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THE MARBLE GOBY *OXYELEOTRIS MARMORATUS* ACTIVATES HEPATIC GLUTAMINE SYNTHETASE AND DETOXIFIES AMMONIA TO GLUTAMINE DURING AIR EXPOSURE

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Summary

Ammonia levels in various tissues of the marble goby *Oxyeleotris marmoratus* remained constant throughout a 72 h period of air exposure. The rate of ammonia excretion in these experimental fish decreased to approximately one-fifth of that of the submerged control. Ammonia was not converted to urea during air exposure because there were no significant increases in urea content in the tissues. Also, urea excretion rate was lowered to one-fiftieth that of the submerged fish. After 24 h of air exposure, there was a significant increase in muscle glutamine content, which peaked at 48 h. The increase in glutamine content could account for the decreases in the amounts of ammonia and urea excretion during air exposure. The specific activities of hepatic glutamate dehydrogenase (amination) and glutamine synthetase in these experimental fish increased threefold and thirtyfold, respectively, in comparison with the submerged controls. Thus, *O. marmoratus* appears to be the first known teleost that responds to air exposure by activating hepatic glutamine synthetase to detoxify internally produced ammonia.

Key words: marble goby, air exposure, *Oxyeleotris marmoratus*, glutamine synthetase, ammonia.

Introduction

The catabolism of amino acids, proteins, nucleotides and other related substances results in the formation of end-products that must be excreted by the animal (Mommsen and Walsh, 1991). Fishes excrete mainly ammonia, with some rare examples that also excrete urea and trimethylamine oxide as nitrogenous end-products (Goldstein and Forster, 1970; Campbell and Anderson, 1991; Anderson, 1995).

The marble goby *Oxyeleotris marmoratus* is a facultative air-breather capable of surviving under terrestrial conditions for up to 7 days (Choo, 1974). In its natural environment, *O. marmoratus* may encounter an absence of water during voluntary emergence or habitat desiccation (Choo, 1974). On land, *O. marmoratus* may face the problem of nitrogenous end-products accumulating to toxic levels in the body. This is due to the absence of a water current to allow the excretion of ammonia through the branchial epithelial surfaces. Thus, attempts were made in this study to determine the amount of ammonia excreted by *O. marmoratus* under submerged and terrestrial conditions.

The accumulation of ammonia to lethal concentrations in the body may be partially prevented by the conversion of some ammonia to urea. This strategy is adopted by certain species of fish exposed to air (Gordon et al., 1969, 1978; Ip et al., 1993), to high levels of ambient ammonia (Olson and Fromm, 1971; Saha and Ratha, 1987; Walsh et al., 1990), to confinement and/or crowding (Walsh et al., 1994; Walsh and Milligan, 1995) or to alkaline environments (Randall et al., 1989; Wood *et al*. 1989; Walsh et al., 1993). Urea production is metabolically expensive, but urea is less toxic than ammonia. Thus, the possibility of *O. marmoratus* producing more urea during air exposure was explored in the present study. Attempts were made to verify the presence of all the components of the urea cycle in the liver of this fish.

Ammonia detoxification may be achieved by other means. Ip et al. (1993) suggested that partial amino acid catabolism, leading to the formation of alanine, may serve to lower the amount of ammonia produced by the mudskipper *Periophthalmodon schlosseri* during air exposure. In addition, glutamine has been found to accumulate in the brain of various teleosts exposed to toxic levels of environmental ammonia (Levi et al., 1974; Arillo et al., 1981; Dabrowska and Wlasow, 1986; Peng et al., 1998). Therefore, we also examined the free amino acid levels and the activities of glutamate dehydrogenase and glutamine synthetase in various tissues of *O. marmoratus*. It is hypothesized that *O. marmoratus* must have adopted one or more of these strategies to allow it to maintain low levels of ammonia in its tissues during air exposure.

Materials and methods

Specimens

Oxyeleotris marmoratus Bleeker (500–800 g body mass) were purchased locally from fish farms at Jalan Kayu, Singapore. Groups of no more than 20 fish were kept submerged in fibreglass tanks (72 cm×55 cm×56 cm; length×width×height) containing 130 l aerated dechlorinated tap water at 25 °C. No attempt was made to separate the sexes. The fish were allowed at least 48 h of acclimation prior to use in experiments. During this period, half the water was changed daily and the fish were not fed. The ammonia concentration in the water was always below 0.02 mmol l^{-1}.

Experimental conditions

Fish submerged in aerated dechlorinated tap water at 25 °C were regarded as controls. Fish exposed to terrestrial conditions at 25 °C were placed in individual plastic tanks (28 cm×18 cm×14 cm; length×width×height) containing a thin layer of tap water (100 ml). These plastic tanks were kept within a moisture-saturated fibreglass tank for the entire period (6 h, 24 h, 48 h or 72 h) of air exposure.

Ammonia, urea and free amino acid contents

Fish submerged in aerated dechlorinated tap water were anaesthetized with 3-aminobenzoic acid ethyl ester (MS 222) (Sigma Chemical Co.) neutralized to pH 7.0 at a final concentration (w/v) of 0.1 % for 10 min; fish exposed to terrestrial conditions were anaesthetized by exposing the fish to diethylether vapour for 10 min. They were then individually killed with a blow on the head. The lateral muscle and liver were excised and immediately freeze-clamped in liquid N_2 using pre-cooled aluminium tongs (Faupel et al., 1972). The whole procedure was carried out within 1 min. Samples were then stored at –80 °C until analyzed.

