EFFECTS OF AMMONIA LOADING ON PORCELLIO SCABER: GLUTAMINE AND GLUTAMATE SYNTHESIS, AMMONIA EXCRETION AND TOXICITY

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Summary
The effects of ammonia loading in the terrestrial isopod Porcellio scaber were studied by exposing animals to atmospheres of high $P_{\text{NH}_3}$. Isopods show a remarkable tolerance of elevated ambient $P_{\text{NH}_3}$, with an LD$_{50}$ of 89 Pa for a 7-day exposure. However, haemolymph total ammonia concentrations generally remained below 5 mmol l$^{-1}$ ($P_{\text{NH}_3}=0.37$ Pa) over the range of ambient ammonia levels used (6.6–165 Pa). Following a 7-day loading period, whole-animal glutamine (Gln) and glutamate (Glu) levels increased in direct proportion to ambient $P_{\text{NH}_3}$, reaching values of 35 $\mu$mol g$^{-1}$ fresh mass for glutamine and 12 $\mu$mol g$^{-1}$ fresh mass for glutamate in 99 Pa $P_{\text{NH}_3}$; these correspond to control levels of 7.5 $\mu$mol g$^{-1}$ fresh mass and 5.9 $\mu$mol g$^{-1}$ fresh mass, respectively. Following transfer to ammonia-free chambers, NH$_3$ excretion rates were augmented five-to-sixfold relative to non-loaded controls. Ammonia volatilization subsequently declined, approaching control levels after 8–9 days. Levels of Gln and Glu showed a concomitant decline to 13.7 $\mu$mol g$^{-1}$ fresh mass and 9.2 $\mu$mol g$^{-1}$ fresh mass, respectively. The results suggest that these amino acids function in ammonia sequestration and, hence, detoxification. Calculations indicate that mobilization of amino groups by deamination of accumulated Gln and Glu could explain 35% of the increased ammonia production. Implications of NH$_3$ volatilization for acid–base balance are discussed.

Introduction
Aquatic crustaceans are primarily ammonotelic, and this mode of nitrogen excretion has been retained by most terrestrial species. In the terrestrial brachyuran crabs Geocarcinus lateralis and Cardisoma guanhumi, the antennal glands produce relatively little nitrogenous waste and the gills are the primary site of ammonia excretion. The urine is passed into the branchial chamber where ions are reabsorbed by the gills. During this process of urine recycling, ammonia concentration increases more than tenfold (Wolcott, 1991). In contrast, the antennal glands of ghost crabs (Ocypode quadrata) produce an acidic urine containing high concentrations of ammonia. As urine is reprocessed by the gills, pH increases and ammonia concentrations decline, suggesting volatilization of NH$_3$ (De Vries and Wolcott, 1991). In Geograpsus grayii the respiratory surfaces are the likely

Key words: isopod, Porcellio scaber, ammonia loading, glutamine, glutamate.
site of ammonia release, and in this crab gaseous ammonia accounts for more than 80% of total nitrogen excretion (Greenaway and Nakamura, 1991).

The terrestrial isopods (Crustacea, Isopoda, Oniscidea) are also primarily ammonotelic (Dresel and Moyle, 1950; Hartenstein, 1968; Wieser et al. 1969; Greenaway, 1991). Most ammonia is volatilized as NH₃ and accounts for over 90% of the total nitrogenous waste (Hartenstein, 1968). Collection of NH₃ in acid traps has revealed that patterns of excretion in Porcellio scaber are irregular and intermittent, with ammonia being volatilized in substantial ‘bursts’ (Wieser et al. 1969; Wieser and Schweizer, 1970). In fasted animals, the incidence of such bursts shows a clear diel rhythm with diurnal maxima, although this is all but abolished in animals taken from populations fed ad libitum (Wieser et al. 1969). Similar diel excretory patterns were reported by Wieser (1972) for the intertidal species Ligia beaudiana Milne-Edwards and by Kirby and Harbaugh (1974) for an idoteid, Idotea granulosa Rathke. Although patterns of excretion have only been studied over considerable time intervals (>30 min), workers have assumed, given the large amounts of ammonia frequently released, that volatilization is probably continuous during individual bursts.

