

The mass of a clam (with shells) was obtained using a Libror EB-280M balance (Shimadzu, Kyoto, Japan) to the nearest 0.01 g. It was then forced open to dissect out the hepatopancreas, adductor muscle, foot muscle and mantle. Results obtained from individual tissues and organs were expressed as percentage of total clam mass (with shells). To determine the water content in tissue samples, the wet masses were recorded to the nearest 0.001 g. They were then dried in an oven at 95°C until constant mass and the dry mass recorded. The water content of a sample was calculated as differences between the wet mass and the dry mass, and expressed as percent of wet mass tissue.

Experimental conditions

For exposure to seawater, groups of 10 clams were transferred from brackish water directly to full strength seawater (30‰) in glass aquaria (43 cm×28 cm×30 cm, L×W×H). They were kept in seawater for 17 days, during which no food was supplied and water was changed daily. For aerial exposure, groups of 10 clams were exposed to terrestrial conditions in an uncovered dry glass aquarium for 17 days (87–90% humidity). Clams kept in brackish water and fasted for the same period served as controls. On day 17, clams were forced open and the haemolymph was collected from the foot muscle with a syringe and needle. After that, the adductor muscle was cut. The hepatopancreas, mantle, adductor muscle and foot muscle were dissected and freeze-clamped with liquid nitrogen-precooled aluminium tongs. Haemolymph samples were deproteinized in two volumes of trichloroacetic acid (TCA) and then centrifuged at 4000 ***g*** for 15 min to obtain the supernatant. Samples collected were kept at –80°C until analysis.

Determination of ammonia and urea excretion rates

To determine the rates of ammonia and urea excretion in *P. expansa* in water, clams were weighed and kept individually in 120 ml of brackish water (10‰) or seawater (30‰) with slight aeration in cylindrical containers (7.5 cm×8.5 cm, D×H), and the external media were changed daily. On days 3, 6, 9, 12 and 17, water samples (2 ml) were collected and acidified with 20 µl of 2 mol l^{-1} HCl for ammonia and urea analyses. Samples were kept at 4°C and analyses were done within a week. Ammonia was determined according to the method of Anderson and Little (1986). Urea content was analyzed as described by Jow et al. (1999). Rates of excretion are presented as µmol day^{-1} g^{-1} clam (with shell). No attempt was made to determine the rates of ammonia and urea excretion in clams exposed to air. Although certain amounts of ammonia and urea could be excreted into the mantle cavity fluid during aerial exposure, the fluid was kept within the animal and was in direct contact with various tissues.

Enzyme assays

The hepatopancreas, mantle, adductor muscle and foot muscle were homogenized three times in 5 volumes (w/v) of ice-cold extraction buffer containing 50 mmol l^{-1} Hepes (pH 7.6), 50 mmol l^{-1} KCl and 0.5 mmol l^{-1} EDTA, using an Ultra Turrax (Janke and Kundel, Staufeni, Staufen, Germany) homogeniser at 24 000 revs min^{-1} for 20 s each separated by intervals of 10 s off. The homogenate was centrifuged at 10 000 ***g*** and 4°C for 15 min to obtain the supernatant, which was subsequently passed through a 10 ml Bio-Rad P-6DG column (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with ice-cold extraction buffer without EDTA. The filtrate was used directly for enzyme assay.

CPS III (E.C. 2.7.2.5) activity was determined in the presence of glutamine, *N*-acetylglutamate and uridine triphosphate as described by Anderson and Walsh (1995). Radioactivity was measured using a Wallac 1414 liquid scintillation counter (Wallac, Oy, Finland). The CPS III activity was expressed as µmol [^{14}C]-urea formed min^{-1} g^{-1} wet mass. GS (E.C. 6.3.1.2) transferase activity was assayed according to the method of Shankar and Anderson (1985). The formation of γ-glutamylhydroxymate was determined at 500 nm using a Shimadzu UV 160 recording spectrophotometer. The GS activity was expressed as µmol γ-glutamylhydroxymate formed min^{-1} g^{-1} wet mass.

Determination of ammonia, urea and free amino acids (FAAs)

The frozen sample was weighed, ground to a powder in liquid nitrogen, and homogenized three times in 5 volumes (w/v) of 6% TCA using an Ultra-Turrax homogenizer at 24 000 revs min^{-1} for 20 s each separated by intervals of 10 s off. The homogenate was centrifuged at 10 000 ***g*** and 4°C for 15 min to obtain the supernatant.