The frozen sample was weighed, powdered under liquid N_2 and homogenized three times in 5 vols of 5 % trichloroacetic acid using an Ultra-Turrax homogenizer at maximum speed (24 000 revs min^{-1}) for 20 s each time with 10 s beween bursts. The homogenate was then centrifuged at 10 000 ***g*** for 10 min in a Beckman J2-21M/E refrigerated centrifuge. The supernatant obtained was used for the analysis of ammonia, urea and free amino acid (FAA) contents.

Plasma samples were collected from another group of anaesthetized fish. The tail of the anaesthetized fish was cut, and the blood exuding from the severed caudal artery was collected in heparinized (Na^+) capillary tubes and centrifuged at 5000 ***g*** for 5 min at 4 °C. The plasma obtained was immediately deproteinized by adding an equal volume of 6 % trichloroacetic acid, followed by centrifugation at 10 000 ***g*** for 10 min. The supernatant fluid was kept at –80 °C until analyzed.

The deproteinized sample was neutralized with 2 mol l^{-1} $KHCO_3$, and the ammonia content was determined according to the method of Kun and Kearney (1974). The change in absorbance at 25 °C and 340 nm was monitored using a Shimadzu UV-160A spectrophotometer. Freshly prepared NH_4Cl solution was used as the standard.

Urea content in 0.2 ml of deproteinized sample was assayed using a Sigma urea assay kit (procedure 535). A blank assay was also performed after 0.2 ml of the sample had been incubated with 0.2 ml of 20 mmol l^{-1} imidazole buffer (pH 7.2) containing 2 units of urease for 15 min at 30 °C. Urea obtained from Sigma Chemical Co. (USA) was used as the standard.

For analysis of FAA content, the deproteinized sample was diluted with an equal volume of 0.2 mol l^{-1} lithium citrate buffer (pH 2.2) and adjusted to pH 2.2 with 4 mol l^{-1} LiOH. Determination of FAA levels was performed using a Shimadzu LC-6A amino acid analysis system with a Shim-pack ISC-07/S1504 Li column. Analytical grade amino acid standard purchased from Sigma Chemical Co. (USA) served as a reference.

Ammonia, urea and FAA contents were expressed as μmol g^{-1} wet mass for muscle and liver and as μmol ml^{-1} for plasma.

Rates of excretion of ammonia and urea

Specimens were submerged individually in plastic aquarium tanks containing 4 l of aerated dechlorinated tap water at 25 °C. After 24 h, a small volume of water was sampled for ammonia and urea analyses.

Subsequently, the same individuals were exposed to terrestrial conditions in plastic tanks containing a thin layer (100 ml) of tap water. After 24 h, the fish were sprayed thoroughly with de-ionized water. The water collected was analyzed for ammonia and urea. The fish were then transferred to other tanks, and the experiment was repeated twice. After 72 h of air exposure, the fish were submerged again to study the rates of ammonia and urea excretion during recovery.

Ammonia was determined colorimetrically according to the method of Anderson and Little (1986). Freshly prepared NH_4Cl solution was used as the standard. Urea was analyzed using a Sigma urea assay kit (Procedure 535) as described above. Ammonia and urea excretion rates were expressed as μmol day^{-1} g^{-1} wet mass of the fish.

Activities of ornithine–urea cycle enzymes

Freshly excised liver samples were homogenized in 10 vols of mitochondrial extraction buffer (285 mmol l^{-1} sucrose, 3 mmol l^{-1} Tris–HCl and 3 mmol l^{-1} EDTA, pH 7.2) using a Glas-Col tissue grinder (Terre Haute, USA) using one stroke at low speed. The homogenized sample was centrifuged at 600 ***g*** for 15 min. The resulting supernatant fluid was centrifuged three times at 10 000 ***g*** for 15 min. Between each spin, the supernatant fluid was discarded and replaced with fresh mitochondrial extraction buffer. After the last spin, the loose pellet was mixed with 1 ml of buffer containing 50 mmol l^{-1} Hepes (pH 7.6), 50 mmol l^{-1} KCl, 0.5 mmol l^{-1} EDTA and 1 mmol l^{-1} DL-dithiothreitol (DTT). This suspension was sonicated three times for 20 s each time with 10 s between bursts. The mitochondrial fraction was obtained by passing the suspension through a 10 ml Econo-Pac 10DG desalting column (Bio-Rad Laboratories Inc., CA, USA) equilibrated with the same buffer.

