

AMMONIA DETOXIFICATION AND LOCALIZATION OF UREA CYCLE ENZYME ACTIVITY IN EMBRYOS OF THE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) IN RELATION TO EARLY TOLERANCE TO HIGH ENVIRONMENTAL AMMONIA LEVELS

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Summary

The present study investigated the role of ammonia as a trigger for hatching, mechanisms of ammonia detoxification and the localization of urea cycle enzymes in the early life stages of freshwater rainbow trout (*Oncorhynchus mykiss*). The key urea cycle enzyme carbamoyl phosphate synthetase III was found exclusively in the embryonic body (non-hepatic tissues); related enzymes were distributed between the liver and embryonic body. 'Eyed-up' trout embryos were exposed either acutely (2 h) to 10 mmol l^{-1} NH_4Cl or chronically (4 days) to 0.2 mmol l^{-1} NH_4Cl . Time to hatching was not affected by either acute or chronic NH_4Cl exposure. Urea levels, but not ammonia levels in the embryonic tissues, were significantly higher than in controls after both acute and chronic NH_4Cl exposure, whereas there were no significant

changes in urea cycle enzyme activities. Total amino acid levels in the embryonic tissues were unaltered by chronic ammonia exposure, but levels of most individual amino acids and total amino acid levels in the yolk were significantly lower (by 34–58%) than in non-exposed controls. The data indicate that trout embryos have an efficient system to prevent ammonia accumulation in embryonic tissue, by conversion of ammonia to urea in embryonic tissues and through elevation of ammonia levels in the yolk.

Key words: amino acid, ammonia, excretion, toxicity, carbamoyl phosphate synthetase III, glutamine synthetase, hatching, urea, yolk, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Recently, significant activities of the ornithine–urea cycle (OUC) enzymes carbamoyl phosphate synthetase III (CPSase III), ornithine transcarbamoylase (OTCase) and arginase, and of the accessory enzyme glutamine synthetase (GSase), have been measured in the embryos of the freshwater rainbow trout (*Oncorhynchus mykiss*) and the marine Atlantic cod (*Gadus morhua*) and halibut (*Hippoglossus hippoglossus*), whereas activities are either low or absent in adults (Wright et al., 1995; Chadwick and Wright, 1999; Terjesen et al., 2000). The localization of these enzymes within the developing embryos has not been determined. Urea synthesis and the OUC typically occur in the liver of ureotelic vertebrates (for reviews, see Campbell, 1991; Anderson, 1995). Recently, significant levels of the first two enzymes in the OUC, CPSase III and OTCase, have been detected in adult skeletal muscle tissue of rainbow trout (Korte et al., 1997), gulf toadfish (*Opsanus beta*) (Julsrud et al., 1998), largemouth bass (*Micropterus salmoides*) (Kong et al., 1998) and most notably the alkaline-lake-adapted tilapia (*Oreochromis alcalicus grahami*) (Lindley et al., 1999). In rainbow trout, CPSase III is completely absent from adult liver tissue (Korte et al., 1997). Does a similar pattern of OUC

expression occur in embryonic trout? In previous studies on whole rainbow trout embryos, OUC enzyme activities were not detected prior to hatching, possibly because intact embryos consist mostly of yolk with a much smaller percentage of embryonic tissue (Wright et al., 1995). We hypothesized that the yolk is devoid of OUC enzyme activities and therefore that previous analysis of enzyme activities in whole embryos underestimated the absolute values of enzyme activities in the embryonic tissues. Moreover, by determining the tissue distribution of the urea cycle enzymes in embryos, we could then optimize the protocol for analyzing enzyme activities in relatively small tissue specimens.

In post-yolk-sac embryos of the viviparous blenny *Zoarces viviparus*, urea is the predominant nitrogenous waste product (Korsgaard, 1994; Korsgaard, 1997). Urea excretion can account for a significant amount of nitrogen excretion in oviparous teleost embryos, but ammonia is the primary nitrogenous waste product (e.g. Torres et al., 1996; Chadwick and Wright, 1999; Pilley and Wright, 2000). Diffusion of ammonia out of the encapsulated embryo may be hindered by the absence of respiratory convection (i.e. gill ventilation) and

indirect contact with bulk water. Indeed, ammonia levels rise steadily in both freshwater (Wright et al., 1995; Terjesen et al., 1997) and marine (Fyhn and Serigstad, 1987; Finn et al., 1996; Chadwick and Wright, 1999) species during embryogenesis.