The physiological mechanism whereby ammonia is volatilized has remained a matter of some speculation. Wieser and Schweizer (1972) measured significant concentrations of ammonia in the haemolymph of P. scaber and proposed that ammonia was transported (actively or passively) across the mitochondria-rich epithelia of the pleopodal endopods into the external pleon fluid that bathes the endopodal surfaces (Verhoeff, 1917, 1920; Unwin, 1931). From there, volatilization of NH₃ could be assisted by alkalization. Subsequently, Hoese (1981) proposed a similar theory for pleon volatilization, involving local elevation of pH to increase the NH₃/NH₄⁺ ratio, but with ammonia being excreted by the paired anterior maxillary glands rather than by the pleopodal endopods; the maxillary urine is conveyed to the pleon via the ventrolateral, infolded pleurae constituting the capillary or water transport system. Hoese’s theory has been accepted by several succeeding workers (e.g. Wieser, 1984; Kümmel, 1984). However, by wrapping individual P. scaber in moist Phenol Red indicator paper, Kirby and Harbaugh (1974) showed that the incidence of ammonia bursts associated exclusively with the pleon was approximately double that of anterior (maxillary) bursts.

More recently, measurements of total ammonia concentrations in the three major body fluids of oniscideans (haemolymph, pleon fluid and maxillary urine) have provided new evidence in favour of direct pleon excretion (Wright and O’Donnell, 1993). These fluids generally contain low ammonia levels (<1.5 mmol l⁻¹) which periodically increase by an order of magnitude or more. The dramatic elevations in total ammonia levels are closely correlated with periods of copious pleon fluid production and ventilatory metachronal beating of the exopods. Bouts of maxillary urination are irregular and show no temporal relationship to the increases in haemolymph ammonia levels, although ammonia levels in the two fluids are probably isosmotic. Urination would thus appear to have only an incidental role in NH₃ volatilization. In vivo pH measurements of the body fluids do not reveal periodic increases in pH that might serve to facilitate NH₃ release. Nevertheless, given a mean pleon fluid (and haemolymph) pH of approximately 7.6 (Wright and O’Donnell, 1993), the periodic ammonia elevations would still generate NH₃
concentrations in the 0.5–1.0 mmol l\(^{-1}\) range, and these would provide a significant partial pressure gradient for volatilization. Although a direct link between total ammonia increases in the haemolymph/pleon fluid and the excretory ‘bursts’ remains to be established, the similar time-courses of the two processes and the association between increased fluid ammonia levels and pleopodal ventilation, which would facilitate volatilization, both indicate that substantial mobilization of ammonia into the haemolymph precedes volatilization.

In the longer term, ammonia excretion in \textit{P. scaber} and \textit{Oniscus asellus} L. shows a clear seasonal pattern, with a spring–summer maximum and an autumn–winter minimum (Wieser \textit{et al.} 1969). The seasonal variations in rates of excretion are inversely correlated with ammonia levels in the somatic tissues, with concentrations ranging from 1.13 to 17.3 mmol l\(^{-1}\) in \textit{P. scaber} (Wieser and Schweizer, 1972). Amino acid analysis indicated substantial levels of both glutamine and glutamate, the former varying directly and the latter inversely with rates of ammonia excretion. In August, the mean concentration of glutamine was 6.46 mmol l\(^{-1}\), representing 7.14\% of total amino acids (AAs), and that of glutamate was 6.2 mmol l\(^{-1}\) (6.12\% of total AAs). The increases in summer glutamine levels are also related to variations in somatic glutaminase activity, which shows an autumn–winter maximum and a spring–summer minimum (Wieser and Schweizer, 1972; Wieser, 1972). Wieser (1972) suggested that the high glutaminase activities observed in winter may relate to the more erratic release of ammonia in this season, volatilization occurring in fewer, more substantial bursts (Wieser \textit{et al.} 1969).

Interpretation of the seasonal variations in components of nitrogen metabolism is complicated by the associated variations in diet and protein metabolism, metabolic rate and reproductive status. Ambient relative humidity (RH) may also be an important factor, since ammonia excretion has been shown to be augmented in high humidities when animals absorb water vapour (Wright and O’Donnell, 1993). Nonetheless, it is clear from the foregoing discussion that glutamine is likely to play a significant role in the nitrogen metabolism of oniscideans. In addition to serving as a source of glutamate for transamination and amino acid synthesis, it may be important in the storage (and hence detoxification) of ammonia between bouts of volatilization (Wieser, 1972; Wright and O’Donnell, 1993).