For ammonia determination, the pH of the supernatant was adjusted to 5.5–6.0 with 2 mol l^{-1} $KHCO_3$. Ammonia was determined according to the methods of Bergmeyer and Beutler (1985). Urea was determined as described by Jow et al. (1999). The difference in absorbance of the sample with and without urease treatment was used to estimate the urea concentration in the sample. For FAA analysis, the supernatant obtained was adjusted to pH 2.2 with 4 mol l^{-1} lithium hydroxide and diluted appropriately with 0.2 mol l^{-1} lithium citrate buffer (pH 2.2). FAAs were analyzed using a Shimadzu LC-6A amino acid analysis system (Kyoto, Japan) with a Shim-pack ISC-07/S1504 Li-type column. Although a complete free amino acid analysis was performed on every sample, only the contents of alanine, glutamate, glutamine, glycine, taurine and total FAA (TFAA) are presented in this report. Results are expressed as µmol g^{-1} wet mass tissue or µmol ml^{-1} haemolymph, as appropriate.

Statistical analyses

Results are presented as means ± the standard error of the mean (S.E.M.). Student's *t*-test or analysis of variance (ANOVA) followed by multiple comparisons using Duncan's procedure was used to evaluate differences between means in groups where appropriate. Arsine transformation was applied to percentage results before statistical analyses. Differences where $P<0.05$ were regarded as statistically significant.

Results

Exposure to seawater or air for 17 days had no significant effects on the water contents of the adductor muscle, foot muscle, hepatopancreas and mantle of *P. expansa* (Table 1).

Ammonia was excreted by *P. expansa* during the 17 days of exposure to brackish water or seawater, but no urea was detected in the external media (Fig. 1). On day 17, there was a significant increase in the rate of ammonia excretion in clams exposed to brackish water (Fig. 1). For clams exposed to seawater, there were significant increases in rates of ammonia excretion on day 15 and day 17, and these rates were

Table 1. *Effects of exposure to brackish water (10‰; control) seawater (30‰) or air (emersion) for 17 days on the water content in various tissues of* Polymesoda expansa

Tissue	Water content (% wet mass)		
	Brackish water (control)	Seawater	Emersion
Adductor muscle	86.9±0.54	85.2±2.01	84.4±1.06
Foot muscle	83.3±0.25	79.2±1.98	79.7±0.48
Hepatopancreas	87.7±0.94	85.2±1.39	87.7±1.91
Mantle	90.4±1.14	87.9±0.34	88.9±0.52

Values are means ± S.E.M. (N=3).

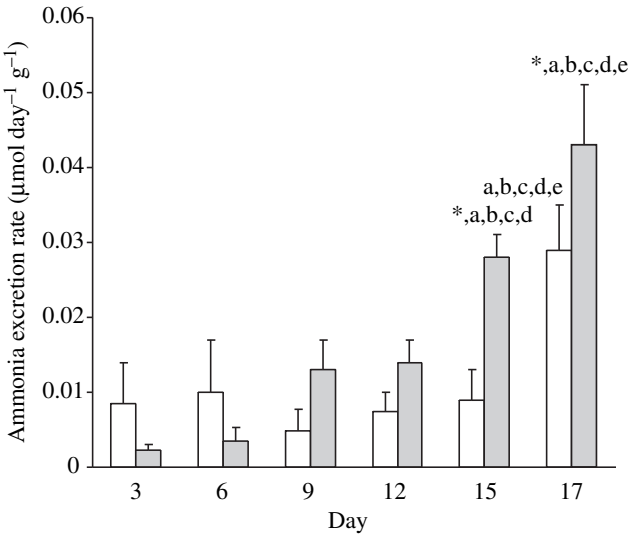

Fig. 1. Time course (17 days) of the effects of exposure to brackish water (10‰, control; white bars) or seawater (30‰; grey bars) on the rate (μmol day^{-1} g^{-1} clam) of ammonia excretion in *Polymesoda expansa*. Values are means ± S.E.M. (N=5). *Significantly different from the corresponding brackish water value (P<0.05); [a]significantly different from the day-3 value (P<0.05); [b]significantly different from the day-6 value (P<0.05); [c]significantly different from the day-9 value (P<0.05); [d]significantly different from the day-12 value (P<0.05); [e]significantly different from the day-15 value (P<0.05).

significantly greater than those of the corresponding control in brackish water (Fig. 1).

There were no detectable CPS III activities (detection limit=0.001 μmol min^{-1} g^{-1} tissue) in hepatopancreas, mantle, adductor muscle and foot muscle of *P. expansa* kept in brackish water, seawater or terrestrial conditions for 17 days. In spite of high background absorbance readings, treatments of samples with urease revealed that no urea was present in these tissues.