Are elevated tissue levels of the potentially toxic ammonia one of the triggers for hatching? Environmental factors (e.g. hypoxia, temperature, light, pH) and developmental stage are important in the initiation of hatching in fishes (DiMichele and Taylor, 1980; Yamagami, 1988). Hatching may facilitate the removal of ammonia from the tissues by enabling the embryos to have direct contact with the external environment, which functions as an unlimited sink for the diffusive loss of ammonia. Thus, a second aim of this study was to examine the effects of elevated external ammonia levels on the time to hatching.

Considering the relative toxicity of ammonia, it is surprising that, compared with their adult forms, the embryos of many teleost species are highly tolerant to external ammonia. Rice and Stokes (Rice and Stokes, 1975) reported that rainbow trout embryos had a 24 h tolerance limit of greater than 3.58 mg l^{-1} of un-ionized ammonia [approximately 2.25 mmol l^{-1} (NH_4SO_4)] compared with the value of 0.097 mg l^{-1} [approximately 0.06 mmol l^{-1} (NH_4SO_4)] in adult fish. Similar trends have been observed in the green sunfish (*Lepomis cyanellus*) (McCormick et al., 1984), the spotted seatrout *Cynoscion nebulosus* (Daniels et al., 1987) and the smallmouth bass *Micropterus dolomieu* Lacepede (Broderius et al., 1985). It is possible that this difference in ammonia tolerance between early and late stages is related to characteristics that are unique to the embryo. The protection afforded by the chorion may not be a factor because just-hatched embryos are less susceptible to ammonia than are encapsulated embryos (Rice and Stokes, 1975; Broderius et al., 1985). In fact, spotted seatrout exhibit an increase in ammonia tolerance over the 4 month period following hatching (Daniels et al., 1987). Embryos may possess efficient biochemical mechanisms by which they convert ammonia to non-toxic substances, such as the non-essential amino acids glutamine and glutamate. Adult rainbow trout (Vedel et al., 1998), mudskippers (*Periophthalmus cantonensis*) (Iwata, 1988) and goldfish (*Carassius auratus*) (Levi et al., 1974) increase glutamine/glutamate production upon exposure to elevated external ammonia levels, particularly in the brain. There is little or no information available on changes in amino acid levels in relation to external ammonia exposure in fish embryos, and therefore this was an area of particular interest in the present study.

Griffith (Griffith, 1991) proposed that the OUC would have been retained and expressed at the embryonic stage to deal with the naturally increasing tissue ammonia levels incurred during embryogenesis. If this is true, then embryos may increase OUC enzyme activity as a detoxifying step in the event of elevated ammonia exposure.

The objectives of this study were threefold: (i) to identify the tissue distribution of CPSase III and other OUC enzymes within the embryo, (ii) to determine whether exposure to high external ammonia levels initiates hatching, and (iii) to

determine whether embryos detoxify ammonia to other nitrogenous compounds (e.g. urea, amino acids). To address the first objective, just-hatched trout embryos were dissected by hand to separate yolk, liver and the embryo proper. The activities of CPSase III, OTCase, arginase and GSase were measured in each of these tissue fractions. For the second and third objectives, two levels of ammonia exposure were used, an acute exposure (2 h) to 10 mmol l^{-1} NH_4Cl and a chronic exposure (4 days) to 0.2 mmol l^{-1} NH_4Cl . The time to hatching, ammonia and urea excretion rates and tissue levels of ammonia and urea were measured in both groups, and tissue levels of amino acids were measured in the chronic exposure group. In these analyses, it was imperative that the relatively large yolk mass was separated from the smaller embryonic tissue mass, particularly for the amino acid measurements, because yolk levels of amino acids are approximately seven times those in the embryonic tissues. Also, to delineate the role of the OUC in ammonia detoxification, the activities of CPSase III, OTCase, arginase and GSase in embryonic tissues were compared between the chronically exposed and control embryos.