In order to explore the roles of glutamine and glutamate in ammonia sequestration, we have developed a novel method for loading animals with high levels of ammonia. This technique involves the exposure of animals to high ambient \(P_{\text{NH}_3}\), and provides a simple and less invasive alternative to haemocoelic injection of ammonium salts or the use of dietary supplements (Shorey \textit{et al.} 1967; Warren and Schenker, 1960). Moreover, ambient \(P_{\text{NH}_3}\) can be selected so as to overcome the outward vapour pressure gradients generated by the animal. In this way it is possible to block NH\(_3\) volatilization completely, thereby enhancing ammonia loading.

\section*{Materials and methods}
\textit{Porcellio scaber} Latreille were maintained in a laboratory culture at 20–25 °C and fed
on a combination of deciduous litter and rabbit chow. Under these conditions, reproduction continued throughout the year.

**Ammonia loading**

Ammonia accumulation in isopods was induced by exposing animals to atmospheres of high $P_{NH_3}$. This procedure is referred to as 'ammonia loading'. The Henderson–Hasselbalch equation can be used to calculate $NH_3$ concentrations for aqueous solutions of known pH and total ammonia concentration ($NH_4^+$$+$$NH_3$):

$$pH = pK_2 - \log\left(\frac{[NH_4^+]}{[NH_3]}\right).$$

Hence:

$$[NH_3] = \frac{([NH_4^+] + [NH_3])}{10^{pK_2 - pH}}.$$  

$NH_4^+$ and $NH_3$ are equimolar at pH 9.25 (i.e. the $pK$ of $NH_4^+$ is 9.25; Lehninger, 1982). Knowing the aqueous $NH_3$ concentration, the $P_{NH_3}$ can be determined by dividing the millimolar concentration by the solubility coefficient of $NH_3$ in water at 20°C (0.303 mmol l$^{-1}$ Pa$^{-1}$; Braker and Mossman, 1971). If animals attain passive equilibration with the loading atmosphere (i.e. $P_{NH_3}$ of the haemolymph equals $P_{NH_3}$ of the loading atmosphere), then the theoretical haemolymph $[NH_3]$ will equal that of the loading solution. The haemolymph total ammonia concentration is then readily determined, knowing haemolymph pH, from the Henderson–Hasselbalch equation. *In vivo* measurements of haemolymph pH for *P. scaber*, using ion-selective micro-electrodes, give a mean value of 7.60 (Wright and O’Donnell, 1993).

Ammonia-loading chambers consisted of sealed, 100 ml glass jars containing 20 ml of aqueous ($NH_4$)$_2$SO$_4$ made up to pre-determined concentrations to give a range of known $P_{NH_3}$ values. The solutions were buffered with 10 mmol l$^{-1}$ Taps (N-tris[hydroxymethyl]methyl-3-amino-propanesulphonic acid) and the pH was adjusted to 8.0 with 0.1 mol l$^{-1}$ H$_2$SO$_4$. Animals were weighed, sexed and transferred in pairs (male and female) to 20 ml glass vials which were placed in the loading chambers.

When animals are volatilizing ammonia, haemolymph total ammonia concentrations can reach levels of 100–150 mmol l$^{-1}$, corresponding to an ambient $P_{NH_3}$ value of between 7.5 and 11.0 Pa. Preliminary observations indicated that significant mortality occurred only in much higher ambient partial pressures and, consequently, for determinations of ammonia tolerance, the range of experimental values was extended. The regulating solutions used are defined in Table 1. Equilibration times of the $P_{NH_3}$ chambers were determined by substituting a piece of glass wool containing 100 μl of deionized water for the animals and measuring the total ammonia concentration of the fluid in replicate controls at hourly intervals. Ammonia was assayed using the Sigma procedure 170-UV, based on the method of Mondzac *et al.* (1965).

