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INCREASES IN TISSUE FREE AMINO ACID LEVELS IN RESPONSE TO PROLONGED EMERSION IN MARINE CRABS: AN AMMONIA-DETOXIFYING PROCESS EFFICIENT IN THE INTERTIDAL *CARCINUS MAENAS* BUT NOT IN THE SUBTIDAL *NECORA PUBER*

FABRICE DURAND*, FABIENNE CHAUSSON AND MICHÈLE REGNAULT

Equipe Ecophysiologie, Observatoire Océanologique de Roscoff (UPMC, CNRS, INSU), Station Biologique, BP 74, F-29682 Roscoff Cedex, France

*e-mail: durand@sb-roscoff.fr

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Summary

Carcinus maenas **and** ***Necora puber*** **were exposed to air for 72 h and 18 h, respectively, at 18 °C. Changes in the free amino acid (FAA) content of their muscle, hepatopancreas and haemolymph were recorded during air-exposure and subsequent reimmersion. Muscle and hepatopancreas urate contents and haemolymph serum protein levels were also measured during emersion.**

In air-exposed ***C. maenas*****, the muscle FAA pool increased significantly within the first 24 h of emersion. This increase was due to an increase in the non-essential amino acid (NEAA) pool only; the essential amino acid (EAA) pool did not change. In haemolymph, the EAA pool decreased during the first 24 h of emersion, whereas the FAA and NEAA pools did not change. However, in this compartment, glutamine levels increased throughout the air-exposure period. No significant changes in FAA, NEAA and EAA contents of the hepatopancreas were observed during the 72 h emersion.**

In air-exposed ***N. puber*****, the FAA pools of muscle and hepatopancreas did not change, although changes in the levels of some amino acids were observed during the 18 h emersion period. In this species, large increases in both the NEAA and EAA pools in the haemolymph were recorded.**

High levels of urate were observed in the muscle and hepatopancreas of immersed ***N. puber*****, but no significant changes occurred during emersion. In contrast, immersed** ***C. maenas*** **exhibited low levels of urate in both compartments, and hepatopancreas urate levels increased slightly during emersion. Haemolymph protein content did not change in air-exposed** ***N. puber*****, whereas it increased in the haemolymph of 72 h emersed** ***C. maenas*****.**

The origin of newly formed NEAAs and their role in ammonia detoxification, particularly in ***C. maenas*****, which is able to regulate its internal ammonia levels during such a prolonged emersion, are discussed.**

Key words: air-exposure, nitrogen metabolism, amino acid, urate, protein, haemolymph, muscle, hepatopancreas, crab, *Carcinus maenas*, *Necora puber*.

Introduction

In aquatic crustaceans, the main end-product of nitrogen catabolism is ammonia, and excretion of this nitrogenous waste product occurs at the gills (Claybrook, 1983; Regnault, 1987; Greenaway, 1991). When in sea water, marine crabs excrete ammonia into the branchial water, which is continuously renewed. Ammonia excretion is a continuous process, and its rapid elimination into the environmental sea water protects all the body compartments from the toxic effects of high ammonia levels. For marine crabs, deprivation of environmental sea water may impede ammonia excretion. This has been observed in two subtidal species, *Cancer pagurus* and *Necora puber*, and in the intertidal crab *Carcinus maenas* (Regnault, 1994; Durand and Regnault, 1998). Moreover, although the ammonia excretion rate was greatly reduced during emersion, these species did not shift to production of other end-products of nitrogen catabolism.

As a general rule, ammoniotelism has also been retained by the terrestrial crustaceans (for a review, see Greenaway, 1991), except for the purinotelic coenobitid *Birgus latro* (Greenaway and Morris, 1989). The requirement for ammonia excretion in aerial conditions has forced terrestrial species to develop different mechanisms of excretion: (i) ammonia excretion into reprocessed urine in the branchial chamber in the crabs *Gecarcoidea natalis* (Greenaway and Nakamura, 1991), *Gecarcinus lateralis* and *Cardisoma guanhumi* (Wolcott, 1991); (ii) ammonia release *via* the urine and reprocessing of this ammonia-enriched urine in the branchial chamber in the ghost crab *Ocypode quadrata* (De Vries and Wolcott, 1993);

and (iii) discontinuous excretion of gaseous ammonia in terrestrial isopods (Wieser and Schweizer, 1970; Kirby and Harbaugh, 1974; Wright and O'Donnell, 1993) and in the crab *Geograpsus grayi* (Varley and Greenaway, 1994). The discontinuous excretion of gaseous ammonia has raised the question of whether nitrogen is sequestered in a less toxic form than ammonia during non-excretory periods. A large increase in tissue free amino acid levels was observed in the isopod *Porcellio scaber* exposed to external ammonia loading (Wright et al., 1994, 1996), and it was suggested that amino acid synthesis was acting as an ammonia-detoxifying process. To our knowledge, sequestration of ammonia as amino acids has not been investigated in terrestrial crabs. However, most land crabs store large amounts of urate in intracellular compartments (Linton and Greenaway, 1997a), and *de novo* synthesis of urate has been demonstrated (Linton and Greenaway, 1997b). According to these authors, urate storage represents either a metabolic nitrogen reserve or a temporary store for nitrogenous wastes when ammonia excretion is prevented by long periods of exposure to dry conditions; the latter hypothesis appears to be more consistent with the available data (Linton and Greenaway, 1998; S. M. Linton, personal communication).

In contrast to terrestrial species, marine crustaceans drastically reduce their rate of ammonia excretion when exposed to air. Consequently, increases in blood ammonia levels generally occur in marine decapods subjected both to short-term emersion (deFur and McMahon, 1984; Vermeer, 1987) and to long-term air-exposure (Regnault, 1992; Schmitt and Uglow, 1997; Durand and Regnault, 1998), although metabolic depression may reduce the rate of ammonia production in some species (Regnault, 1992).

A previous study of the effects of prolonged emersion on the nitrogen excretion of two portunid crabs, *Carcinus maenas* and *Necora puber* (Durand and Regnault, 1998), has shown that both species reduce their rate of ammonia excretion by 95 % in these conditions. A large increase in ammonia level was observed in the blood (from 0.1 to 1.3 mmol l^{-1}) and muscle (from 7.5 to 10.4 mmol l^{-1}) of the subtidal *Necora puber* exposed to air for 18 h. In contrast, the intertidal *Carcinus maenas* exhibited a marked ability to regulate both blood and muscle ammonia levels during a 72 h air-exposure. Following reimmersion, rates of ammonia excretion were enhanced for only 3 h in *N. puber*, whereas high ammonia excretion rates were observed throughout the recovery period (24 h) in *C. maenas*. In this species, the regulation of blood and muscle ammonia levels during emersion, the pattern of ammonia excretion after reimmersion and the recovery of blood and muscle ammonia levels suggested that unexcreted ammonia was either stored in an unidentified body compartment or recycled through biosynthesis of nitrogenous compounds during emersion. As indicated above, amino acids are good candidates for ammonia sequestration, and their synthesis in response to emersion has previously been observed in the amphibious fish *Periophthalmus cantonensis* (Iwata, 1988).