Materials and methods

Animals

Rainbow trout *Oncorhynchus mykiss* (Walbaum) embryos were purchased from Rainbow Springs Trout Farm (Thamesford, Ontario, Canada) and held in continuous-flow incubation trays with mesh bottoms supplied with local well water ($10\text{--}12^\circ\text{C}$; pH 7.9; water hardness 411 mg l^{-1} as CaCO_3 ; Ca^{2+} , $5.24 \text{ mequiv l}^{-1}$; Cl^- , $1.47 \text{ mequiv l}^{-1}$; Mg^{2+} , $2.98 \text{ mequiv l}^{-1}$; K^+ , $0.06 \text{ mequiv l}^{-1}$; Na^+ , $1.05 \text{ mequiv l}^{-1}$) at Hagen Aqualab, University of Guelph, Guelph, Ontario, Canada. Incubation trays were shielded from the light during the entire incubation period. For series I experiments, hatching occurred between 32 and 34 days post-fertilization. For series II and III, experimentation was initiated at the 'eyed-up' stage (13 days post-fertilization in series II, 30 days post-fertilization in series III), when the pigmented eye was first clearly visible. Embryos for each series were obtained from four or more females. For the three series of experiments, separate batches of embryos were used. Hence, comparisons were not made between series, only within series. Mortality was 15 % over the experimental period, and there was no significant difference in mortality between control and experimental animals.

Experimental protocol

Series I: localization of urea cycle enzymes in embryos

Just-hatched embryos were anaesthetized in 0.15 g l^{-1} MS-222 for 10 min, rinsed with water and placed under a dissecting microscope at $12.5\times$ magnification. The yolk sac was teased away from the embryonic body and intact liver, and the outer yolk membrane was cut. The intact yolk sac was then removed with forceps, placed in an aluminium container and frozen in liquid nitrogen. Both the yolk and the yolk sac membrane were included as the yolk sac fraction. Next, the liver was separated

from the embryonic body by cutting the primary hepatic artery and the other connecting tissues. Visual observation indicated minimal loss of tissue during this process and no cross-contamination of samples taken from individual fish. The embryonic body and isolated liver were frozen as separate fractions in liquid nitrogen. Five pooled samples of each tissue were collected, and each sample contained the tissues from 30–50 individuals. Samples were weighed and then stored at -80°C . Controls were included to confirm that MS-222 treatment did not affect enzyme activities.

Series II: recovery from acute exposure to $10\text{ mmol l}^{-1}\text{ NH}_4\text{Cl}$

Thirty-two embryos were placed into perforated polystyrene chambers. Each chamber (approximately 50 ml) represented a pooled sample ($N=1$). In preliminary experiments, several concentrations of NH_4Cl were tested to determine what external ammonia concentration was required to induce a significant elevation of internal ammonia levels. The level of $10\text{ mmol l}^{-1}\text{ NH}_4\text{Cl}$ was chosen because whole-embryo ammonia levels were significantly elevated (control $1.83\pm 0.80\text{ }\mu\text{mol N g}^{-1}$ versus treated $3.87\pm 0.13\text{ }\mu\text{mol N g}^{-1}$; $P=9.5\times 10^{-8}$, single factor ANOVA) after 2 h of exposure. Six treated chambers ($10\text{ mmol l}^{-1}\text{ NH}_4\text{Cl}$) and six control chambers (fresh water) were assigned to each time period, 0, 1, 6, 24, 48 and 120 h post-treatment. Water in the experimental chambers was aerated throughout the experiment.

Embryos were exposed to either treatment or control water for 2 h and then returned to fresh water. The pH of the NH_4Cl solution was adjusted to match that of the control solution (pH 7.94). Immediately following treatment (0 h), a sub-sample of 10 embryos from each of six treated and six control chambers was collected, blotted dry, weighed and immediately frozen in liquid nitrogen for later tissue analysis of urea and ammonia levels. Ammonia and urea excretion rates over 2 h were measured at the end of each time period (as described previously, Wright et al., 1995).

In eight separate chambers (four treated, four control) containing 25 embryos each, post-treatment or control animals were monitored for time to hatching. Hatching was recorded on a daily basis until it was complete.