Pairs of animals were exposed to known $P_{NH_3}$ for periods of seven days. The pH of the regulating ($NH_4$)$_2$SO$_4$ solutions remained stable over this period. Control animals were maintained in humidified, ammonia-free chambers. During the course of the week, mortalities were noted and dead animals removed. Cumulative mortalities for the different loading treatments were analysed by Probit transformation (Finney, 1964). At the end of incubation, haemolymph from the animals was sampled using a pulled glass
micropipette. Sample volumes were measured in Drummond micro-capillaries using an eyepiece micrometer. Each sample was then assayed for total ammonia. Individual animals could be removed from the chamber and sampled within 5 min, after which they were promptly killed by decapitation and prepared for the total glutamine/glutamate assay.

**Glutamine and glutamate assays**

Separation of free amino acids and precipitation of proteins from tissue samples followed the procedure of Lund (1986). Freshly killed animals were transferred to 1.5 ml Eppendorf tubes and their tissues were cut up finely using iridectomy scissors. They were then treated with 50 μl of ice-cold 1.7 mol l⁻¹ perchloric acid, agitated for 2 min, and diluted to a net volume of 400 μl with double distilled water. Each sample was subsequently neutralized to pH 7.0 with a solution containing 4 mol l⁻¹ KOH and 0.4 mol l⁻¹ KCl to favour precipitation of perchlorate (Lund, 1986). Restriction of acid precipitation to a 2 min period minimizes breakdown of glutamine. After neutralization, samples were sonicated for two bouts of 2 min in an XL 2020 sonicator cell disruptor (Heat Systems Ultrasonics Inc., NY) to disrupt cell membranes, and centrifuged at 3000 g for 10 min. Samples of 125 μl of supernatant were removed for glutamine and glutamate assays following the Sigma procedure GLN-1.

For glutamine and glutamate assays, a standard curve was compiled using glutamine concentrations ranging from 0 to 1.0 mmol l⁻¹. Two samples of 125 μl were used for each assay, one pre-treated with 375 μl glutaminase (10 i.u. ml⁻¹) in 100 mmol l⁻¹ acetate buffer (pH 5.0) at 37 °C for 1 h to yield glutamate through deamination of glutamine, the other treated in the same way but without glutaminase so as to measure endogenous glutamate only. Half of each pre-treated sample (250 μl) was then added to 750 μl of assay solution, made up to give the following final concentrations (in mmol l⁻¹): 50 Tris, 1 EDTA, 300 hydrazine, 1.5 NAD and 0.5 ADP. Initial absorbances were read at 340 nm on a Perkin-Elmer-3 spectrophotometer. Each sample was then treated with 0.01 ml L-GDH (1200 i.u. ml⁻¹), mixed by inversion, and left for 40 min at room temperature before

<table>
<thead>
<tr>
<th>Total ammonia (mmol l⁻¹)</th>
<th>NH₃ (mmol l⁻¹)</th>
<th>PNH₃ (Pa)</th>
<th>Haemolymp total ammonia (mmol l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.6</td>
<td>2</td>
<td>6.6</td>
<td>89</td>
</tr>
<tr>
<td>88.9</td>
<td>5</td>
<td>16.5</td>
<td>223</td>
</tr>
<tr>
<td>177.8</td>
<td>10</td>
<td>33.0</td>
<td>447</td>
</tr>
<tr>
<td>268.6</td>
<td>15</td>
<td>49.5</td>
<td>670</td>
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<tr>
<td>358.2</td>
<td>20</td>
<td>66.0</td>
<td>893</td>
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<tr>
<td>447.8</td>
<td>25</td>
<td>82.5</td>
<td>1117</td>
</tr>
<tr>
<td>537.4</td>
<td>30</td>
<td>99.0</td>
<td>1340</td>
</tr>
<tr>
<td>895.6</td>
<td>50</td>
<td>165.0</td>
<td>2234</td>
</tr>
</tbody>
</table>

Haemolymp total ammonia concentrations are theoretical values assuming a pH of 7.6 and passive equilibration with the loading PNH₃.
reading the absorbance again. The absorbance shift represents the accumulation of
NADH through the oxidative dehydrogenation of L-glutamate to α-ketoglutarate.
Glutamate and glutamine concentrations were then determined from the standard curve;
this was very close to a perfect linear fit (r²=0.995). Tissue concentrations
(mmol g⁻¹ fresh mass) are determined from the test sample concentration by multiplying
by the dilution factor involved in deproteination (400 µl per animal volume in µl).