To examine this possibility, the present study investigated the qualitative and quantitative changes in the amino acid pool of *C. maenas* and *N. puber* during prolonged air-exposure and subsequent recovery periods. Twenty amino acids were studied to determine those primarily concerned in the response of the crabs to air-exposure. They were analysed in three body compartments (muscle tissue, haemolymph and hepatopancreas). Because urate may also contribute to nitrogen sequestration, as it does in semi-terrestrial species, the urate contents of muscle and hepatopancreas were measured during prolonged emersion. Additionally, changes in levels of haemolymph proteins, which are known to contribute to the free amino acid pool of the tissues by their synthesis/degradation equilibrium, were measured.

In this study, crabs were exposed to prolonged emersions of 18 h (*Necora puber*) or of 72 h (*Carcinus maenas*) in the same conditions as used previously (Durand and Regnault, 1998).

Materials and methods

Crabs

Carcinus maenas (L.) (70–110 g wet mass) and *Necora puber* (L.) (70–100 g) were collected in summer in the low intertidal area and the sublittoral zone of Roscoff (North Brittany, France), respectively. Animals were kept in running sea water (open system) at ambient temperature (18–19 °C) and salinity 33–34 ‰ for 1 week before use; they were fed every 2 days with pieces of thawed fish (*Trachurus trachurus*). Only male crabs in the intermoult stage were used for experiments.

Experimental conditions

Crabs were emersed in the experimental apparatus as described previously (Durand and Regnault, 1998). Briefly, after their last feed, crabs were placed into individual compartments and kept immersed in running sea water for 24 h (prior to experimentation). Emersions (without any handling of the crabs) were performed at room temperature (18±1 °C), under natural photoperiod and in a highly hydrated atmosphere. Air-exposure was imposed for 72 h in *C. maenas* and for only 18 h in *N. puber* because of its high rate of mortality for longer exposure periods (Johnson and Uglow, 1985; Durand and Regnault, 1998).

Five groups of 12 crabs were used for the emersion experiment for *C. maenas*. Four groups were emersed and sampled after 12 h, 24 h, 48 h or 72 h of air-exposure. Crabs in the fifth group (control) were sampled at the time when the experimental crabs were deprived of environmental water. This pre-emersion value (PE) is the control value. In addition, some crabs were kept immersed for 48 h to estimate the effects of fasting for 48 h on the variables studied. For reimmersion experiments, four new groups of crabs previously emersed for 72 h in the same conditions, but without any sampling disturbance, were used. One group was sampled at the end of the air-exposure period (72 h), and the other three groups were sampled following 6 h, 12 h or 24 h of reimmersion.

For *N. puber*, four groups of crabs (6–10 animals) were used. One group of control crabs was sampled only at the time when

the other crabs were deprived of environmental water, and the other three groups were emersed and sampled after 6 h, 12 h or 18 h of air-exposure. Crabs in a fifth group (three specimens) were emersed for 18 h, then reimmersed and sampled after 6 h of reimmersion.

Tissue sampling

Prebranchial haemolymph (approximately 1 ml) was collected through the arthropodial membrane at the base of the fourth pereiopod using a 1 ml sterilised syringe and a 21 gauge needle. Blood samples were transferred into refrigerated Eppendorf microtubes; 500 µl subsamples were immediately frozen in liquid nitrogen and stored at −30 °C until analysis of their amino acid content; further 500 µl subsamples were centrifuged at 3500 ***g*** (4 °C, 10 min), and the supernatants collected (blood serum) were analysed for their protein content using the method of Lowry et al. (1951).

One cheliped was removed by forced autotomy, and the propodus muscle was dissected out rapidly on ice. Hepatopancreatic tissue was also dissected out. Each tissue was partitioned into several 50 mg samples. These were rapidly weighed, transferred into refrigerated Eppendorf microtubes and immediately frozen in liquid nitrogen. Samples were stored at −30 °C until analysis.

Analysis of urate in tissues

Frozen muscle and hepatopancreas samples (50 mg) were disrupted in 200 µl of ice-cold twice-distilled water by sonication at 20 kHz (4×30 s periods) at 4 °C; 200 µl of twice-distilled water was added to the homogenate, which was mixed and kept on ice for 30 min. Homogenates were then centrifuged for 30 min at 10 000 ***g*** and 4 °C. For muscle, supernatants were collected and used directly for urate measurement using Sigma kit no. 685. In the case of hepatopancreatic tissue, supernatants were ultrafiltered using molecular-mass cut-off filters (30 000 M_r; Centricon; Amicon, Inc., Beverly, USA) before analysis, because undefined substances were interfering with the reaction of the Sigma kit. The results obtained using the present method were similar to those obtained using the high-performance liquid chromatography (HPLC) method (Wynants et al., 1987) used in the laboratory of Professor M. K. Grieshaber (M. C. De Cian, personal communication).

Analysis of free amino acids

Frozen tissue (50 mg) or haemolymph (200 µl) was disrupted in 500 µl of ice-cold 80 % (v/v) methanol/water solution (Harris and Andrews, 1985) by sonication at 20 kHz (4×30 s periods) at 4 °C using a Vibra Cell 72446 sonicator (Bioblock Scientific). A sample of the methanol/water solution (500 µl) was added to the homogenate and mixed, and extraction was performed on ice for 1 h. The homogenates were then centrifuged for 15 min at 10 000 ***g*** and 4 °C. The supernatants were used immediately for amino acid analysis.

Concentrations of amino acids were measured by reverse-phase HPLC using the method of precolumn derivatization described by Lindroth and Mopper (1979). Supernatants were diluted from 1/250- to 1/1000-fold, depending on the tissue, using twice-distilled water. Derivatization was carried out using ortho-phthaldialdehyde and 2-mercaptoethanol for 3 min at room temperature (20 °C); 100 µl of the derivatized sample was injected into the HPLC column. Separation of amino acids was achieved by gradient flow (1 ml min^{-1}) of two eluents (A, 100 % methanol; B, 10 % methanol/90 % citrate buffer, pH 7.23) through a C18 column (Ultratech 5 ODS) at 30 °C during 75 min (Table 1). The fluorescence of derivatized primary amino acids was detected using a post-column fluorodetector (LDC FluoroMonitor III, Milton Roy). Since only primary amino acids were derivatized, imines such as proline and hydroxyproline were not detected by this method. Cysteine was poorly detected using this method.

Table 1. *Gradient conditions for HPLC analysis*

Time (min)	% A	% B
0	10	90
3	10	90
10	15	85
20	15	85
30	19	81
40	25	75
45	33	67
50	46	54
60	48	52
65	80	20
75	90	10

A, 100 % methanol; B, 10 % methanol/90 % citrate buffer, pH 7.23.

The areas under the amino acid absorbance peaks were recorded and integrated using the Boreal Chromatography Package program (Flotec Software). Amino acids were identified according to their retention times compared with a standard solution, run each day, containing 20 amino acids.

Expression of results and statistical analyses

The amino acid content of tissues is expressed as µmol g^{-1} wet mass. Haemolymph amino acid content is expressed as mmol l^{-1} of blood. In the present study, free amino acids (FAAs) are the sum of the 20 amino acids measured. Non-essential amino acids (NEAAs) are aspartate, asparagine, glutamate, glutamine, serine, glycine, taurine, alanine, arginine, ornithine and tyrosine (Stryer, 1995). Essential amino acids (EAAs) are threonine, histidine, methionine, tryptophan, valine, phenylalanine, isoleucine, leucine and lysine. The urate content of the tissues is expressed as µmol g^{-1} wet mass, and blood protein content is expressed as g l^{-1} of blood serum.