Series III: chronic exposure to $0.2\text{ mmol l}^{-1}\text{ NH}_4\text{Cl}$

In the chronic ammonia exposure experiment, it was necessary to choose a concentration of external NH_4Cl well below the level in the acute experiment, but sufficient to cause a small but marked disturbance in internal ammonia levels. We chose an external level of $0.2\text{ mmol l}^{-1}\text{ NH}_4\text{Cl}$ on the basis of previous experiments (Wright and Land, 1998). Forty embryos were placed in perforated polystyrene chambers. Each chamber held a volume of approximately 250 ml and represented one pooled sample ($N=1$). Twelve chambers were allocated to either the control (fresh water, $N=6$) or treated ($0.2\text{ mmol l}^{-1}\text{ NH}_4\text{Cl}$, $N=6$) groups. As before, chambers were continuously aerated throughout the 4 day treatment period. The pH of the NH_4Cl solution was adjusted to match that of the control solution (pH 7.86). Solutions were changed in both groups

every 12 h to avoid accumulation of endogenously excreted ammonia (levels remained at $<20\text{ }\mu\text{mol l}^{-1}$).

Nitrogen excretion rates over 3 h were measured on day 0 (at the onset of treatment) and on days 1, 2 and 4 of treatment in both the control and treated embryos, as described for series II. Tissue samples were taken at the end of day 4 of the treatment period. Embryos were removed from the chambers, rinsed briefly in fresh water, blotted dry and frozen in liquid nitrogen for later tissue analysis of ammonia, urea and amino acid levels and urea cycle enzyme activities.

Hatching was monitored as described for series II.

Analytical techniques

Water samples were stored at -20°C for up to 4 weeks prior to analysis. Water samples from both series II and III were analyzed for ammonia content using colorimetric assays (as described by Verdouw et al., 1978). The detection limits of this assay were $0.1\text{--}100\text{ }\mu\text{mol l}^{-1}$ ammonia. For series III, water samples from treated chambers ($0.2\text{ mmol l}^{-1}\text{ NH}_4\text{Cl}$) were diluted 2.5-fold before being assayed for ammonia concentration. These samples were also assayed in triplicate to reduce error associated with a high background level of ammonia. Urea content in series III was measured using colorimetric assays (as described by Rahmatullah and Boyde, 1980). The detection limits of this assay are $0.2\text{--}200\text{ }\mu\text{mol l}^{-1}$ urea. All spectrophotometric measurements in this study were performed using a Perkin Elmer UV/VIS spectrophotometer (Lambda 2) (Perkin Elmer Corp., Norwalk, CT, USA). Excretion rates were calculated as the difference in concentration between the initial and final water sample multiplied by the volume of the chamber and divided by the total wet mass of the animals in the chamber and the excretion time.

To separate the embryonic tissue (including the yolk sac membrane) from the yolk for further analyses of ammonia, urea and amino acid levels and enzyme activities, a centrifugation method was used (A. Shashavarani, Z. Thomas, J. S. Ballantyne and P. A. Wright, in preparation). The chorions of five embryos per sample were first removed manually using fine forceps. The embryos were diluted in five times their mass:volume of 50 mmol l^{-1} imidazole/ 50 mmol l^{-1} potassium chloride buffer (pH 8, 4°C). Samples were gently inverted until all the yolk was completely dissolved in the buffer and the remaining embryos were floating freely in the solution and were centrifuged briefly (3 s). The supernatant was removed and stored as the yolk fraction at -80°C . Embryos were rinsed again in imidazole/KCl buffer and frozen at -80°C .

Yolk and embryo fractions were stored at -80°C for up to a week prior to analysis. The embryo fractions were ground using a mortar and pestle under liquid nitrogen, deproteinized and neutralized as described by Wright et al. (Wright et al., 1995) with the following exceptions; 10 volumes of ice-cold 8% perchloric acid (PCA) was used to deproteinize each sample, followed by centrifugation for 10 min at $16\text{ }000\text{ g}$. Yolk fractions ($250\text{ }\mu\text{l}$ of the total fraction) were deproteinized using twice the volume of ice-cold 8% PCA and centrifuged for

10 min at 16 000 g. Ammonia content was measured in all fractions using an enzymatic Sigma diagnostic kit (171-C, Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada), and urea concentration was analyzed using the method of Rahmatullah and Boyde (Rahmatullah and Boyde, 1980). The detection limits of the Sigma diagnostic kit are 1–882 $\mu\text{mol l}^{-1}$ ammonia.