Ammonia contents in the adductor muscle, foot muscle, hepatopancreas, mantle and haemolymph of *P. expansa* kept in seawater for 17 days were comparable to those of clams kept in brackish water (control) for the same period (Fig. 2). In contrast, there were significantly greater levels of ammonia in all these tissues in clams after 17 days of aerial exposure

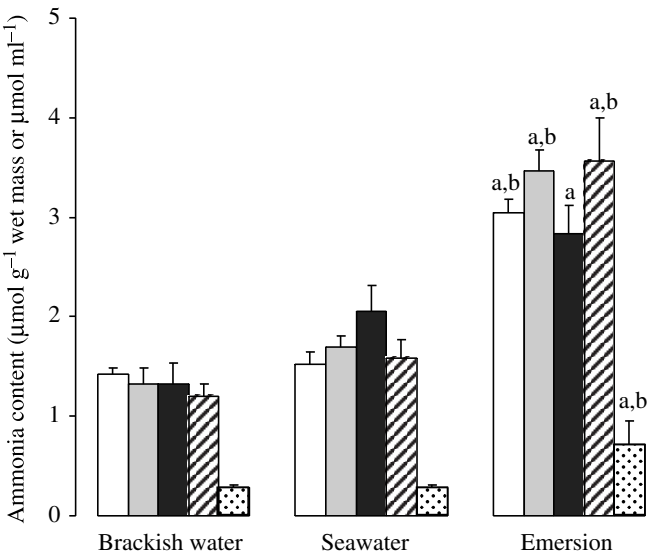

Fig. 2. Effects of exposure to brackish water (10‰, control), seawater (30‰) or air (emersion) for 17 days on ammonia contents in the adductor muscle (white bars), foot muscle (grey bars), hepatopancreas (black bars), mantle (hatched bars) and plasma (dotted bars) of *Polymesoda expansa.* Values are means ± S.E.M. (N=5). [a]Significantly different from the brackish water value (P<0.05); [b]significantly different from the seawater value (P<0.05).

compared with those exposed to brackish water or seawater (Fig. 2).

The contents of alanine in the adductor muscle, foot muscle, hepatopancreas and mantle (Fig. 3) were greater than those of glycine (Fig. 4), glutamate (Fig. 5) and glutamine (Fig. 6) in *P. expansa* exposed to all three experimental conditions. Taurine content in the adductor muscle, foot muscle, hepatopancreas and mantle of clams in brackish water as 2.67±0.31, 2.34±0.55, 2.03±0.48, 0.76±0.05, respectively. Thus, alanine was a major contributor to the TFAA pool (Fig. 7) in *P. expansa*. The contents of alanine (Fig. 3) and glycine (Fig. 4) in the adductor muscle, foot muscle, hepatopancreas and mantle in clams exposed to seawater for 17 days were significantly greater than those in clams exposed to brackish water or terrestrial conditions for a similar period. Exposure to seawater induced greater glutamate content only in the hepatopancreas and mantle (Fig. 5), and had no effect on the glutamine content of any of the tissues studied (Fig. 6). In contrast, aerial exposure for 17 days resulted in significantly greater glutamine content in the adductor muscle, foot muscle, hepatopancreas and mantle of *P. expansa* (Fig. 6). Both seawater and air exposure had only minor effects on taurine content in these tissues (results not shown). Overall, exposure to seawater, but not to air, led to significantly greater TFAA levels in all the tissues of *P. expansa* examined as compared with exposure to brackish water (Fig. 7).

GS (transferase) activities were detected in the adductor muscle, foot muscle, hepatopancreas and mantle of *P. expansa* (Fig. 8). The activities of GS in these tissues in *P. expansa* were unaffected by exposure to seawater for 17 days (Fig. 8),