To obtain the cytosolic fraction, the freshly excised liver was

homogenized with 5 vols of mitochondrial extraction buffer (see above) and centrifuged at 10 000 ***g*** for 15 min. The resulting supernatant was collected and passed through a 10 ml Econo-Pac 10DG desalting column equilibrated with Hepes buffer. Samples were immediately assayed for carbamoyl phosphate synthetase (CPS) II activity. CPS activity was detected using the method described by Korte *et al.* (1997). Preliminary studies showed that CPS activities in both the cytosolic and mitochondrial samples were low in the presence of NH_4^+, but high when glutamine was present. The standard reaction mixture contained 50 mmol l^{-1} Hepes (pH 7.6), 50 mmol l^{-1} KCl, 5 mmol l^{-1} EDTA, 20 mmol l^{-1} ATP, 25 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} $NaHCO_3$, 22.5 mmol l^{-1} phosphoenolpyruvate (PEP), 2 units of pyruvate kinase (PK), 74 MBq ml^{-1} [^{14}C]$NaHCO_3$, 20 mmol l^{-1} glutamine and 0.1 ml of the sample in the presence or absence of 1.67 mmol l^{-1} *N*-acetylglutamate (AGA) or 1.67 mmol l^{-1} AGA + 1.67 mmol l^{-1} UTP. Radioactivity was measured using a Wallac 1414 liquid scintillation counter. CPS activity was expressed as μmol [^{14}C]urea min^{-1} g^{-1} wet liver mass.

The activity of ornithine transcarbamoylase was determined according to Xiong and Anderson (1989). Ornithine transcarbamoylase activity was expressed as μmol citrulline min^{-1} g^{-1} wet liver mass.

Argininosuccinate synthetase + lyase activities were determined together assuming that both were present by measuring [^{14}C]fumarate formation from [^{14}C]aspartate following the method of Cao et al. (1991). Radioactivity was measured using a Wallac 1414 liquid scintillation counter. Argininosuccinate synthetase + lyase activity was expressed as μmol [^{14}C]fumarate min^{-1} g^{-1} wet liver mass.

Arginase activity was assayed according to Casey and Anderson (1982). The urea formed was determined colorimetrically as described above. Arginase activity was expressed as μmol urea min^{-1} g^{-1} wet liver mass.

Activities of glutamate dehydrogenase (GDH), alanine aminotransferase (ALT) and glutamine synthetase

The frozen liver and muscle samples were homogenized three times in 5 vols of ice-cold buffer containing 50 mmol l^{-1} imidazole (pH 7.4), 50 mmol l^{-1} NaF, 3 mmol l^{-1} EGTA, 3 mmol l^{-1} EDTA and 0.5 mmol l^{-1} phenylmethylsulphonyl-fluoride (PMSF) at maximum speed (24 000 revs min^{-1}) for 20 s each with 10 s between bursts. The resulting homogenate was centrifuged at 10 000 ***g*** for 15 min at 4 °C. The supernatant fluid was passed through a 10 ml Econo-Pac 10DG desalting column equilibrated with the buffer without PMSF. The resulting fluid was used for enzymatic analysis.

Enzyme activities were recorded at 25 °C and 340 nm unless otherwise stated. All chemicals and coupling enzymes were obtained from Sigma Chemical Company (MO, USA). Enzyme activities are expressed in μmol NADH min^{-1} g^{-1} wet tissue mass unless otherwise stated.

GDH activities were assayed in the aminating and deaminating directions according to the method of Peng et al. (1994). GDH activity in the deaminating direction was recorded at 492 nm and is expressed as μmol formazan min^{-1} g^{-1} wet tissue mass. ALT activity in the direction of alanine degradation was determined following the method of Peng et al. (1994). Glutamine synthetase activity was measured according to Shankar and Anderson (1985). Glutamine synthetase activity was measured at 500 nm and is expressed as μmol γ-glutamylhydroxymate min^{-1} g^{-1} wet tissue mass.

Statistical analysis

Results are presented as means ± S.E.M. One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple-range test was used to compare differences between means where applicable. Differences with $P<0.05$ were regarded as statistically significant.

Results

Air exposure had no significant effect on the ammonia levels in the muscle of *O. marmoratus* (Table 1). In the liver and plasma, the ammonia level rose transiently to a significantly higher value after air exposure, but returned to

Table 1. *Total ammonia content in the muscle, liver and plasma of* Oxyeleotris marmoratus *exposed to submerged conditions or terrestrial conditions for 6, 24, 48 or 72 h*

Conditions	Muscle (μmol g^{-1} wet mass)	Liver (μmol g^{-1} wet mass)	Plasma (μmol ml^{-1})
Submerged	3.01±0.12 (5)	0.76±0.17 (6)	0.129±0.015 (4)
Terrestrial			
6 h	2.08±0.44 (5)	1.06±0.25[a] (5)	0.016±0.004[a] (4)
24 h	2.13±0.28 (5)	1.74±0.23[a,b] (5)	0.175±0.013[a,b] (4)
48 h	1.95±0.38 (5)	0.26±0.08[b,c] (4)	0.053±0.007[a,c] (4)
72 h	3.04±0.15 (5)	0.44±0.11[b,c] (4)	0.090±0.021[b,c] (4)

Results represent means ± S.E.M. with the number of determinations in parentheses.
[a]Significantly different from the submerged value ($P<0.05$).
[b]Significantly different from the value of 6 h terrestrial exposure ($P<0.05$).
[c]Significantly different from the value of 24 h terrestrial exposure ($P<0.05$).