Values are expressed as means ± S.E.M. Individual variations in amino acid levels are given as mean difference ± 95 % confidence interval. Mean values at every sampling time during air-exposure were compared with the pre-emersion value (PE) of the control crabs. Values obtained during the recovery period were compared with those recorded at the end

of the air-exposure period in crabs emersed without any sampling disturbance. Differences between mean values were tested statistically using one-way analysis of variance (ANOVA). *Post-hoc* testing was carried out using Dunnett's method for multiple comparisons with a control group. Pairwise comparisons were also carried out using the Tukey–Kramer method for comparison of multiple means when samples were of unequal size.

Values were considered significantly different when $P<0.05$.

Results

Free amino acids

Carcinus maenas

Muscle tissue. The total pool of free amino acids (FAAs) measured in muscle of control crabs was 206.3±6.0 μmol g^{-1} wet mass (N=11). Non-essential amino acids (NEAAs, 187.9±5.9 μmol g^{-1} wet mass, N=11) and essential amino acids (EAAs, 18.3±5.9 μmol g^{-1} wet mass, N=11) accounted for 91.1 % and 8.9 % of FAAs, respectively. In control crabs kept immersed for 48 h, similar NEAA and EAA pools were observed, although levels of some amino acids (asparagine, methionine and valine) were significantly reduced.

During the first 24 h of air-exposure, the muscle FAA pool increased significantly ($P<0.001$), reaching 262.9±6.7 μmol g^{-1} wet mass (N=12) (Fig. 1A). This increase is due to a significant increase ($P<0.001$) in the NEAA pool only; no significant changes were observed in the EAA pool. The increase in the NEAA pool was initiated during the first 12 h of emersion, and glycine concentration increased significantly ($P<0.05$) (from 65.2±3.0 to 75.5±3.2 μmol g^{-1} wet mass, N=11). Glycine concentration increased further during the next 12 h, and at this time the muscle contents of alanine, glutamine and arginine were also significantly increased.

When emersion was prolonged beyond 24 h, the FAA pool exhibited no more changes. However, an increase of 13.5 μmol g^{-1} wet mass in NEAAs together with a significant decrease ($P<0.05$) in EAAs from 21.4±2.6 (N=10) to 12.9±1.2 μmol g^{-1} wet mass (N=11) were observed between 24 h and 48 h of air-exposure. At this time, the muscle glutamate content was significantly higher (by 1.73±1.32 μmol g^{-1} wet mass) than its pre-emersion value. No significant changes in the NEAA and EAA pools were observed between 48 h and 72 h of emersion.

The result of the 72 h air-exposure period was an increase of approximately 47.6±14.5 μmol g^{-1} wet mass in the muscle NEAA pool. The amino acids responsible for this increase were (Fig. 2A) glycine (+28.5±7.4 μmol g^{-1} wet mass), alanine (+8±5.4 μmol g^{-1} wet mass), glutamine (+7.2±3.4 μmol g^{-1} wet mass) and arginine (+6.3±4.5 μmol g^{-1} wet mass).

Following reimmersion, a large decrease in total NEAA levels was observed between 6 h and 12 h (Fig. 1A). This decrease was due to significant decreases in glycine, alanine and arginine levels. No major changes in the levels of any amino acid were observed during the next 12 h. At the end of the reimmersion period, the FAA pool (223.2±11.4 μmol g^{-1} wet mass, N=8) and the NEAA pool (211.8±10.3 μmol g^{-1} wet mass, N=8) were similar ($P>0.05$) to their respective pre-emersion levels. It was also observed that the glutamine level reached at the end of emersion was maintained throughout the reimmersion period.

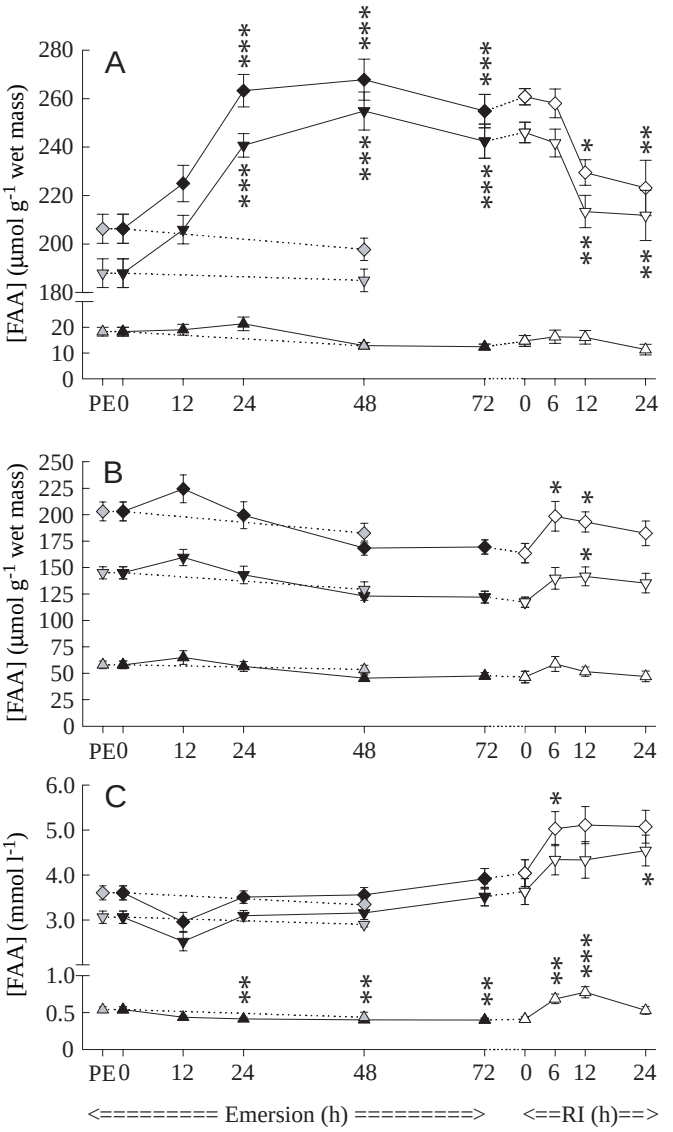

Fig. 1. Changes in amino acid content of muscle (A), hepatopancreas (B) and haemolymph (C) of *Carcinus maenas* during a 72 h air-exposure and subsequent recovery. Total free amino acids, FAAs (diamonds); non-essential amino acids, NEAAs (▼, ▽); essential amino acids, EAAs (▲, △). Values are means ± S.E.M., N=10–12 for the emersion period (filled symbols) and N=8 for the reimmersion period (RI; open symbols). The control value was given by immersed controls (PE, pre-emersion value; shaded symbols) and by 48 h-immersed controls (N=4). Significant changes during emersion according to the PE value are indicated with an asterisk; significant changes during reimmersion with reference to the value observed at the end of the emersion are also indicated with an asterisk. Significance levels: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