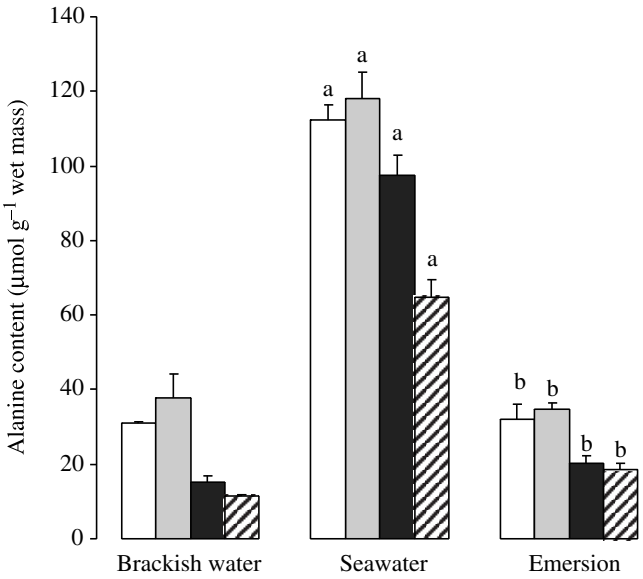

Fig. 3. Effects of exposure to brackish water (10‰, control), seawater (30‰) or air (emersion) for 17 days on the alanine contents in the adductor muscle (white bars), foot muscle (grey bars), hepatopancreas (black bars) and mantle (hatched bars) of *Polymesoda expansa.* Values are means ± S.E.M. (N=4). [a]Significantly different from the brackish water value (P<0.05); [b]significantly different from the seawater value (P<0.05).

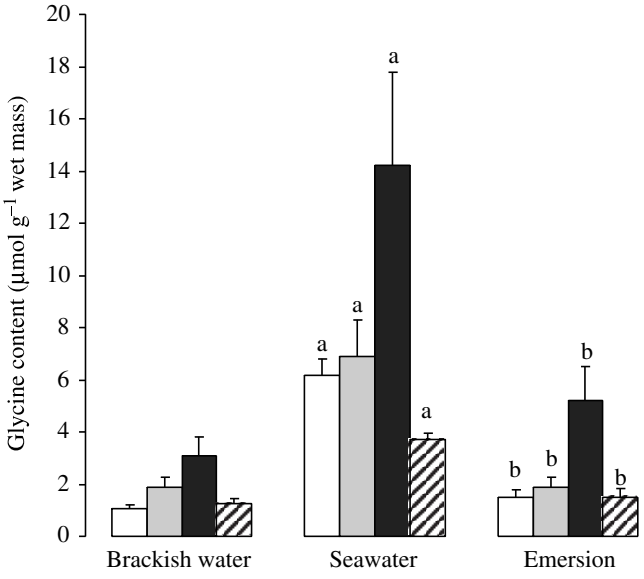

Fig. 4. Effects of exposure to brackish water (10‰, control), seawater (30‰) or air (emersion) for 17 days on the glycine contents in the adductor muscle (white bars), foot muscle (grey bars), hepatopancreas (black bars) and mantle (hatched bars) of *Polymesoda expansa.* Values are means ± S.E.M. (N=4). [a]Significantly different from the brackish water value (P<0.05); [b]significantly different from the seawater value (P<0.05).

but aerial exposure led to significant greater activities of GS in the adductor muscle and hepatopancreas (Fig. 8).

Discussion

P. expansa *in brackish water*

There was no detectable CPS III activity in the hepatopancreas, mantle, adductor muscle or foot muscle of *P. expansa*. Thus, similar to other bivalves (Andrews and Reid, 1972), *P. expansa* is non-ureogenic. In addition, no urea was detected in the ambient brackish water. Therefore, *P. expansa* is ammonotelic, excreting nitrogenous wastes mainly as ammonia.

FAAs are substrates for energy metabolism, protein synthesis and osmoregulation. Changes in salinity and/or exposure to environmental contaminants may affect the concentration and composition of FAAs in bivalves (Bayne et al., 1976a,b; Livingstone, 1985). It has been reported that the FAA levels in the tissues of brackish water bivalves (e.g. *P. caroliniana* and *Rangia cuneata*) contribute 25–30% of the intracellular solute at 10–20‰ salinity (Allen, 1961; Fyhn, 1976; Gainey, 1978a,b; Henry et al., 1980), and alanine is the major contributor to the FAA pool (Virkar and Webb, 1970; Pierce, 1971; Gainey, 1978b; Henry et al., 1980; Matsushima et al., 1984). Indeed, similar to other bivalves, alanine (43–48%) was the major FAA in *P. expansa* in brackish water (also in seawater and in air).