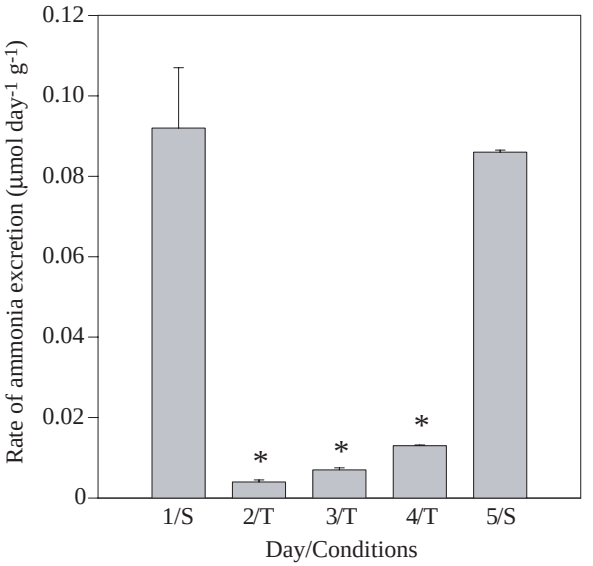

Fig. 1. The time course of the effects of air exposure on the rate of ammonia excretion (μmol day^{-1} g^{-1} wet mass) of *Oxyeleotris marmoratus*. Values are means + S.E.M. Day 1, *N*=10; day 2, *N*=9; day 3, *N*=8; days 4/5, *N*=4. S, submerged; T, terrestrial. Asterisks mark values that are significantly different from the value for day 1/S (*P*<0.05).

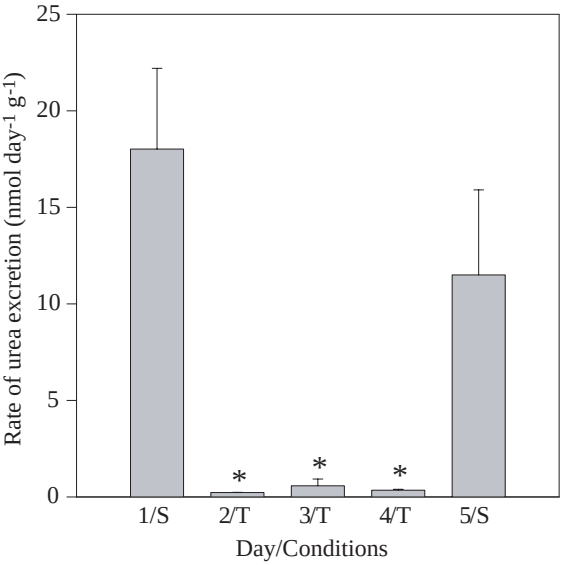

Fig. 2. The time course of the effects of air exposure on the rate of urea excretion (nmol day^{-1} g^{-1} wet mass) of *Oxyeleotris marmoratus*. Values are means + S.E.M. Days 1/2, *N*=9; day 3, *N*=7; days 4/5, *N*=4. S, submerged; T, terrestrial. Asterisks mark values that are significantly different from the value for day 1/S (*P*<0.05).

the control (submerged) value thereafter. The rate of ammonia excretion by *O. marmoratus* during air exposure was significantly lower than that when the fish was submerged (Fig. 1). When the fish was resubmerged, this rate returned to the initial control value.

The urea content of the muscle of *O. marmoratus* rose significantly after 6 h of air exposure but returned to the control (submerged) level thereafter (Table 2). Air exposure did not affect the concentration of urea in the plasma. However, the urea content of some of the liver samples collected after 24 h, 48 h and 72 h of air exposure became undetectable. Air exposure also significantly lowered the rate of urea excretion by *O. marmoratus* (Fig. 2). Upon resubmergence, this rate returned to the initial control value.

Using glutamine as a substrate, CPS activity was detected in the hepatic mitochondrial and cytosolic fractions. CPS activity in the mitochondrial fraction was activated by AGA but this stimulated activity was unaffected by UTP (Table 3). In contrast, CPS activity in the cytosol was unaffected by AGA but was inhibited by UTP. Air exposure had no effect on the activity of CPS in either fraction. Ornithine transcarbamoylase, argininosuccinate synthetase + lyase and arginase activities were detected in the liver of *O. marmoratus* (Table 4). These activities were unaffected by air exposure, except for arginase

Table 2. *Urea content in the muscle, liver and plasma of* Oxyeleotris marmoratus *exposed to submerged conditions and terrestrial conditions for 6, 24, 48 or 72 h*

Conditions	Muscle (μmol g^{-1} wet mass)	Liver (μmol g^{-1} wet mass)	Plasma (μmol ml^{-1})
Submerged	2.04±0.28 (5)	2.22±0.37 (5)	2.40±0.56 (4)
Terrestrial			
6 h	5.22±0.81[a] (5)	3.68±0.73 (5)	2.46±0.52 (5)
24 h	1.95±0.23[b] (5)	2.12±1.08 (3)[1]	3.39±0.49 (5)
48 h	2.61±0.68[b] (5)	2.10 (2)[2]	2.33±0.41 (5)
72 h	2.72±0.41[b] (5)	2.03 (1)[3]	2.31±0.22 (5)

Results represent means ± S.E.M. with the number of determinations in parentheses.
[a]Significantly different from the submerged value (*P*<0.05).
[b]Significantly different from the value of 6 h terrestrial exposure (*P*<0.05).
[1]Values of two determinations were not detectable.
[2]Values of three determinations were not detectable.
[3]Values of four determinations were not detectable.