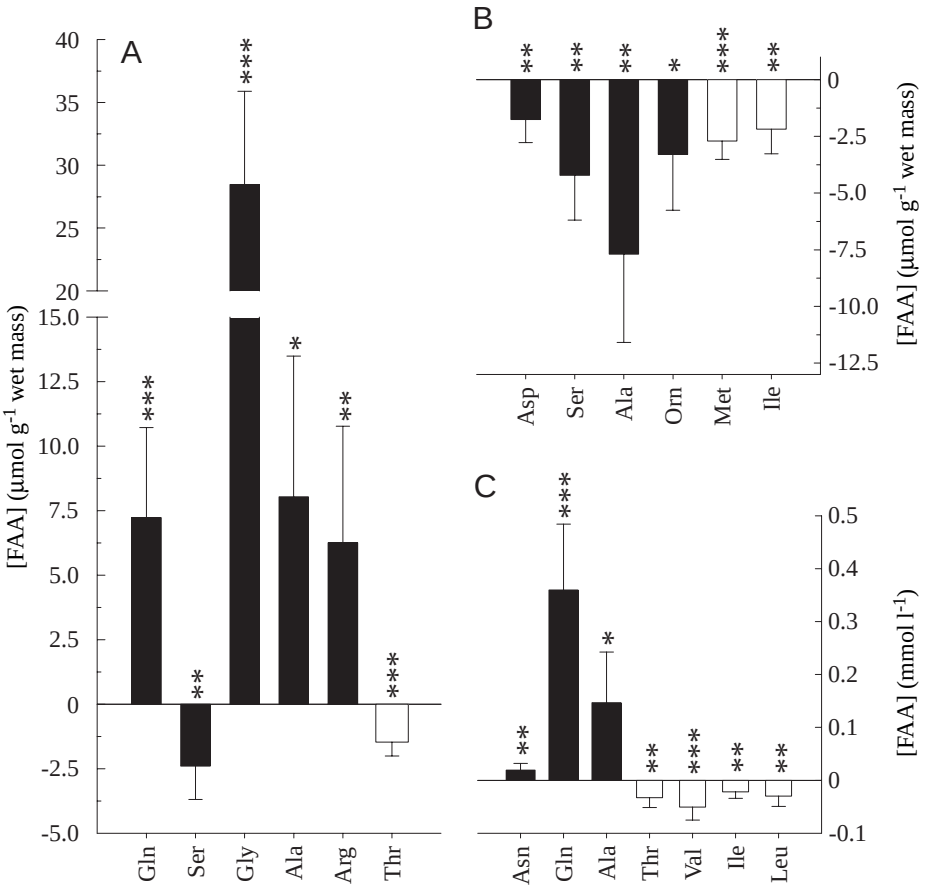

Fig. 2. Changes in the levels of individual amino acids resulting from a 72 h air-exposure period in muscle (A), hepatopancreas (B) and haemolymph (C) of *Carcinus maenas*. Values are mean differences + 95 % confidence interval. Filled columns, non-essential amino acids (NEAAs); open columns, essential amino acids (EAAs). Significant changes are indicated with an asterisk. Significance levels: **P*<0.05, ***P*<0.01, ****P*<0.001.

Hepatopancreas. The total pool of FAAs in hepatopancreas of control crabs was 203.1±8.9 μmol g^{-1} wet mass (*N*=11). NEAAs accounted for 71.4 % of FAAs (145.2±5.8 μmol g^{-1} wet mass, *N*=11) and EAAs for 28.6 % of FAAs (57.9±3.6 μmol g^{-1} wet mass, *N*=11). In crabs immersed for 48 h, no significant changes in the NEAA and EAA pools were observed, although significant decreases in alanine and isoleucine levels and a significant increase in arginine level were recorded.

In this tissue, no significant changes in either the NEAA or the EAA pool were observed during the 72 h air-exposure period (Fig. 1B). However, levels of some NEAAs (aspartate, serine, alanine and ornithine) and of two EAAs (methionine and isoleucine) decreased significantly after 72 h of emersion (Fig. 2B).

Following reimmersion, the FAA pool increased significantly within the first 6 h (Fig. 1B). This increase was mainly due to a significant (*P*<0.05) increase in the NEAA pool (Fig. 1B).

Haemolymph. The FAA pool in haemolymph of control crabs was 3.6±0.2 mmol l^{-1} (*N*=11). NEAAs accounted for 85 % of FAAs (3.06±0.14 mmol l^{-1}, *N*=11) and EAAs for 15 % of FAAs (0.54±0.04 mmol l^{-1}, *N*=11). No significant change in these pools was observed in controls after 48 h of immersion.

During emersion, no significant changes in the FAA and NEAA pools were observed, although a small transient decrease in these pools was recorded after 12 h of emersion (Fig. 1C). The EAA pool was significantly reduced (*P*<0.01) after 24 h of emersion, and levelled off at approximately 0.4 mmol l^{-1} until the end of the 72 h air-exposure period. At this time, levels of four EAAs (threonine, valine, isoleucine and leucine) had decreased significantly (Fig. 2C).

Although the NEAA pool did not change during emersion, glutamine, asparagine and alanine levels had increased significantly at the end of the 72 h emersion period (Fig. 2C). It was also observed that the glutamine level increased consistently throughout the emersion period, the changes being significant at every sampling time.

At reimmersion, a significant increase in both FAA and EAA pools was observed during the first 6 h (Fig. 1C). The EAA pool returned to its pre-emersion level after 24 h of reimmersion, at which time the NEAA pool was significantly larger than its pre-emersion level.

Necora puber

Muscle tissue. In this species, the muscle FAA pool of control crabs was 252.4±7.6 μmol g^{-1} wet mass (*N*=8). NEAAs accounted for 91.2 % of FAAs (232.1±5.3 μmol g^{-1} wet mass, *N*=8) and EAAs for 8.8 % of FAAs (20.3±3.6 μmol g^{-1} wet mass, *N*=8).

No significant changes in the FAA, NEAA and EAA pools were observed during the 18 h air-exposure period (Fig. 3A). However, the muscle contents of both glutamine and ornithine