In order to assess the effects of ammonia loading on subsequent rates of NH₃ excretion,
some animals were loaded for 7 days in a P_{NH₃} of 99 Pa and then transferred to sealed jars
containing a humidifying wick and a 10 ml vial containing an acid trap to collect
volatilized NH₃. Acid traps consisted of a small piece of glass wool soaked with 1 ml of
0.1 mol l⁻¹ H₂SO₄. These were replaced daily, diluted tenfold with deionised water, and
assayed for total ammonia. Control animals were placed in humidified, ammonia-free
chambers for 7 days prior to the trapping of volatilized NH₃ to test for the effects of
fasting over the experimental period. Following 8–10 days, animals were killed and
assayed for Gln and Glu as before.

Results

Times required for equilibration of P_{NH₃} within the loading chambers were determined
for values of P_{NH₃} spanning the experimental range: 6.6 Pa, 82.5 Pa and 165.0 Pa (N=3 for
each P_{NH₃}). In all chambers, P_{NH₃} reached 90 % of the equilibrium value within 14 h.

Ammonia tolerance and haemolymph ammonia levels following loading

Animals exposed to elevated P_{NH₃} showed a rapid general suppression of locomotory
activity and accompanying sensory inhibition; activity was quickly restored on transfer
to ammonia-free chambers. The isopods displayed remarkable tolerance of the elevated
external ammonia levels: appreciable mortalities (>50 % over the 7-day period) were
noted only when ambient P_{NH₃} values exceeded 82.5 Pa. Cumulative mortalities in
different P_{NH₃} are illustrated in Fig. 1; data are pooled for males and females since
there was no significant difference between the sexes. Probit transformations of this
data set permit determination of the values of LT₅₀ (the duration of exposure to a given
P_{NH₃} inducing 50 % mortality) and the LD₅₀ for the 7-day exposure (the P_{NH₃} inducing
50 % mortality over this loading period). LT₅₀ for the 165 Pa treatment was 1.52 days,
and 2.44 days for the 99 Pa treatment. The LD₅₀ for the 7-day exposure was 89.2 Pa. If
animals were to attain passive equilibrium with this P_{NH₃}, assuming a haemolymph pH
of 7.6, the requisite total haemolymph ammonia concentration would be
1240 mmol l⁻¹.

In fact, total ammonia assays of haemolymph samples following 7 days of loading
showed only small increases in comparison with fasted controls (Fig. 2). With four
exceptions, haemolymph total ammonia concentrations were below 5 mmol l⁻¹
(0.22 Pa) for all ambient ammonia levels (6.6–165.0 Pa). Animals thus maintain
haemolymph ammonia concentrations far below levels consistent with passive
equilibration.
Glutamine and glutamate accumulation in response to ammonia loading

Determinations of whole-animal Gln and Glu concentrations following 7 days of NH\textsubscript{3} loading are illustrated in Figs 3 and 4. The graphs also indicate levels of Gln and Glu in control animals maintained in ammonia-free chambers for the same 7-day period. The control level of Gln was 7.52±1.20 μmol g\textsuperscript{-1} fresh mass and of Glu was 5.89±0.48 μmol g\textsuperscript{-1} fresh mass (N=17) and these did not differ significantly from levels measured in feeding animals taken directly from culture (8.49±0.75 and 7.58±0.57 μmol g\textsuperscript{-1} fresh mass, respectively; N=12). These values are in good agreement with the data of Wieser and Schweizer (1972). Under NH\textsubscript{3}-loading conditions, both Gln and Glu were accumulated in direct proportion to ambient P\textsubscript{NH\textsubscript{3}}. Taken together with the finding that haemolymph ammonia levels do not change significantly in response to loading, these observations suggest that accumulated ammonia is sequestered, at least in part, as Gln and Glu. The slopes of the regression lines in Figs 3 and 4 indicate accumulation of Gln and Glu in a 4:1 ratio. A somewhat lower ratio (2.9:1) is indicated from product-moment correlation analysis of paired Gln and Glu measurements for individual animals (r=0.65; P<0.001).