Table 3. *Effect of 72 h of air exposure on the enzyme activities of carbamoyl phosphate synthetase from the mitochondrial and cytosolic fractions of the liver of* Oxyeleotris marmoratus

Conditions	Substrate and/or effector present	Mitochondria (nmol min^{-1} g^{-1} wet mass)	Cytosol (nmol min^{-1} g^{-1} wet mass)
Submerged	G	0.548±0.375	1.50±0.94
	G + AGA	7.10±3.68	1.83±1.16
	G + AGA + UTP	5.58±2.85	ND
Terrestrial	G	0.173±0.042	1.77±0.96
	G + AGA	5.07±0.63	1.48±0.59
	G + AGA + UTP	4.45±0.92	ND

Results represent means ± S.E.M., N=4.
G, glutamine; AGA, *N*-acetyglutamate; UTP, uridine triphosphate; ND, not detectable.

from the cytosolic fraction, which exhibited significantly lower activity.

After 72 h of air exposure, the glutamine level in the muscle of *O. marmoratus* increased threefold (Table 5). Small but insignificant changes were detected for aspartate, phenylalanine and threonine (Table 5). The total free amino acid (TFAA) content of the muscle remained unchanged during air exposure. In the liver, significant increases in the contents of glutamine, histidine, isoleucine, leucine, serine, threonine and valine were observed after 24 h of air exposure (results not shown). However, by 72 h, these values had either returned to control values (glutamine, histidine, leucine and threonine) or fallen to levels lower than that of the control (isoleucine, serine and valine) (Table 5). In the blood, few changes in FAA content were detected and TFAA remained unchanged during air exposure (Table 5).

A time course study of the glutamine level in the muscle (Fig. 3) showed that glutamine rose to peak levels by 48 h of air exposure and levelled off thereafter. In contrast, the glutamine level of the liver reached its peak at approximately 24 h, when it was more than five times the control value (Fig. 3). Subsequently, at 48 h, it returned to the control level.

There was no significant difference between GDH (amination) activities from the muscle of *O. marmoratus* exposed to submerged and terrestrial conditions (Table 6). No GDH activity in the deamination direction was detected in the muscle of *O. marmoratus*. There was a threefold increase in GDH (amination) activity in the liver of fish exposed to terrestrial conditions, while deamination levels remained unchanged. Hence, air exposure led to an increase in the amination:deamination ratio of GDH in the liver of *O. marmoratus* (Table 6).

ALT activities from both muscle and liver were unaffected by air exposure (Table 7). There was a significant increase in the hepatic glutamine synthetase activity of *O. marmoratus* after 48 h and 72 h of air exposure (Table 7; Fig. 4). The activity of hepatic glutamine synthetase remained low when *O. marmoratus* was exposed to terrestrial conditions for up to approximately 24 h, after which the activity increased approximately thirtyfold (Fig. 4). In contrast, no such effect was observed for muscle glutamine synthetase (Table 7).

Discussion

In contrast to the amphibious mudskippers (*Periophthalmus cantonensis* and *Boleophthalmus pectinirostris*, Morii, 1979; *Boleophthalmus boddaerti* and *Periophthalmodon schlosseri*, Ip et al., 1993), the ammonia level in the muscle of *O. marmoratus* never increased during air exposure. In the liver and plasma of *O. marmoratus*, ammonia content rose only transiently after air exposure to 1.74 μmol g^{-1} and 0.18 μmol ml^{-1}, respectively. These are very small increases compared with the values of 3.46 μmol g^{-1} and 0.92 μmol ml^{-1}, respectively, in air-exposed *P. schlosseri* (Ip et al., 1993). Since the ammonia excretion rate in *O. marmoratus* exposed to air was one-fifth of that of the submerged controls, and since

Table 4. *Effect of 72 h of air exposure on the enzyme activities of ornithine transcarbamoylase, argininosuccinate synthetase + lyase and arginase from the mitochondrial and cytosolic fractions of the liver of* Oxyeleotris marmoratus

Conditions	OTC Mitochondria (μmol min^{-1} g^{-1} wet mass)	ARGS + L Cytosol (μmol min^{-1} g^{-1} wet mass)	Arginase	
			Mitochondria (μmol min^{-1} g^{-1} wet mass)	Cytosol (μmol min^{-1} g^{-1} wet mass)
Submerged	18.3±1.6 (6)	6.52±2.95 (4)	369±33 (4)	46.4±6.1 (5)
Terrestrial	18.4±1.1 (4)	9.18±4.11 (4)	456±83 (4)	25.1±4.2 (4)*

Results represent means ± S.E.M. with the number of determinations in parentheses.
OTC, ornithine transcarbamoylase; ARGS, arginosuccinate synthetase; L, arginosuccinate lyase.
*Significantly different from the submerged value (P<0.05).