More importantly, our results confirm that glutamine contributed significantly (8.5–16.1%) to the TFAA pool of *P. expansa* in brackish water. This contribution was greater than that of glycine (1.6–8.8%) and comparable to that of glutamate (9.3–13.5%). The high levels of glutamine in the tissues of *P. expansa* indicate that this marsh clam was capable of synthesizing glutamine. Indeed, we verified the presence of GS (transferase) activities in all the tissues examined, with the greatest activity in the hepatopancreas. Due to the paucity of information in this area, generalizations about glutamine biosynthetic capabilities of bivalves cannot be made. However, studies with ^{14}C-labelled precursor molecules (glutamate, Krebs cycle intermediates and glucose) in *M. edulis* and other bivalve species confirm the absence of any glutamine biosynthetic capacity (Wijsman et al., 1977; Baginski and Pierce, 1978; Collicutt and Hochachka, 1977). In contrast, it has been reported that giant clams that harbour symbiotic zooxanthellae are capable of glutamine synthesis (Rees et al., 1994), and that a reasonably rapid glutamine synthesis and turnover occurs in species that excrete reasonable amounts of purines (terrestrial pulmonate snails and some prosobranch gastropods; see review by Bishop et al., 1983). Although GS has been found in tissues of several gastropod species (Campbell and Bishop, 1970; Reddy and Swami, 1975; Horne, 1977), there has been no report of the activity in tissues of non-symbiotic bivalves. Thus, this is the first report of the presence of glutamine synthetic capacity in a non-symbiotic intertidal bivalve, and therefore we made an effort to elucidate its functional role in *P. expansa* (see below).

Since food was withheld for 20 days (3 days prior to and 17 days during the experiment), the rate of proteolysis was

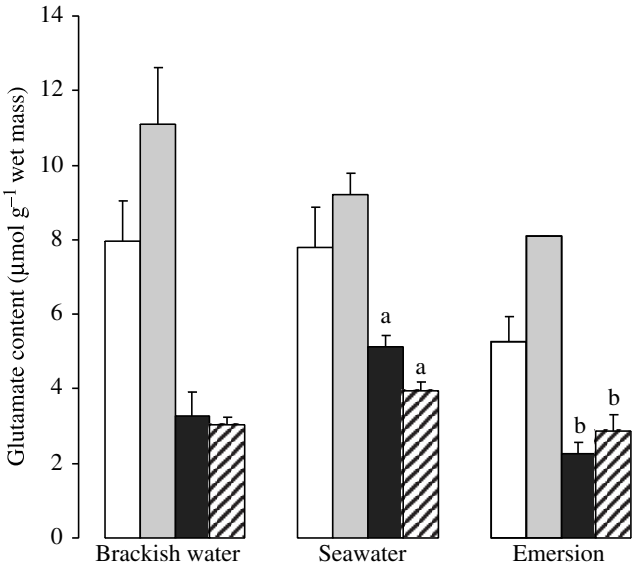

Fig. 5. Effects of exposure to brackish water (10‰, control), seawater (30‰) or air (emersion) for 17 days on the glutamate contents in the adductor muscle (white bars), foot muscle (grey bars), hepatopancreas (black bars) and mantle (hatched bars) of *Polymesoda expansa.* Values are means ± S.E.M. (*N*=4). [a]Significantly different from the brackish water value (P<0.05); [b]significantly different from the seawater value (P<0.05).

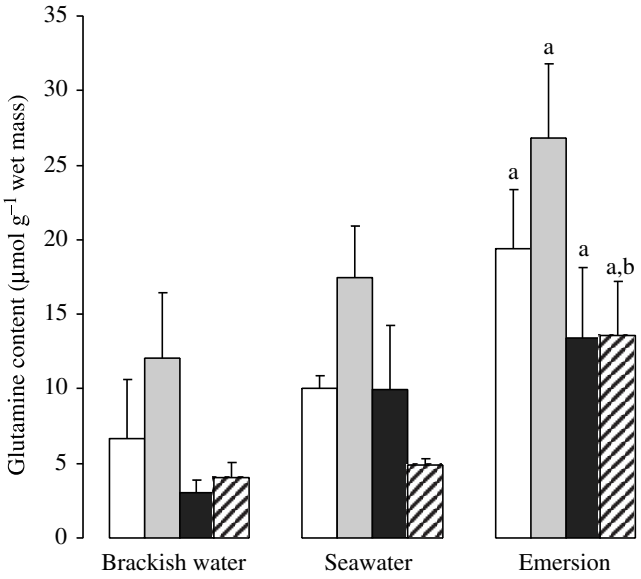

Fig. 6. Effects of exposure to brackish water (10‰, control), seawater (30‰) or air (emersion) for 17 days on the glutamine contents in the adductor muscle (white bars), foot muscle (grey bars), hepatopancreas (black bars) and mantle (hatched bars) of *Polymesoda expansa.* Values are means ± S.E.M. (*N*=4). [a]Significantly different from the brackish water value (P<0.05); [b]significantly different from the seawater value (P<0.05).

likely to be greater than the rate of protein synthesis, and net protein degradation would have occurred. Protein degradation together with increased amino acid catabolism would lead to an increase in the production of ammonia. Indeed, there was a significant increase in the rate of ammonia excretion in clams maintained in brackish water for 17 days. These results indicate that *P. expansa* could effectively excrete the excess ammonia produced during this period of fasting in brackish water.