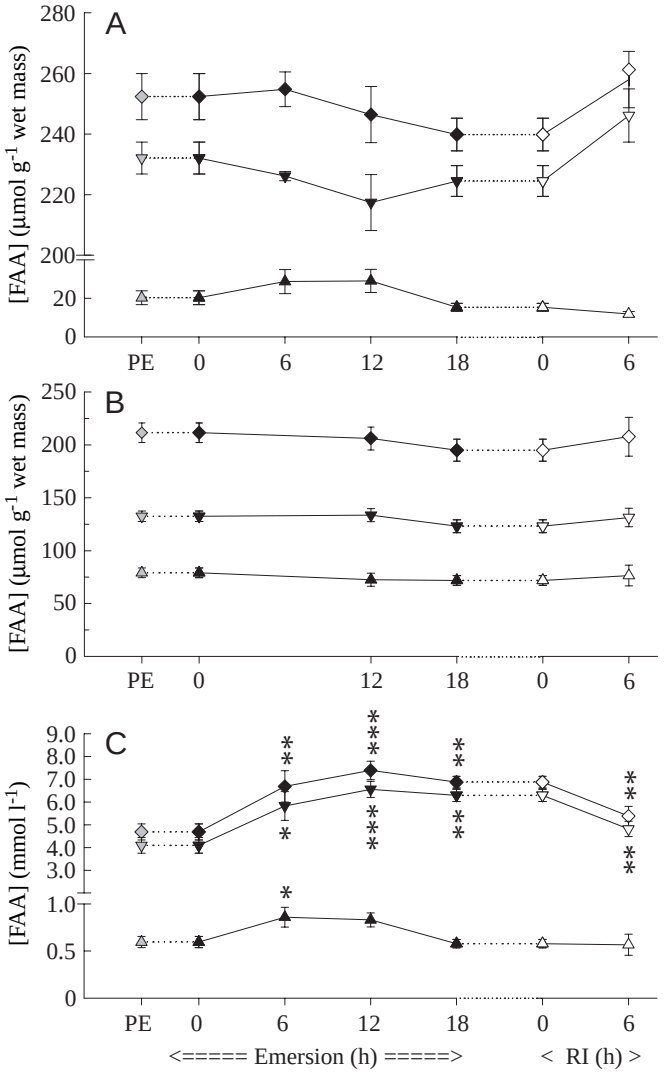

Fig. 3. Changes in amino acid content of muscle (A), hepatopancreas (B) and haemolymph (C) of *Necora puber* during an 18 h air-exposure and subsequent recovery. Total free amino acids, FAAs (diamonds); non-essential amino acids, NEAAs (▼, ▽); essential amino acids, EAAs (▲, △). Values are means ± S.E.M., N=6–10 for the emersion period (filled symbols) and N=3 for the reimmersion period (RI, open symbols). The control value was given by immersed controls (PE, pre-emersion value; shaded symbols). Significant changes during the emersion with respect to the PE value are indicated with an asterisk. Significance levels: *P<0.05, **P<0.01, ***P<0.001.

were significantly reduced at the end of the emersion period (Fig. 4A).

After 6 h of reimmersion, the FAA level in muscle was similar to its pre-emersion value, but glutamate and ornithine levels had not returned to their pre-emersion levels.

Hepatopancreas. The FAA pool in the hepatopancreas of control crabs was 211.5±9.3 μmol g^{-1} wet mass (N=8). NEAAs accounted for 62.7 % of FAAs (132.6±4.9 μmol g^{-1} wet mass, N=8) and EAAs for 37.4 % of FAAs (78.9±4.6 μmol g^{-1} wet mass, N=8).

No changes in the NEAA and EAA pools were observed as a result of an 18 h emersion (Fig. 3B), but significant changes occurred in the levels of some amino acids in these pools (Fig. 4B). Levels of glutamate, arginine and ornithine and the level of the EAA lysine decreased significantly, whereas the level of alanine increased significantly (+12.1±4.3 μmol g^{-1} wet mass).

Following 6 h of reimmersion, only ornithine had returned to its pre-emersion level.

Haemolymph. The FAA pool in the haemolymph of control crabs was 4.69±0.36 mmol l^{-1} (N=8). NEAAs accounted for 87.2 % of FAAs (4.09±0.35 mmol l^{-1}, N=8) and EAAs for 12.8 % of FAAs (0.59±0.06 mmol l^{-1}, N=8).

During emersion, significant increases (P<0.01) in the NEAA pool and in the EAA pool (P<0.05) were observed during the first 6 h (Fig. 3C). The NEAA pool then levelled off at 6.29±0.27 mmol l^{-1}, while the EAA pool returned to its pre-emersion level. At the end of emersion, only the NEAA pool had increased significantly and this was due to significant increases in glycine, alanine, arginine and ornithine levels (Fig. 4C). In this species, the level of glutamine in the haemolymph was reduced in response to emersion.

After 6 h of reimmersion, all NEAA levels, except for alanine, had returned to their pre-emersion value.

Nitrogen balance

Total nitrogen accumulated as amino acids was estimated on the basis of the nitrogen content of each amino acid. This was 1 N-atom for most amino acids, but 2 N-atoms for glutamine, asparagine, lysine, tryptophan and ornithine, 3 N-atoms for histidine and 4 N-atoms for arginine. For the calculations, the haemolymph and muscle were assumed to represent 30 % and 25 % of body mass, respectively, and the hepatopancreas, 5 % (Heath and Barnes, 1970; Lallier and Walsh, 1991). For both species, values were calculated for a 100 g crab.

Carcinus maenas

In response to a 72 h-emersion, 1805 and 23 μmol of nitrogen accumulated as amino acids in muscle and haemolymph, respectively, and 125 μmol of nitrogen was liberated from the hepatopancreas FAAs (Fig. 5). Thus, the net increase in nitrogen stored as amino acids was 1703 μmol for a 100 g crab exposed to air for 72 h in our experimental conditions.

At reimmersion, 1290 μmol of nitrogen was liberated from the muscle, while 18 μmol of nitrogen accumulated in the haemolymph. Thus, 1272 μmol of nitrogen would be liberated in a 100 g crab during a re-immersion period of 24 h.

Necora puber

In response to an 18 h air-exposure, 101 μmol of nitrogen accumulated as amino acids in the haemolymph (Fig. 5). The significant decreases in amino acid levels in the muscle and hepatopancreas would liberate 92 and 151 μmol of nitrogen, respectively. Thus, in this species, nitrogen liberated from

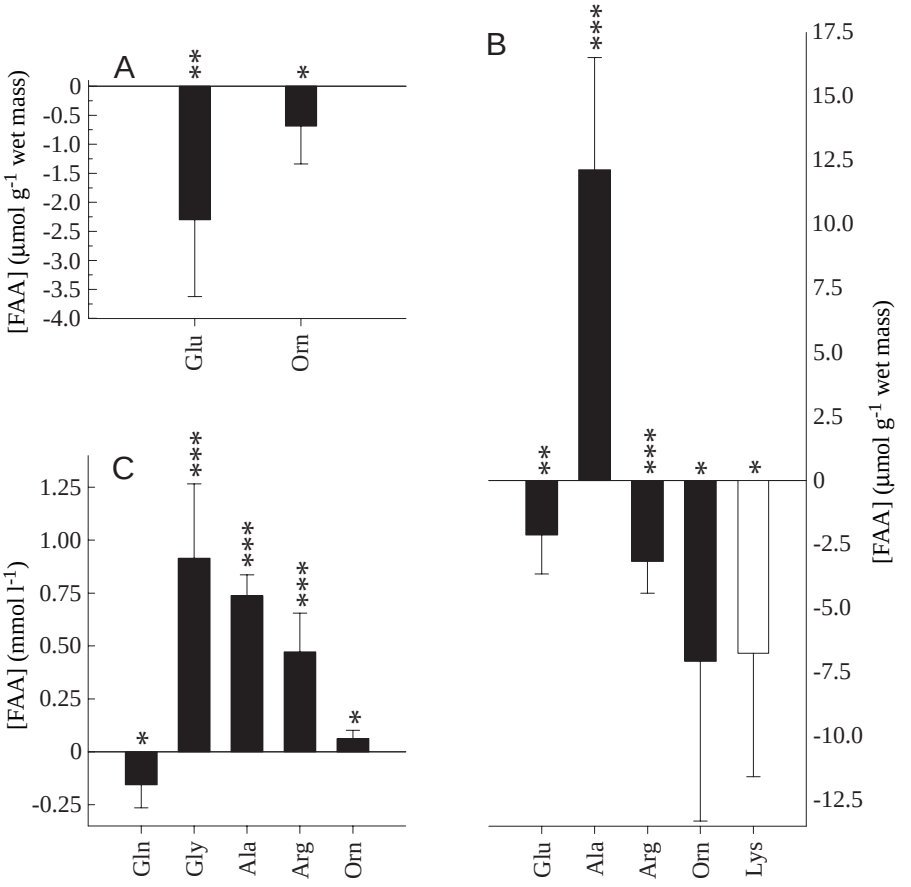

Fig. 4. Changes in the levels of individual amino acids resulting from an 18 h air-exposure period in muscle (A), hepatopancreas (B) and haemolymph (C) of *Necora puber*. Values are mean differences + 95 % confidence interval. Filled columns, non-essential amino acids (NEAAs); open columns, essential amino acids (EAAs). Significant changes are indicated with an asterisk. Significance levels: **P*<0.05, ***P*<0.01, ****P*<0.001.