Table 5. *Effects of 72 h of air exposure on the contents of various free amino acids and total free amino acids in the muscle, liver and plasma of* Oxyeleotris marmoratus

	Muscle		Liver		Plasma	
FAA	Submerged (μmol g^{-1} wet mass)	Terrestrial (μmol g^{-1} wet mass)	Submerged (μmol g^{-1} wet mass)	Terrestrial (μmol g^{-1} wet mass)	Submerged (μmol l^{-1})	Terrestrial (μmol l^{-1})
Ala	1.467±0.375	1.894±0.524	0.945±0.449	0.862±0.300	0.224±0.024	0.082±0.003
Arg	0.063±0.017	0.047±0.004	ND	ND	ND	ND
Asg	0.234±0.035	0.366±0.082	ND	ND	0.039±0.006	0.016±0.002[a]
Asp	1.037±0.317	0.440±0.216	0.287±0.107	0.119±0.028	0.033±0.006	0.020±0.003
Cys	0.027±0.009	0.019±0.003	0.051±0.006	0.043±0.003	0.110±0.005	0.005±0.001
Gln	0.471±0.039	1.194±0.187[a]	0.274±0.095	0.513±0.090	0.092±0.014	0.052±0.013
Glu	0.376±0.112	0.930±0.356	4.779±0.413	3.126±0.624	0.068±0.002	0.071±0.013
Gly	1.245±0.242	2.245±0.900	0.571±0.144	0.201±0.036	ND	ND
His	0.631±0.113	1.226±0.337	0.338±0.060	0.159±0.039	0.076±0.010	0.033±0.003
Ile	0.223±0.036	0.146±0.030	0.091±0.033	0.020±0.005[a]	0.084±0.018	0.034±0.005
Leu	0.384±0.067	0.302±0.064	0.174±0.062	0.044±0.008	ND	ND
Lys	1.535±0.207	1.570±0.307	0.240±0.077	0.073±0.018	0.103±0.017	0.052±0.013
Met	0.048±0.021	0.032±0.015	0.054±0.012	0.030±0.004	0.010±0.002	0.009±0.001
Phe	0.146±0.013	0.120±0.017	0.161±0.017	0.045±0.013	0.046±0.013	0.055±0.012
Pro	0.182±0.041	0.335±0.065	0.339±0.015	0.114±0.020	0.037±0.003	0.016±0.005
Ser	0.394±0.079	0.623±0.118	0.104±0.018	0.031±0.008[a]	0.045±0.002	0.026±0.003
Tau	16.36±0.86	14.29±0.95	1.844±0.503	1.546±0.536	0.277±0.052	0.181±0.017
Thr	0.488±0.076	0.888±0.189	0.351±0.077	0.081±0.016	0.117±0.032	0.041±0.008
Try	0.049±0.004	0.047±0.006	ND	ND	0.025±0.005	0.032±0.014
Tyr	0.087±0.016	0.100±0.015	0.100±0.031	0.076±0.020	0.043±0.008	0.109±0.023
Val	0.312±0.057	0.271±0.056	0.179±0.041	0.030±0.006[a]	0.128±0.026	0.063±0.009
Total FAA	23.37±2.47	27.08±2.93	10.88±1.02	6.94±0.71	1.454±0.098	0.894±0.126

Results represent means ± S.E.M., N=4.
FAA, free amino acid; ND, not detectable.
[a]Significantly different from the corresponding submerged value (P<0.05).

the ammonia excretion rate did not increase above the control value upon resubmersion, this fish must be capable of reducing its rates of protein and/or amino acid catabolism, or detoxifying ammonia to other less toxic products, or both, during air exposure.

Buttle et al. (1996) reported that the African catfish *Clarias gariepinus* excreted more ammonia during resubmersion only if the period of air exposure was short (<5 min). Between 5 and 180 min of air exposure, *C. gariepinus* resorted to decreasing the rate of ammonia production and converting ammonia to urea. The period of air exposure imposed on *C. gariepinus* was short, possibly because it was unable to survive on land like *O. marmoratus* for 72 h or more.

O. marmoratus excreted a large proportion of its nitrogenous waste as urea in the control submerged condition. The ratio of ammonia:urea excreted was approximately 5.1, which is lower than values (7.6–33.4) for the sockeye salmon *Oncorhynchus nerka* (Brett and Zala, 1975), for *Blennius pholis* (Davenport and Sayer, 1986) and for five other facultative air-breathers, *Amphipnous cuchia*, *Clarias batrachus*, *Heteropneustes fossilis*, *Anabas testudineus* and *Channa punctatus* (Saha and Ratha, 1989). Although *O. marmoratus* did not detoxify ammonia to urea during air exposure, all the five enzymes involved in the ornithine–urea cycle were present in the liver