P. expansa *in seawater*

Exposure to seawater for 17 days did not induce CPS III activity or accumulation of urea in the hepatopancreas, mantle, adductor muscle and foot muscle of *P. expansa*. Thus, urea did not act as an osmolyte in this marsh clam.

In invertebrates, FAAs are major intracellular osmolytes, contributing effectively to cell volume regulation (Pierce and Greenberg, 1972). Concentrations of intracellular FAAs of marine or brackish water bivalves fluctuate in response to changes in the ambient salinity (Pierce, 1971; Gainey, 1978a,b). In response to hypo-osmotic stress, intracellular levels of FAAs decrease as a result of their efflux to extracellular compartments (Pierce and Greenberg, 1972, 1973) and/or transformation to other substances inside cells (Matsushima et al., 1986). Conversely, in response to hyperosmotic stress, intracellular FAA concentrations are elevated as a result of protein degradation (Baginski and Pierce, 1978; Livingstone et al., 1979; Henry et al., 1980). Indeed, the TFAA content in the adductor muscle, foot muscle, hepatopancreas and mantle increased significantly by 2.6-, 2.3-, 4.2- and 3.6-fold, respectively, in *P. expansa* kept in seawater for 17 days. These changes were not a result of changes in water contents of the tissues. Simultaneously, the alanine content increased by 3.1- to 6.4-fold, and that of glycine by 3.0- to 5.9-fold. However, in terms of absolute quantity, alanine was the major contributor, because its percentage contribution to the TFAA pool increased from 43.0–48.0% to 61.7–73.1% in various tissues. Thus, as suggested before for other brackish and marine bivalves (Bayne et al., 1976a; Bishop et al., 1983), the functional role of alanine in *P. expansa* is mainly connected with intracellular osmoregulation.

The steady-state concentrations of FAAs in tissues are determined and maintained by the rates of their degradation and production (through proteolysis and/or synthesis). Alteration of these two rates would lead to changes in FAA concentration. The supply of FAAs for osmoregulatory purposes through protein degradation in clams in high salinity is confirmed by studies on enzymes involved in amino acid metabolism e.g. transaminase (Greenwalt and Bishop, 1980) and peptidase/proteinase (Bayne et al., 1981; Deaton et al., 1984). Since the ammonia excretion rates in *P. expansa* exposed to seawater were significantly greater than in clams exposed to brackish water on day 15 and day 17, it can be deduced that the rates of amino acid catabolism in the former were greater than those in the latter. In addition, since there were greater TFAA contents in tissues of the former than the latter, it can be concluded that a greater rate of protein

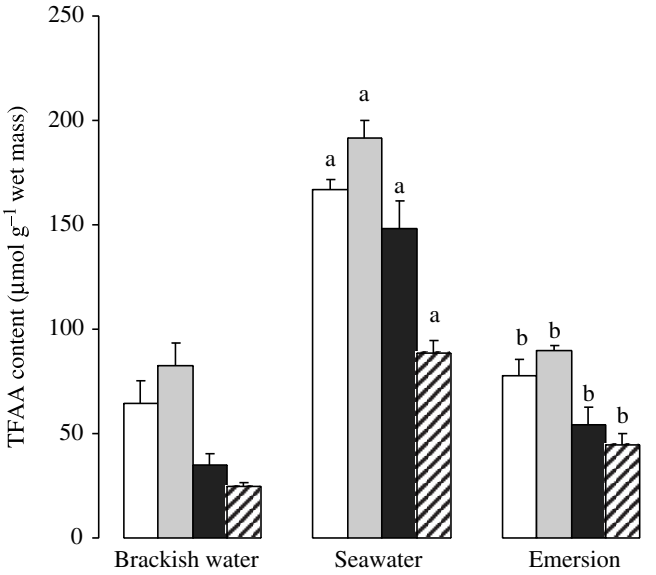

Fig. 7. Effects of exposure to brackish water (10‰, control), seawater (30‰) or air (emersion) for 17 days on the total free amino acid (TFAA) contents in the adductor muscle (white bars), foot muscle (grey bars), hepatopancreas (black bars) and mantle (hatched bars) of *Polymesoda expansa.* Values are means ± S.E.M. (N=4). [a]Significantly different from the brackish water value (P<0.05); [b]significantly different from the seawater value (P<0.05).