FAAs in the three compartments studied would represent 142 μmol of nitrogen for a 100 g crab during an 18 h air-exposure period.

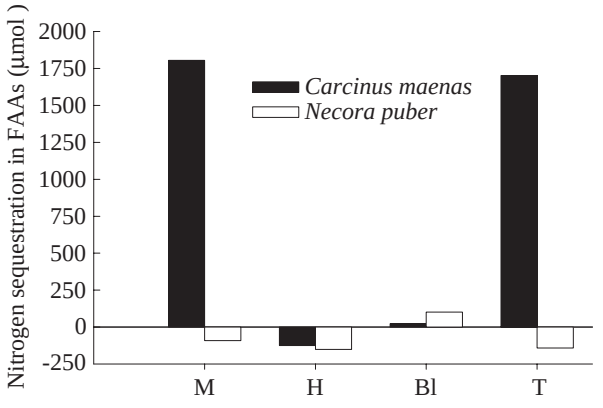

Fig. 5. Contributions of various tissues to nitrogen sequestered as free amino acids and the total amount of nitrogen sequestered as the result of a 72 h emersion in *Carcinus maenas* (filled columns) and an 18 h emersion in *Necora puber* (open columns). Values are calculated for a 100 g crab (see Results). Negative values indicated nitrogen production from amino acids of the tissue. Bl, haemolymph; H, hepatopancreas; M, muscle; T, total.

Urate content of muscle and hepatopancreas

Carcinus maenas

In control crabs, the urate contents of the muscle and hepatopancreas were 0.043±0.011 μmol g^{-1} wet mass (*N*=12) and 0.059±0.005 μmol g^{-1} wet mass (*N*=8), respectively.

During emersion, the urate content of the muscle decreased significantly (*P*<0.05) after 24 h of emersion and levelled off at approximately 0.025 μmol g^{-1} wet mass at 48 h of air-exposure (Fig. 6A). At the end of the 72 h emersion period, the muscle urate content was 0.029±0.003 μmol g^{-1} wet mass (*N*=12).

In contrast, the level of urate in the hepatopancreas increased in response to prolonged emersion (Fig. 6A), with significantly higher values being recorded after 48 h of air-exposure (0.093±0.014 μmol g^{-1} wet mass, *N*=11).

Necora puber

In control crabs, the urate contents of the muscle and hepatopancreas were 0.224±0.059 μmol g^{-1} wet mass (*N*=8) and 0.555±0.190 μmol g^{-1} wet mass (*N*=8), respectively. In this species, individual values of urate content were widely variable, ranging from 0.078 to 0.612 μmol g^{-1} wet mass in muscle and from 0.075 to 1.707 μmol g^{-1} wet mass in the hepatopancreas.

In response to emersion, no significant changes in urate

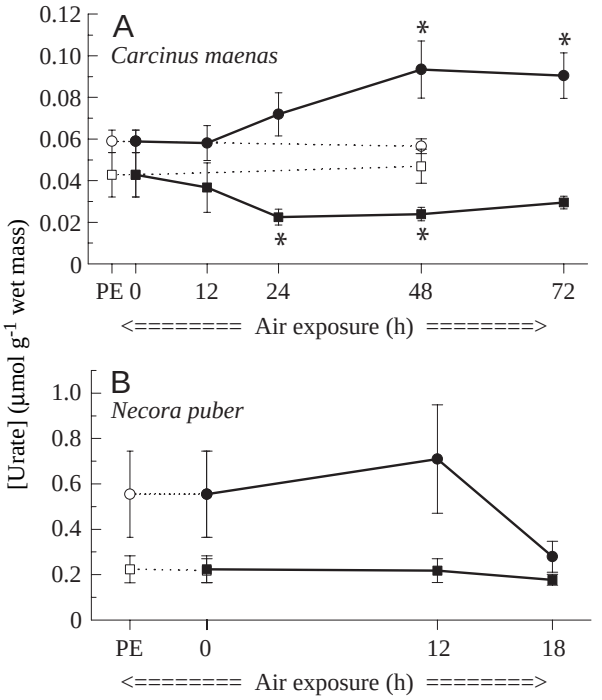

Fig. 6. Changes in urate content (µmol g^{-1} wet mass) of muscle (filled squares) and hepatopancreas (filled circles) during prolonged air-exposure. (A) *Carcinus maenas* exposed to air for 72 h; values are means ± S.E.M., *N*=8–12. (B) *Necora puber* exposed to air for 18 h; values are means ± S.E.M., *N*=7–10. The urate content of both tissues in control immersed crabs at the onset of air-exposure (PE, pre-emersion value) is indicated (open symbols). For *Carcinus maenas*, the urate content of both tissues in 48 h-immersed controls (*N*=4) is also indicated. Significant changes (*P*<0.05) between control (PE) and experimental crabs are indicated with an asterisk.

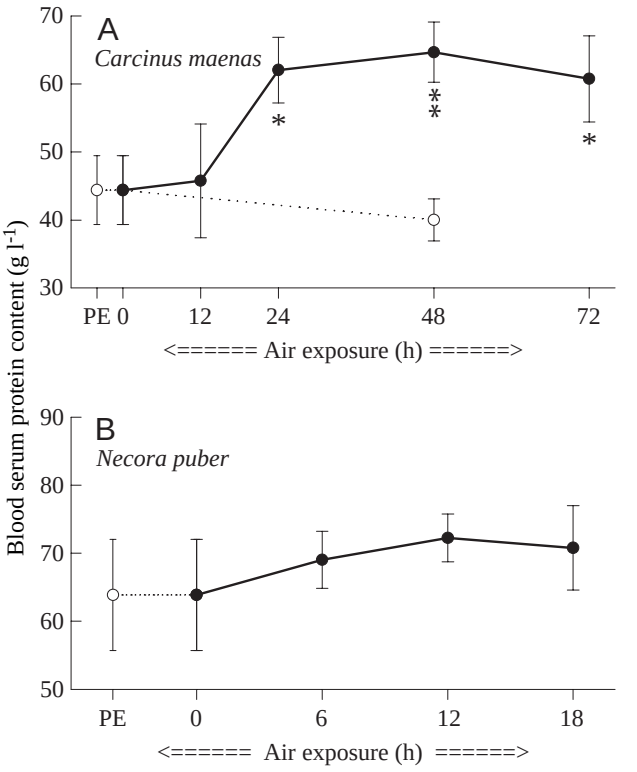

Fig. 7. Changes in protein content (g l^{-1}) of haemolymph serum during prolonged air-exposure. (A) *Carcinus maenas* exposed to air for 72 h (filled symbols); values are means ± S.E.M., *N*=11–16. (B) *Necora puber* exposed to air for 18 h (filled symbols); values are means ± S.E.M., *N*=8–10. The protein content of haemolymph serum in control immersed crabs at the onset of air-exposure (PE, pre-emersion value) is indicated (open symbols). For *Carcinus maenas*, the protein content of haemolymph in 48 h-immersed control (*N*=4) crabs is also indicated. Significant changes are indicated with an asterisk. Significance levels: **P*<0.05, ***P*<0.01.

content of either muscle or hepatopancreas were observed (Fig. 6B).