NH\textsubscript{3} excretion following ammonia loading

A dramatic elevation in ammonia excretion was evident following ammonia loading in 99.0 Pa P\textsubscript{NH\textsubscript{3}} (Fig. 5). This response suggests the release of sequestered ammonia that had accumulated during the loading period. For the first 2 days following transfer to saturated (100 % RH) ammonia-free chambers, mean excretion rates were augmented five- to sixfold relative to non-loaded controls. Ammonia excretion subsequently
declined, approaching control levels after 8–9 days. Although cumulative ammonia production over this period varied widely between individuals, animals showed a consistent pattern of ammonia ‘unloading’. This is evident if data for the individual animals are normalized ($N = (E_x - E_9) / (E_1 - E_9)$; where $E_x$, $E_1$ and $E_9$ are the ammonia excretion rates at days $x$, 1 and 9 respectively). Normalized excretion rates are shown in Fig. 5B; data are excluded for two animals in which NH$_3$ production on day 9 exceeded that on day 1, thus precluding normalization.

The cumulative ammonia excretion measured for the 9-day period following loading in Fig. 5 is 204.1 mmol g$^{-1}$ fresh mass. Subtraction of the extrapolated cumulative excretion for the controls (74.0 mmol g$^{-1}$ fresh mass) gives a net increase in ammonia production of 130.1 mmol g$^{-1}$ fresh mass. This value can be compared with the net mobilization of amino groups determined from changes in Gln and Glu concentrations. Concentrations immediately following loading are 35.2 mmol g$^{-1}$ fresh mass for Gln and 11.7 mmol g$^{-1}$ fresh mass for Glu, as determined from the regression lines of Figs 3 and 4. Levels measured following the 9-day period of ammonia release declined to 13.70±2.35 μmol g$^{-1}$ fresh mass for Gln and 9.17±1.36 μmol g$^{-1}$ fresh mass for Glu. The difference in concentrations before and after the 9-day period of ammonia release yields an estimated decline in Gln and Glu of 21.50 mmol g$^{-1}$ fresh mass and 2.53 mmol g$^{-1}$ fresh mass, respectively. Given that there are two amino groups per Gln, this represents a total nitrogen sequestration of 45.53 mmol g$^{-1}$ fresh mass, which can account for approximately 35% of the cumulative measured increase in ammonia excretion.
The potential contribution of Gln and Glu to ammonia sequestration can also be assessed from the relationship between net ammonia excretion and the concentration of each amino acid in the whole animal. For these experiments, cumulative ammonia

**Fig. 3.** Whole-animal glutamine levels (μmol g\(^{-1}\) fresh mass) as a function of ambient \(P_{\text{NH}_3}\). The dashed line indicates glutamine levels (7.52±1.20 μmol g\(^{-1}\) fresh mass; \(N=17\)) in the control group. The solid line was fitted by linear regression analysis; related statistics are shown. The \(P\) value refers to the significance of the regression \(t\)-test for the null hypothesis, \(\beta=0\), where \(\beta\) is the slope.

\[
y = 1.02 + 0.59x \\
r = 0.78, P<0.001
\]

**Fig. 4.** Whole-animal glutamate levels (μmol g\(^{-1}\) fresh mass) as a function of ambient \(P_{\text{NH}_3}\). The dashed line indicates glutamate levels (5.89±0.48 μmol g\(^{-1}\) fresh mass; \(N=17\)) in the control group. The solid line was fitted by linear regression analysis. The data point at 165 Pa was excluded from the regression on the basis of residual analysis (Sokal and Rohlf, 1969).
excretion and Gln and Glu levels were assayed in pre-loaded animals (N=18) which had subsequently been transferred to ammonia-free chambers and allowed to release ammonia over a 3-day period. This duration was chosen as a compromise between the need for a sufficient period for significant ammonia release and the need to measure Gln and Glu concentrations whilst these remained substantially elevated above control levels. Glutamate levels show a sharp decline as a function of NH₃ production (Fig. 6). This indicates a rapid breakdown of accumulated Glu during the early stages of ammonia release. No significant relationship was evident between glutamine levels and net ammonia production on the basis of product–moment correlation analysis (P>0.1). Possible explanations for this finding are discussed below.

Discussion

It is clear from this study that Porcellio scaber displays a remarkable tolerance of elevated ambient ammonia levels. Haemolymph ammonia levels remain low under such conditions, but concentrations of Glu and Gln increase dramatically. These results suggest a probable role for these amino acids in sequestration and hence detoxification of accumulated ammonia.