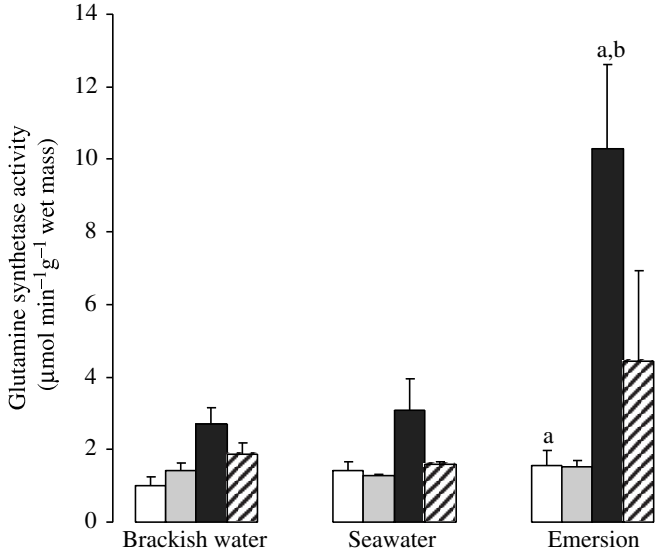

Fig. 8. Effects of exposure to brackish water (10‰, control), seawater (30‰) or air (emersion) for 17 days on the glutamine synthetase activity in the adductor muscle (white bars), foot muscle (grey bars), hepatopancreas (black bars) and mantle (hatched bars) of *Polymesoda expansa.* Values are means ± S.E.M. (N=4). [a]Significantly different from the brackish water value (P<0.05); [b]significantly different from the seawater value (P<0.05).

degradation had occurred in clams exposed to seawater compared with those exposed to brackish water.

Protein degradation leads to the release of FAAs. FAAs can be further catabolized, releasing ammonia. Increases in ammonia concentration would push reactions catalysed by glutamate dehydrogenase and alanine aminotransferase towards the synthesis of glutamate and alanine (Newsholme and Leech, 1983), leading to their accumulation. Indeed, there were minor but significant increases in levels of glutamate in the hepatopancreas and mantle of *P. expansa*, indicating that the glutamate dehydrogenase reaction had been perturbed. Furthermore, certain amino acids (e.g. arginine, glutamine, histidine and proline) can be converted to glutamate. Glutamate can undergo transamination with pyruvate, catalyzed by alanine aminotransferase, producing α-ketoglutarate without the release of ammonia (Ip et al., 2001a,c; Chew et al., 2003). α-Ketoglutarate is then channelled into the tricarboxylic acid (TCA) cycle for partial metabolism. The removal of malate from the TCA cycle can give a continuous supply of pyruvate. Transamination of pyruvate would produce alanine continuously, facilitating the oxidation of carbon chains of certain amino acids without releasing ammonia.

In contrast to alanine, there were no significant increases in glutamine levels in various tissues of *P. expansa* in seawater. As a result, there were decreases in the percentages contribution of glutamine to the TFAA pools in the adductor muscle, foot muscle, hepatopancreas and mantle of clams exposed to seawater (6.0, 9.1, 6.8 and 5.5, respectively) compared with those exposed to brackish water (10.4, 14.7, 8.5 and 16.1, respectively). Thus, it can be concluded that the physiological significance of glutamine synthesis in *P. expansa* is unrelated to osmoregulation.

P. expansa *in air*

Emersion inhibits several metabolic functions, such as feeding, respiration and excretion, in bivalves. In many cases, oxygen consumption rates are reduced during air exposure. In response to a lack of oxygen, the energy requirements of clams are satisfied through anaerobic glucose catabolism, for which alanine is one of the major end-products (de Zwaan, 1977; Widdows et al., 1979; Henry et al., 1980; Zurburg and de Zwaan, 1981). However, *P. expansa* was exceptional because there were no significant increases in alanine content in any of the tissues studied after 17 days of aerial exposure. This could be related to the unique capability of *P. expansa* to maintain a normal O_2 utilization rate during emersion (see Introduction). In spite of maintaining aerobic respiration, the tissues of *P. expansa* were not exposed to dehydration during emersion.

Although *P. expansa* does not encounter a lack of oxygen in air, it is confronted with problems associated with ammonia excretion when water is lacking. During aerial exposure, ammonia can be excreted into the mantle cavity fluid (Shick et al., 1988), but ammonia concentrated therein would build up and lead to accumulation of ammonia in its tissues. Indeed, the ammonia contents in various tissues increased between 2.1-fold and 3.0-fold in *P. expansa* exposed to air for 17 days. Because there was no detectable CPS III activity and no accumulation of urea in various tissues of *P. expansa* exposed

to air for 17 days, it can be concluded that, unlike in some gastropods (see review by Bishop et al., 1983), ammonia accumulated during emersion was not detoxified to urea in this marsh clam.