Blood serum proteins

The protein content of blood serum in control *C. maenas* was 44.4±5.1 g l^{-1} (*N*=16). In response to emersion, a large and significant increase in blood serum protein level was observed between 12 h and 24 h of air-exposure, reaching 64.7±4.4 g l^{-1} (*N*=11, *P*<0.01) after 48 h of emersion (Fig. 7A). No further changes were observed until the end of the air-exposure period.

The protein content of blood serum in control *N. puber* was 63.9±8.2 g l^{-1} (*N*=8), and no significant changes in serum protein levels were observed throughout the 18 h air-exposure period (Fig. 7B).

Discussion

A previous study has shown that, under similar conditions of air-exposure, *Carcinus maenas* and *Necora puber* have to face a potential ammonia overload because their rate of ammonia excretion is greatly reduced (Durand and Regnault, 1998). It was observed that the intertidal species *C. maenas* was able to regulate both its blood ammonia content and its muscle ammonia content, whereas the subtidal species *N. puber* could not and was overloaded with the end-products of nitrogen metabolism. Since amino acid synthesis is known to be an ammonia-detoxifying process, its enhancement in response to prolonged emersion was investigated in both species.

The present study shows that, in *C. maenas*, the amino acid content of muscle increased greatly within the first 24 h of emersion (Fig. 1A). Amino acids that accumulated in this tissue could have different origins: (i) from the diet, (ii) from protein degradation, (iii) from the transfer of amino acids from some other body compartment to the muscle and (iv) from *de novo* synthesis of amino acids. The first hypothesis can be discarded because crabs were kept unfed for 24 h before the experiments and were not fed during emersion. In addition, short-term starvation has been observed to have no major effects on the amino acid content of tissues in *C. maenas* immersed for 48 h. Similar observations were made by Dall and Smith (1987) in muscle tissue of *Penaeus esculentus* after

5 days of starvation and by Taylor et al. (1987) in the free amino acid pool of haemolymph and muscle in *Palaemon elegans* after 6 h, 12 h and 24 h of starvation. Moreover, Harris and Andrews (1985) observed no changes in the content of ninhydrin-positive substances in the haemolymph of immersed and unfed *Carcinus maenas* throughout a 144 h experiment.

With regard to the second hypothesis (ii), an increase in the FAA pool has been observed in *C. maenas* in response to hyperosmotic stress (Siebers et al., 1972). According to these authors, most new free amino acids originated from the degradation of haemolymph proteins, mainly from haemocyanin (Gilles, 1977). In the present study, no decrease in the protein content of the haemolymph of *C. maenas* was recorded throughout the air-exposure experiment. Besides, given the biochemical composition of *C. maenas* haemocyanin (Boone and Schoffeniels, 1979), its degradation would lead to an equivalent release of NEAAs and EAAs; only the NEAA pool of muscle increased in this study and no increase in EAA levels was observed in any of the compartments studied. This also indicated that the increase in the FAA pool was not the result of tissue dehydration.

With regard to the third hypothesis (iii), although levels of some amino acids decreased in the hepatopancreas (Fig. 2B), the total amount of amino acids (110 μmol) provided by this tissue during the second and the third days of emersion could not account for the large increase in the amount of free amino acids in the muscle compartment (1150 μmol) that occurred during emersion. The decrease in levels of some EAAs in the haemolymph, observed during the first 24 h of air-exposure (Fig. 1C), could nevertheless contribute to the increase in muscle FAA levels, but the amounts of these amino acids transferred were negligible.

Thus, only *de novo* synthesis of amino acids (hypothesis iv) could explain the observed increase in muscle amino acid levels; this conclusion is corroborated by the increase in the NEAA pool and the constant level of the EAA pool. In our previous study of air-exposed *C. maenas*, the transient increase in muscle ammonia content after 12 h of emersion together with the general metabolic responses of *C. maenas* to emersion and during the subsequent recovery period strongly suggested that muscle could be a compartment for ammonia storage as well as a site for ammonia detoxification (Durand and Regnault, 1998). The present results provide strong evidence that muscle tissue is contributing through enhanced amino acid synthesis to reducing the high internal ammonia levels.

Such an increase in NEAA levels has been observed in other ammoniotelic species facing various conditions of ammonia excretion impairment. A large increase in the levels of NEAAs was observed in the isopod *Porcellio scaber* exposed to an atmosphere containing high levels of gaseous ammonia. Two body compartments, the hepatopancreas and body wall, contributed most to ammonia sequestration by synthesis/accumulation of amino acids (Wright et al., 1996). In the amphibious fish *Periophthalmus cantonensis*, a large increase in levels of NEAAs was observed when this species was subjected to either a 48 h air-exposure or an environmental ammonia stress (Iwata, 1988), and muscle was the main body compartment implicated in this ammonia-detoxifying process.

In *C. maenas*, some NEAAs appeared to play a major role in the detoxification of unexcreted ammonia: glutamine, glycine, alanine and arginine. Evidence for the *de novo* synthesis of amino acids was provided by the relative changes in levels of particular amino acids: a concomitant increase in glycine level with a decrease in levels of its precursor serine (after an initial increase in the first 12 h of emersion) may favour the *de novo* synthesis of glycine. In addition, a transient increase in glutamate level was observed after 48 h of emersion, and glutamate synthesis *via* NADH-dependent glutamate dehydrogenase is known to be the main route for incorporation of inorganic ammonia into the NEAA pool (Claybrook, 1983). The activity of NADH-dependent GDH was also observed to increase in the muscle of *Cancer pagurus* following 12 h of air-exposure (Regnault, 1992). The transient increase in the level of this amino acid could be explained by its role as an amino donor for all other NEAAs (alanine, serine, glycine, etc.), its conversion to some other NEAAs, such as proline (not quantified here), and its additional amination to form glutamine. The level of glutamine, commonly implicated in the ammonia detoxification process, was observed to increase in muscle and in haemolymph (Fig. 2C). In this compartment, glutamine level increased throughout the 3 day air-exposure period at a constant rate of approximately $0.13\,\text{mmol}\,\text{l}^{-1}\,\text{day}^{-1}$, and an increase such as this has never been observed for any haemolymph metabolites. Glutamine represented a non-toxic form for ammonia transport during prolonged air-exposure. Also, it could provide amino groups for glucosamine synthesis (which is enhanced in response to prolonged air-exposure; Regnault, 1996) and for the subsequent synthesis of glycosaminoglycans, the level of which increased in gill tissue of *C. maenas* under the present conditions of emersion (Regnault and Durand, 1998).

An unexpected increase in arginine level was also observed during emersion. A large increase in arginine level, which accounted for approximately 25 % of net nitrogen sequestration, has previously been observed in ammonia-stressed *Porcellio scaber* (Wright et al., 1996), and these authors suggested that *de novo* synthesis of arginine was occurring in this species. In *C. maenas*, the accumulation of arginine in muscle tissue is all the more surprising because it is usually considered to be an essential amino acid in crustaceans. Furthermore, its production *via* the urea cycle appears to be unlikely since a functional urea cycle is lacking in this phyla (Claybrook, 1983). During emersion, arginine would originate from dephosphorylation of phosphoarginine rather than from *de novo* synthesis:

$$\text{Phosphoarginine} + \text{ADP} \leftrightarrow \text{Arginine} + \text{ATP}\,.$$

ATP thus generated would supply an additional energy source, because most of the carbon skeletons of newly formed amino acids originate from glycolytic and tricarboxylic acid cycle intermediates, limiting the supply of energy substrates and reducing energy production. During reimmersion, both alanine

and glycine were rapidly degraded and are very probably used as energy substrates. The resulting ATP production, together with the high arginine levels, would restore phosphagen reserves by the reverse reaction.