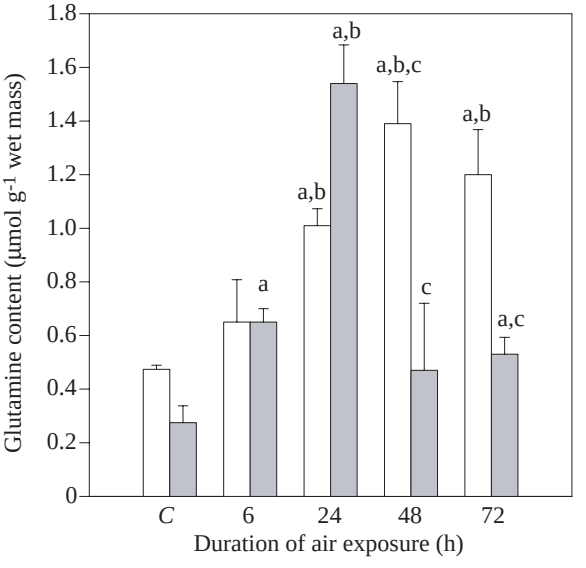

Fig. 3. The time course of the effects of air exposure on the glutamine content (μmol g^{-1} wet mass; N=4) of the muscle (open bars) and liver (filled bars) of *Oxyeleotris marmoratus*. Values are means + S.E.M. a marks values significantly different from the corresponding control (C) value (P<0.05), b marks values significantly different from the corresponding value at 6 h (P<0.05), c marks values significantly different from the corresponding value at 24 h (P<0.05).

Table 6. *Effects of 6 h or 72 h of air exposure on the activities of glutamate dehydrogenase in the amination (μmol NADH oxidised min^{-1} g^{-1} wet mass) and deamination (μmol formazan formed min^{-1} g^{-1} wet mass) reactions, and their ratios (amination/deamination) from the muscle and liver of* Oxyeleotris marmoratus

Tissues	Conditions	Amination (μmol min^{-1} g^{-1} wet mass)	Deamination (μmol min^{-1} g^{-1} wet mass)	Amination/deamination
Muscle	Submerged	0.055±0.009	ND	NA
	6 h	0.063±0.010	ND	NA
	72 h	0.049±0.008	ND	NA
Liver	Submerged	0.883±0.158	0.106±0.026	10.2±3.8
	6 h	0.798±0.123	0.048±0.005	16.5±2.9
	72 h	2.435±0.432[a,b]	0.072±0.027	36.5±5.7[a,b]

Results represent means ± S.E.M., *N*=4.
NA, not available; ND, not detectable.
[a]Significantly different from the submerged value (P<0.05).
[b]Significantly different from the value of 6 h terrestrial exposure (P<0.05).

of *O. marmoratus*. CPS III, which is responsible for urea synthesis in the majority of fish species (Mommsen and Walsh, 1991), was detected only at low levels (approximately 5 nmol min^{-1} g^{-1}) in this fish. CPS II, which does not require AGA for activity and is inhibited by UTP (Campbell and Anderson, 1991), was found in the cytosol.

Air exposure exhibited no significant effect on the activities of CPS III, ornithine transcarbamoylase and argininosuccinate synthetase + lyase from the liver of *O. marmoratus*. However, arginase activity in the cytosol was significantly lower at 72 h of air exposure. Incidentally, the rate of urea excretion in *O. marmoratus* exposed to the terrestrial condition decreased to one-fiftieth of the submerged value, leading to a higher ammonia:urea excretion ratio of 20. After all, it is metabolically expensive to produce urea. Four moles of ATP are required per mole of urea formed from two moles of ammonia (Mommsen and Walsh, 1991).

Superficially, it would appear that *O. marmoratus* suppressed both ammonia and urea production during air exposure. Taking the submerged ammonia excretion rate to be 0.09 μmol ammonia day^{-1} g^{-1} wet mass, and assuming that the rate dropped to one-fifth of this value during air exposure, approximately 0.072 μmol ammonia day^{-1} g^{-1} wet mass would theoretically have been accumulated in the tissues of this fish. Since ammonia levels in the muscle exposed to the submerged and terrestrial conditions were comparable, a 500 g fish would have reduced ammonia production by approximately 108 μmol in 72 h. The decrease in urea excretion for the entire 72 h period of air exposure was approximately 5.3×10^{-2} μmol g^{-1}. Since urea content in the muscle was not affected by air exposure, the fish had decreased overall urea production by the same amount (5.3×10^{-2} μmol g^{-1}) during this period. Again, a 500 g

Table 7. *Effects of 6 h or 72 h of air exposure on the activities of glutamine synthetase and alanine aminotransferase from the muscle and liver of* Oxyeleotris marmoratus

Tissues	Conditions	Glutamine synthetase (μmol min^{-1} g^{-1})	Alanine aminotransferase (μmol min^{-1} g^{-1})
Muscle	Submerged	0.018±0.002 (4)	1.39±0.17 (4)
	6 h	0.015±0.004 (4)	1.09±0.13 (4)
	72 h	0.022±0.007 (4)	1.60±0.45 (4)
Liver	Submerged	0.021±0.002 (5)	15.4±0.6 (5)
	6 h	0.023±0.002 (4)	17.7±0.8 (4)
	72 h	0.617±0.068[a,b] (4)	15.6±1.8 (4)

Results represent means ± S.E.M. with the number of determinations in parentheses.
[a]Significantly different from the submerged value (P<0.05).
[b]Significantly different from the value of 6 h terrestrial exposure (P<0.05).