Since reactions catalyzed by transaminases and glutamate dehydrogenase are near equilibrium *in vivo* (Newsholme and Leech, 1983), substrate concentrations (e.g. ammonia) determine whether transamination and deamination of amino acids or amination of α-keto acids occurs. An increase in tissue ammonia concentration would push the equilibrium towards amination of α-keto acids through the reaction catalyzed by glutamate dehydrogenase. This would theoretically result in an accumulation of glutamate and transaminable non-essential FAAs, including alanine (see above), in the tissues. However, there were no increases in alanine content in any of the tissues studied in *P. expansa* exposed to air for 17 days. Hence, alanine was not a product ammonia detoxification in *P. expansa*. Our results confirm that ammonia was detoxified to glutamine in *P. expansa* during 17 days of aerial exposure. That means glutamate formed from NH_4^+ and α-ketoglutarate reacted further with ammonia, in the presence of ATP, to produce glutamine. The contents of glutamine in the adductor muscle, foot muscle, hepatopancreas and mantle increased 2.9-, 2.5-, 4.5- and 3.4-fold, respectively. Simultaneously, there were significant increases in GS activities in the adductor muscle (1.56-fold) and hepatopancreas (3.8-fold). This is the first report on the upregulation of GS and accumulation of glutamine in a clam in response to aerial exposure. Such a phenomenon may be peculiar to those bivalves that can maintain aerobic energy metabolism during emersion, because synthesis of glutamine is ATP-dependent and production of glutamate requires NAD(P)H. It would be energetically uneconomical for an animal to increase glutamine and glutamate syntheses while undergoing anaerobic energy metabolism due to a lack of oxygen supply, because there would be a decrease in ATP production and problems associated with redox balance. Indeed, it has been reported recently that the swamp eel *Monopterus albus* does not accumulate glutamine after 40 days of aestivation in hypoxic mud, but accumulates glutamine to high levels in its tissues during 6 days of aerial exposure (Chew et al., 2005a).

Sokolowski et al. (2003) determined FAA contents in the clam *Macoma balthica* L. from brackish waters of the southern Baltic Sea, and reported that the overall temporal pattern of variations in the concentration of glutamine in the period analysed resembled, in general, that of alanine, with high values in the winter and low in spring. Sokolowski et al. (2003) suggested that the physiological roles of these two amino acids were similar within a seasonal cycle, despite the lack of evidence for such metabolic connections. Contrary to the proposition of Sokilowski et al. (2003), our results reveal that the functional role of glutamine was distinctly different from that of alanine in *P. expansa*. However, how alanine formation and glutamine synthesis, both involving glutamate as a substrate, are regulated in *P. expansa* in response to different environmental conditions (exposure to seawater *vs* exposure to air) is uncertain at present.

Conclusion

Returning to the initial impetus of this study, the results obtained with *P. expansa* demonstrate that the adoption of glutamine synthesis, similar to the adoption of urea synthesis, for ammonia detoxification first took place among invertebrates. Similar to other bivalve species, *P. expansa* is incapable of urea synthesis *de novo*, but is capable of detoxifying ammonia to glutamine during emersion. The capacity for glutamine synthesis was present in a variety of tissues, including the hepatopancreas, mantle, adductor muscle and foot muscle, in *P. expansa*. This capacity apparently extends to the lower vertebrates, because some air-breathing fishes are capable of synthesizing glutamine in their liver, muscles, and digestive tracts as a major strategy to defend against ammonia toxicity during aerial (Jow et al., 1999; Ip et al., 2001b; Chew et al., 2001; Tay et al., 2003) or environmental (Peng et al., 1998; Anderson et al., 2002; Ip et al., 2004a; Lim et al., 2004) ammonia exposure. Brains of vertebrates, from fish (Campbell and Anderson, 1991; Ip et al., 2001a, 2004b; Chew et al., 2005b) to mammals (Cooper and Plum, 1987; Brusilow, 2002; Felipo and Butterworth, 2002), are capable of detoxifying ammonia to glutamine. However, higher vertebrates such as mammals do not adopt glutamine synthesis as a major strategy to deal with ammonia toxicity in extra-cranial tissues, because they have evolved to depend mainly on urea synthesis through the hepatic OUC to detoxify ammonia (Cooper and Plum, 1987).

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