Separated yolks and embryos (series III) were stored at -80°C for up to 2 weeks prior to amino acid analysis. Analysis was performed using high-performance liquid chromatography (HPLC). Yolk samples (a 200 μl fraction of each pooled yolk sample) were analyzed using the solvents and method described by Barton et al. (Barton et al., 1995) for plasma. Embryo samples were analyzed using the same solvents, but samples were processed by grinding approximately 125 mg of tissue in 500 μl of 0.5% trifluoroacetic acid in methanol. Samples were brought up to 750 μl using double-distilled water. A 6 mmol l^{-1} internal standard of L-norvaline and L-azetidine-2-carboxylic acid was used for the embryo samples (12.5 μl per sample). Samples were then centrifuged for 5 min at 4°C at 16 500 g, after which 50 μl of 1 mol l^{-1} sodium acetate and 25 μl of 1 mol l^{-1} sodium hydroxide was added to each sample. Samples were then centrifuged for an additional 25 min at 4°C and 16 500 g. The resulting supernatant was analyzed using a Hewlett Packard Series II 1090 liquid chromatograph (Hewlett-Packard Company, Avondale, PA, USA).

For urea cycle enzyme analysis (series III), embryos were separated as described above except that 14 embryos per sample (0.15–0.25 g) were used and the yolks were discarded. Embryos used for enzyme analysis were stored overnight at -80°C prior to analysis. Samples were homogenized in approximately 20 volumes of ice-cold extract buffer (0.05 mol l^{-1} Hepes buffer, pH 7.5, 0.05 mol l^{-1} KCl, 0.5 mmol l^{-1} EDTA, 1 mol l^{-1} DL-dithiothreitol) and sonicated. Homogenates were centrifuged at 4°C for 10 min at 14 000 g. Because of the relatively low mass of samples, the supernatant from the centrifuged tissue homogenate was not passed through a Sephadex column as described previously (Felskie et al., 1998). To account for the presence of endogenous substrates in the homogenate, controls without exogenous substrate were included in each enzyme assay.

All enzyme activities were measured at 26°C . OTCase activity was determined by measuring the production of citrulline using the reaction mixture described by Wright et al. (Wright et al., 1995) and the colorimetric assay method described by Xiong and Anderson (Xiong and Anderson, 1989) from 0 to 40 min. GSase activity was quantified as the production of γ -glutamyl hydroxamate from 0 to 40 min using the method described by Shankar and Anderson (Shankar and Anderson, 1985). Arginase activity was measured using the reaction mixture described by Felskie et al. (Felskie et al., 1998) and the colorimetric assay described by Rahmatullah and Boyde (Rahmatullah and Boyde, 1980). The reaction mixture used for CPSase activity measurement was as described in Chadwick and Wright (Chadwick and Wright, 1999), and the

reaction was conducted for 60 min, after which [^{14}C]carbamoyl phosphate production was measured using the technique described by Anderson et al. (Anderson et al., 1970).

For series I, an additional set of reactions was included in which NH_4Cl was substituted for glutamine (at the same concentration) to compare the efficacies with which CPSase III uses ammonia or glutamine as substrates. Although it is generally accepted that ammonia is not a physiologically significant nitrogen-donating substrate for the urea-cycle-related CPSase in fish (Felskie et al., 1998), recent work on fish tissues has shown that ammonia can be as important as or more important than glutamine (Saha et al., 1997; Lindley et al., 1999). Activities were expressed as the number of μmoles of product formed per gram of tissue per minute ($\mu\text{mol g}^{-1} \text{min}^{-1}$). CPSase III activity was defined as the activity in the presence of glutamine (the substrate), N-acetylglutamate (AGA, a required positive effector) and uridine-3'-triphosphate (UTP, a CPSase II inhibitor). The estimated limit of detection for this assay is 0.1 $\text{nmol g}^{-1} \text{min}^{-1}$. CPSase II was defined as activity in the presence of glutamine and the absence of AGA.

Statistical analyses

The differences between control and ammonia-treated values for the yolk and embryonic tissue analysis (ammonia, urea and enzyme activities) and time to hatching within each series were compared using single-factor analysis of variance (ANOVA). Significant differences were declared if $P \leq 0.05$. Excretion data were analyzed using repeated-measures ANOVA and amino acid data using a General Linear Models procedure, both using the SAS system (version 6.12; SAS Institute Inc., Cary, NC, USA). The Tukey test was applied after significant differences had been identified between treatment and control animals ($P \leq 0.05$). Results are presented as means \pm S.E.M.