Moreover, when arginine nitrogen is omitted from the calculation of the nitrogen balance, the amounts of nitrogen sequestered in all other amino acids during the 72 h emersion period and liberated during the 24 h reimmersion period were 1048 μmol per 100 g crab and 725 μmol per 100 g crab, respectively. These values are close to those calculated in our previous study (1130 μmol per 100 g crab and 810 μmol per 100 g crab, respectively; Durand and Regnault, 1998), indicating that arginine did not contribute to the sequestration of unexcreted ammonia during prolonged air-exposure.

In *C. maenas*, amino acid synthesis was initiated during the first 12 h of emersion and continued during the next 12 h of emersion (Fig. 1A). It also occurred later, between 24 h and 48 h of air-exposure, but at an apparently lower rate, because the NEAA pool increased by only approximately 14 μmol g^{-1} wet mass. During this last period, a concomitant and significant decrease in the pool of EAAs suggests that some of the newly synthesised amino acids and the EAAs were used for protein synthesis. The large increase in blood serum protein level supported this assumption (Fig. 7A). This increase could not be attributable to water loss from the haemolymph by dehydration because, in the highly hydrated atmosphere of our experimental conditions, water loss in *C. maenas* represents approximately 2–3 % of body mass, and this water loss is at the expense of branchial water (Ahsanullah and Newell, 1977). Moreover, blood serum protein levels did not change in *Necora puber*, although this species is more sensitive than *Carcinus maenas* to dehydration (Johnson and Uglow, 1985). Furthermore, in semi-terrestrial species, air-exposure in hydrated air did not induce water loss (Taylor and Greenaway, 1994).

Synthesis of proteins, using newly synthesised amino acids, has also been observed, using ^{15}N incorporation, in *Periophthalmus modestus* exposed to external ammonia loading (Iwata and Deguchi, 1995). In the case of *C. maenas*, protein synthesis would contribute to the regulation of the pool of free amino acids in muscle, which cannot support unlimited increases in these active osmotic compounds without facing some physiological disruption. The present results suggested that the haemolymph was a storage compartment for these newly synthesised proteins.

During the recovery period of *C. maenas* emersed for 72 h, deamination of muscle amino acids, possibly then used as metabolic substrates at reimmersion, was strongly suggested by the large increase in ammonia content of muscle and the high level of haemolymph ammonia recorded at this time (Durand and Regnault, 1998). However, at reimmersion, the high levels of amino acids recorded in prebranchial haemolymph indicate that part of the amino acids synthesised in the muscle was released into the haemolymph and very likely degraded at the gills. In addition, at reimmersion, the simultaneous increase in NEAA and EAA levels in the haemolymph (Fig. 1C) corroborated our previous conclusions (i) that newly formed NEAAs and endogenous EAAs were used for protein synthesis during the emersion period, and (ii) that newly formed proteins were degraded at reimmersion.

In contrast to the intertidal species *C. maenas*, the subtidal species *Necora puber* did not exhibit an ability to store unexcreted ammonia as amino acids. Although a large increase in FAA levels was observed in the haemolymph (Fig. 3C), this compartment contributed in only a minor way to ammonia storage. Moreover, the simultaneous increase in NEAA and EAA levels suggested that these amino acids originated partly from protein breakdown. Thus, in this species, amino acid synthesis as an ammonia-detoxifying process appears to be inefficient and, as previously observed, a large internal ammonia overload resulted from an 18 h air-exposure. Furthermore, the large increase in alanine level observed in the hepatopancreas (Fig. 4B) may reflect the requirements of anaerobic metabolism more than an active process for ammonia detoxification. This result confirmed previous reports of a large increase in blood lactate level in *N. puber* indicating that this species relies on anaerobic metabolism for its energetic needs (Johnson and Uglow, 1985; Durand and Regnault, 1998).

Storage of urate in the body of terrestrial crabs has been observed in many species (Gifford, 1968; Greenaway, 1991; Linton and Greenaway, 1997a) and a *de novo* purine synthesis pathway has been demonstrated in *Gecarcoidea natalis* (Linton and Greenaway, 1997b). Although the role of urate storage is still under study, it has been proposed that urate is a nitrogenous compound for ammonia detoxification under dry conditions that affect ammonia excretion (Linton and Greenaway, 1998).

In *Necora puber*, surprisingly high levels of urate, given its solubility, were recorded in the muscle and hepatopancreas, indicating that urate was probably stored in a solid form. Intracellular urate granules have been found to be widely distributed among the spongy connective tissue associated with various organs in *Gecarcoidea natalis* (Linton and Greenaway, 1997a). The importance of urate storage in land crabs was observed to be dependent on the nitrogen content of their dietary source (Wolcott and Wolcott, 1984, 1987; Linton and Greenaway, 1997a). In immersed *N. puber*, the large range of values recorded in the muscle and hepatopancreas may reflect the dietary history of the crabs. However, these levels did not indicate whether the urate originated from purine catabolism or from *de novo* synthesis. No increase in urate content of either compartment was observed in response to prolonged emersion. In contrast, there was a tendency (close to the significance level) for urate content to decrease in the hepatopancreas between 12 h and 18 h of air-exposure (Fig. 6B). Because blood urate content increased markedly throughout the period of air-exposure in this species (up to 650 μmol l^{-1}; Durand and Regnault, 1998), urate liberated from the hepatopancreas would be a possible origin for haemolymph urate. Urate is known to increase the oxygen affinity of

haemocyanin (Morris et al., 1985), and this process would help this species to cope with hypoxic conditions (Lallier et al., 1987).

In contrast to *N. puber*, the urate contents of the muscle and hepatopancreas of *C. maenas* were similar to those measured in the haemolymph (Durand and Regnault, 1998). In response to air-exposure, a significant increase in urate content of the hepatopancreas was observed during the second day of emersion (Fig. 6A). Uricase is inhibited when oxygen availability is reduced (Dykens, 1991), and inhibition of urate breakdown rather than enhancement of uricogenesis as a process for ammonia detoxification would explain the increase in urate level in the hepatopancreas. Furthermore, the amount of nitrogen that accumulated as urate in this compartment (0.6 μmol) during the air-exposure period would account for only a minor part of the total sequestered nitrogen. The synthesis of 1 mol of urate requires 4 mol of amino acid precursors. Thus, this mode of ammonia detoxification allows much more effective long-term sequestration of nitrogen. Urate synthesis may represent an additional stage in the adaptation of the nitrogen metabolism of terrestrial crustaceans during their transition to land.

In summary, this study shows that the intertidal crab *C. maenas* has developed an efficient ammonia-detoxifying process based on amino acid synthesis to deal with the potential ammonia overload caused by prolonged air-exposure. This process, together with the known respiratory abilities of emersed *C. maenas*, could make it a transition species between the fully aquatic crabs, such as *N. puber*, and the semi-terrestrial crabs.

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