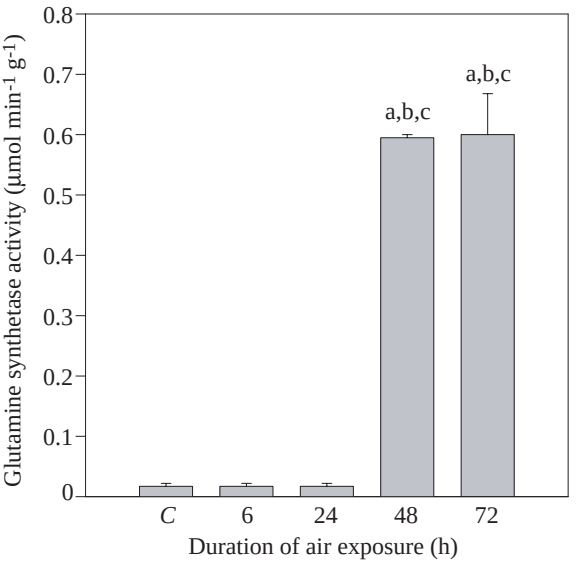

Fig. 4. The effects of air exposure on the enzyme activities (μmol γ-glutamylhydroxamate min^{-1} g^{-1} wet liver mass) of glutamine synthetase from the liver of *Oxyeleotris marmoratus*. Values are means + S.E.M. Control, *N*=5; all other times, *N*=4. a marks values significantly different from the control (*C*) value (P<0.05), b marks values significantly different from the value at 6 h (P<0.05), c marks values significantly different from the value at 24 h (P<0.05).

fish would have produced 26.5 μmol less urea. Two ammonia molecules are utilized during the formation of urea; hence, the fish would have reduced production by approximately 53 μmol of ammonia-equivalent during 72 h of air exposure. A 500 g *O. marmoratus* would have decreased its total ammonia production by 161 μmol (108+53 μmol) during this period.

However, the glutamine content of the muscle of *O. marmoratus* exposed to air increased threefold in 72 h. Glutamine may be produced from glutamate and NH_4^+ catalyzed by glutamine synthetase in the muscle and/or liver. Glutamate may, in turn, be produced from α-ketoglutarate (αKG) and NH_4^+ catalyzed by GDH, or from αKG and other amino acids catalyzed by various transaminases. In other words, the formation of one glutamine molecule allows the uptake of two ammonia molecules (Campbell, 1973). The hepatic glutamine content peaked at 24 h of air exposure, while that in the muscle peaked only after 48 h. This suggests that the glutamine formed in the liver was perhaps later shuttled to the muscle, which acted as a reservoir for glutamine accumulation. Consequently, the steady-state glutamine level in the plasma was maintained.

Given that the glutamine level rose by approximately $0.7\,\mu mol\,g^{-1}$ in the muscle after 72 h of air exposure, and that approximately 60 % of a 500 g *O. marmoratus* is muscle, approximately 210 μmol of glutamine would have been accumulated. Since two NH_4^+ are needed to produce one glutamine molecule, 420 μmol of ammonia would have been detoxified in this manner. This would be more than enough to account for the apparent decrease of 161 μmol of ammonia produced during the entire period. Therefore, it was unlikely that amino acid catabolism had been reduced; instead, the ammonia produced was channelled towards the formation of glutamine. Protein or amino acid catabolism in *O. marmoratus* may, in fact, have been increased during air exposure because glutamine accumulated to levels far in excess of that needed to detoxify the ammonia produced during this period.

To the best of our knowledge, glutamine has been found to play a role in ammonia detoxification in fish only in response to high environmental ammonia concentrations (Levi et al., 1974; Arillo et al., 1981, Dabrowska and Wlasow, 1986; Mommsen and Walsh, 1992; Peng et al., 1998). Thus, *O. marmoratus* may represent the first known case in which a fish is able to detoxify internally generated ammonia in this way.

The increase in hepatic GDH (amination) activity in *O. marmoratus* exposed to the terrestrial condition did not lead to any increase in glutamate or alanine content, as in *P. schlosseri* (Ip et al., 1993), in its tissues. Under the same conditions, hepatic glutamine synthetase activity rose from $0.021\,\mu mol\,min^{-1}\,g^{-1}$ to $0.62\,\mu mol\,min^{-1}\,g^{-1}$. This was an increase of approximately thirtyfold. Hence, the glutamate produced must be channelled into the formation of glutamine. There was a good correlation between the peak of hepatic glutamine synthetase activity and the peak of glutamine accumulation in the muscle of the fish exposed to terrestrial condition.

In other cases where environmental ammonia is detoxified to glutamine (Levi et al., 1974; Arillo et al., 1981; Peng et al., 1998), neither cerebral nor hepatic glutamine synthetase activity was found to be significantly different from control values. This suggests that pre-existing glutamine synthetase is sufficient to account for the increased glutamine formation (Walsh et al., 1993; Wright et al., 1993). Although Peng et al. (1998) observed some increases in the specific activities of cerebral glutamine synthetase in two species of mudskipper in response to ammonia loading, those from the liver remained unchanged. In the gulf toadfish *Opsanus beta* kept under confinement/crowding conditions, hepatic glutamine synthetase activity increased several-fold, leading to an induction of ureotely (Walsh et al., 1994; Walsh and Milligan, 1995).

Thus, *O. marmoratus* appears to be the first known teleost that responds to air exposure by activating hepatic glutamine synthetase and detoxifying internally produced ammonia to glutamine.

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