Results

Localization of enzyme activities

No CPSase activity was detected in the yolk sac of rainbow trout (Fig. 1). The absence of CPSase II was indicated by the lack of activity in the presence of substrate, and the absence of CPSase III was indicated by the lack of activation by AGA. In the embryonic body, there was substantial CPSase III activity, as indicated by the activation of activity (106%) in the presence of AGA (glutamine alone, $0.79 \pm 0.12 \text{ nmol g}^{-1} \text{min}^{-1}$ versus glutamine+AGA, $1.63 \pm 0.14 \text{ nmol g}^{-1} \text{min}^{-1}$, $N=5$). CPSase II activity, but not CPSase III activity, was detected in the liver. In the embryonic body and liver, the activity of CPSase II with ammonia as a substrate was as high as that with glutamine. The absence of activation by AGA (ammonia alone, $0.69 \pm 0.09 \text{ nmol g}^{-1} \text{min}^{-1}$ versus ammonia+AGA, $0.78 \pm 0.08 \text{ nmol g}^{-1} \text{min}^{-1}$, $N=5$) indicated that ammonia was not used as a substrate by CPSase III. With respect to the whole animal, CPSase III activity in the embryonic body (minus the liver) accounted for 100% of the total CPSase III activity.

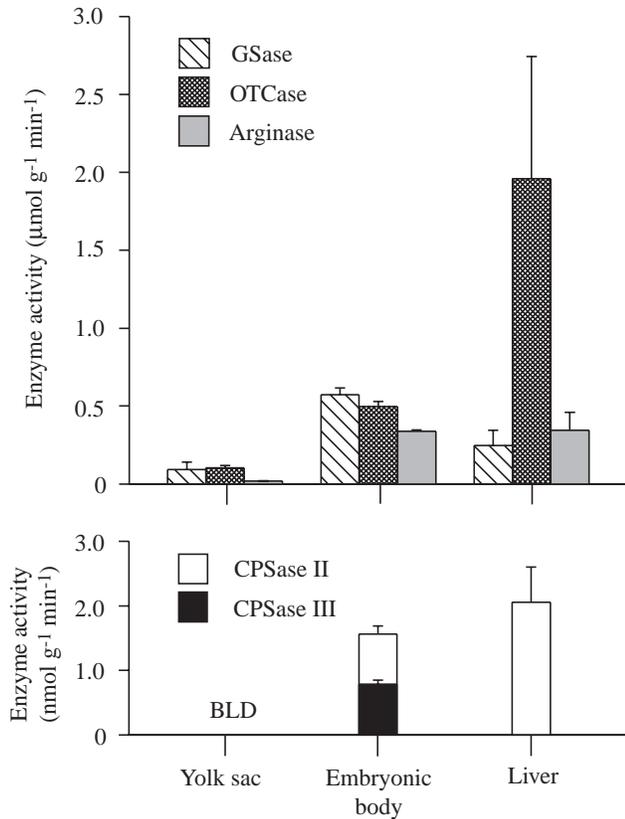


Fig. 1. Activities of glutamine synthetase (GSase), ornithine transcarbamoylase (OTCase), arginase and carbamoyl phosphate synthetases (CPSases) II and III in the yolk sac, embryonic body and liver fractions of rainbow trout just after hatching (38 days post-fertilization). Values are means + S.E.M. of five measurements (30–50 individuals were pooled for each measurement). BLD, below levels of detection.

CPSase II activity was almost three times greater in the liver than in the embryonic body (Fig. 1).

GSase, OTCase and arginase activity was detected in all fractions, but the activities of these enzymes were relatively low in the yolk sac (Fig. 1). OTCase activity was four times greater in the liver than in the embryonic body (Fig. 1).

Time to hatching

Hatching in series II began 23 days post-fertilization and took 6 days to complete, whereas in series III, hatching began 41 days post-fertilization and took 7 days to complete. Time to hatching was not affected by treatment with NH_4Cl in either series (Fig. 2).