Our data show that P. scaber withstands chronic exposure to an ambient P₉₉ of at least 99.0 Pa. This is more than three orders of magnitude greater than typical LD₅₀ values for teleost fish (Rice and Stokes, 1975; Campbell, 1991). Several mammals tolerate even greater ambient ammonia levels; guano cave bats withstand prolonged exposure to 300–400 Pa (Mitchell, 1963, 1964), but this depends on efficient filtration of inhaled NH₃.
by respiratory mucus (Studier, 1969). Although isopods in their natural habitat are probably not exposed to the extreme $P_{\text{NH}_3}$ levels used in this study, tolerance of significantly elevated ambient levels may be adaptive. Crowding of animals in restricted diurnal refuges, as a result of orienting responses (Warburg, 1968; Cloudsley-Thompson, 1977) and thigmokinesis (Warburg, 1968; Takeda, 1984), is likely to generate substantial local concentrations of gaseous ammonia (Sutton, 1980).

Our previous study showed that $P.\ scaber$ displays dramatic periodic increases in haemolymph ammonia levels, apparently associated with ammonia volatilization. During these periods, total ammonia concentrations may exceptionally exceed 100 mmol l$^{-1}$, representing NH$_3$ levels higher than 2.2 mmol l$^{-1}$ or approximately 7.3 Pa (Wright and O’Donnell, 1993). These rank among the highest circulating levels reported for Metazoa, exceeded only by those reported for the oplophorid shrimp *Notostomus gibbosus* (Sanders and Childress, 1988). Between periods of volatilization, however, haemolymph total ammonia levels in *P. scaber* typically remain low, between 1 and 1.5 mmol l$^{-1}$.

Ammonia generated by metabolism of proteins and nucleic acids must, therefore, be sequestered during these often prolonged, non-excretory periods. The fact that haemolymph ammonia levels do not increase significantly when animals are exposed to loading conditions indicates that efficient sequestration is maintained even in near-lethal values of $P_{\text{NH}_3}$.

Ammonia sequestration apparently depends, as in the vertebrate liver, on transamination of amino acids to glutamate and subsequent glutamine synthesis. Whole-animal levels of Gln and Glu increase in direct proportion to ambient $P_{\text{NH}_3}$ during ammonia loading. In control animals, the levels of the two amino acids are similar

![Fig. 6. Variations in whole-animal glutamate concentrations ($\mu$mol g$^{-1}$ fresh mass) as a function of the amount of NH$_3$ released over a 3-day period. Data points represent individual animals which were previously ammonia-loaded for 7 days in $P_{\text{NH}_3}$ of 99 Pa. The dotted line indicates the glutamate concentration in animals maintained in saturated, ammonia-free chambers for the same 7-day period.](image)
(5–8 mmol l\(^{-1}\)), but the proportionate increase in Gln during loading is 3–4 times greater than that of Glu. Since every molecule of glutamine contains two amino groups, this corresponds to a six- to eightfold difference in accumulation of amino groups.

When animals are returned to an ammonia-free atmosphere following a period of loading, initial ammonia production is elevated by as much as sixfold and subsequently declines to control levels over the course of 8–9 days. A similar release of accumulated ammonia following exposure to high ambient \(P_{NH_3}\) has been reported for cave bats (Studier, 1966). The net increase in ammonia excretion indicates a corresponding mobilization of sequestered amino groups. Much of this can be accounted for by deamination of Gln and Glu, since their concentrations similarly decline to near-control values after 9 days. The steep decline in Glu levels as a function of net ammonia production, illustrated in Fig. 6, indicates a rapid breakdown over the first few hours. No such relationship was observed for glutamine. This may be attributable to high variance in the initial (post-loading) Gln levels. Variance is exacerbated by the relative instability of Gln, and the possibility of some breakdown during acid precipitation of proteins (Lund, 1986). Such breakdown would lead to a consequent underestimate of Gln accumulation. The absence of a clear relationship between Gln breakdown and ammonia release may also be attributable to a low rate of deamination over the initial 3-day period. When measured over a 9-day period following loading, however, the clear reduction in both Gln and Glu levels can explain approximately 35% of the augmented ammonia production during this period. Preliminary amino acid analyses of ammonia-loaded versus non-loaded isopods indicate that alanine, arginine and glycine also contribute to ammonia storage (J. C. Wright, S. Caveney, M. J. O’Donnell and J. Reichert, unpublished data).

The proposed roles of Gln and Glu in the periodic storage and release of ammonia depend on the presence of appropriate synthetic and degradative enzyme pathways. Significant glutaminase activities have been reported for the somatic tissues (body wall) of \(P.\ scaber\) (Wieser and Schweizer, 1972; Wieser, 1972), although glutaminase activities are low in \(O.\ asellus\) (Hartenstein, 1968). In this oniscidean, deamination depends primarily upon \(L\)-amino acid oxidases, requiring \(H_2O_2\) (Hartenstein, 1968; Lehninger, 1982). Substrates for these enzymes include alanine, asparagine, aspartate, glutamine, glutamate, cysteine, serine and tyrosine. Glutamate dehydrogenase is present in both \(P.\ scaber\) and \(O.\ asellus\). Glutamine synthetase activity was undetectable in \(O.\ asellus\) (Hartenstein, 1968) but no comparable assays have been performed for \(P.\ scaber\). The rapid and large-scale changes in circulating ammonia levels associated with volatilization (Wright and O’Donnell, 1993) require appropriate shifts in enzyme activities and reaction equilibria. The allosteric basis for regulation of glutamate dehydrogenase and glutamine synthetase activities are well known in mammals (Frieden, 1976), although details of the associated regulatory pathways in isopods remain to be determined.

Implications of ammonia volatilization for acid–base balance

Ammonotely in oniscideans creates potential problems for acid–base regulation, as Wieser and Schweizer (1972) have noted. For every molecule of \(NH_3\) volatilized, a
proton is left in solution. The complete volatilization of ammonia from 20 mmol l\(^{-1}\) NH\(_4^+\), a typical haemolymph level measured during excretion, would generate a pH of 1.7 or a drop in pH of approximately 5.9 units in the absence of buffering. Owing to the high pK for ammonia, even modest acidification of the pleon fluid would lead to a dramatic decline in [NH\(_3\)], effectively eliminating the partial pressure gradient for volatilization. In fact, haemolymph and pleon fluid pH drops by less than 0.2 of a unit during ammonia volatilization (J. C. Wright and M. J. O’Donnell, unpublished observations). For an initial ammonia concentration of 20 mmol l\(^{-1}\) NH\(_4^+\), this would require a haemolymph buffering capacity of 20/0.2=100 mmol l\(^{-1}\) per pH unit, a value substantially higher (4- to 40-fold) than reported measurements of buffering capacities for insects (Harrison et al. 1990) or crustaceans (Truchot, 1976; Heisler, 1986).

Given that measured acidosis during NH\(_3\) volatilization is so small, and requisite buffering capacity is improbably large, deamination may be accompanied by bicarbonate synthesis. Protons combining with HCO\(_3^-\) in the pleon fluid would generate CO\(_2\) which would volatilize down its partial pressure gradient, along with NH\(_3\). The excretion of accumulated acid equivalents could thus be spatially and temporally coupled to ammonia excretion. One way in which bicarbonate synthesis could be achieved is through the oxidation of \(\alpha\)-ketoglutarate via gluconeogenesis or the trichloracetic acid (TCA) cycle (Tannen, 1978; Walser, 1986):
\[
2^{-}\text{OOCCHO}_{2}\text{CH}_{2}\text{COO}^{-} + 8\text{O}_2 \rightarrow 6\text{CO}_2 + 4\text{HCO}_3^- + 2\text{H}_2\text{O}.
\]
Allowing for the additional NH\(_4^+\) generated by deamination of glutamine, this would produce a 1:1 ratio of bicarbonate to acid equivalents generated by ammonotely. A precedent for similar compensation of systemic acidosis has been proposed for the mammalian kidney, involving increased flux through the \(\alpha\)-ketoglutarate dehydrogenase reaction (Schoolwerth and LaNoue, 1983; Schoolwerth et al. 1988). Isopods may, alternatively, derive bicarbonate from reduction of CO\(_3^{2-}\) stored in the exoskeleton, as proposed for gastropods (Speeg and Campbell, 1968; Barnhardt, 1992). Acid–base regulation in animals excreting gaseous ammonia remains an intriguing problem for future research.

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References

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