Exposure to elevated environmental ammonia levels

In the series II experiments, ammonia excretion was significantly higher (+62%) in ammonia-treated embryos (1 h) than in control animals immediately after the treatment (time 0 h). Ammonia excretion rates remained significantly higher in treated embryos up to 24 h post-treatment, but declined to match those of controls by 48 h (Fig. 3A). Urea excretion rates

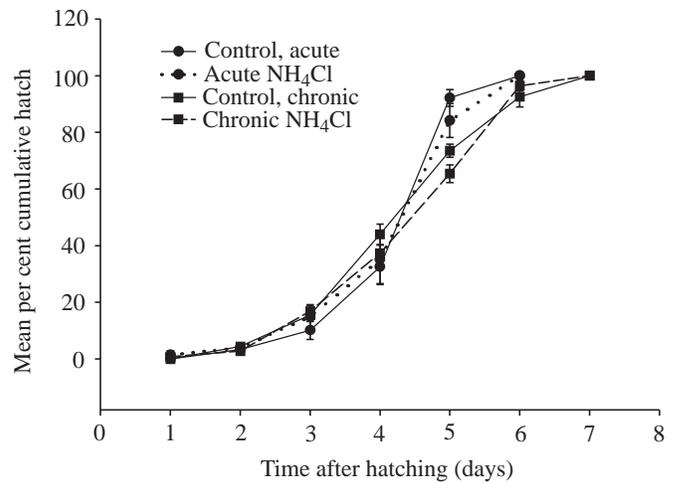


Fig. 2. Mean percentage cumulative hatch (means \pm S.E.M.) of rainbow trout embryos exposed either acutely (2 h) to 10 mmol l^{-1} NH_4Cl or chronically (4 days) to 0.2 mmol l^{-1} NH_4Cl ($N=6$).

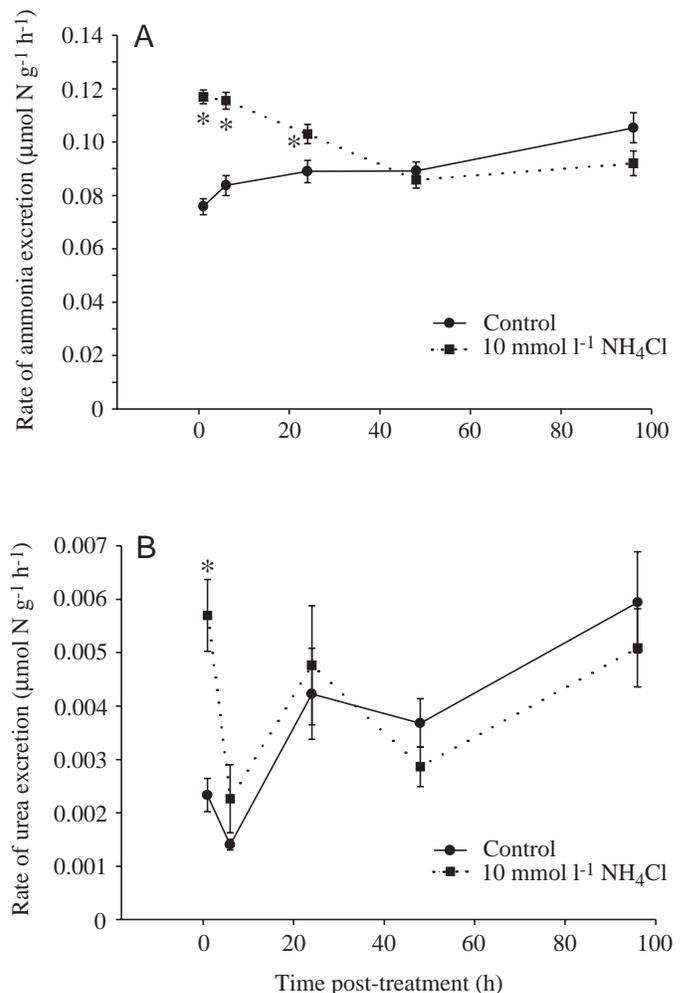


Fig. 3. Rates of ammonia (A) and urea (B) excretion ($\mu\text{mol N g}^{-1} \text{ h}^{-1}$) of rainbow trout embryos at several times following treatment with 10 mmol l^{-1} NH_4Cl (means \pm S.E.M., $N=6$). An asterisk indicates a significant difference from controls.

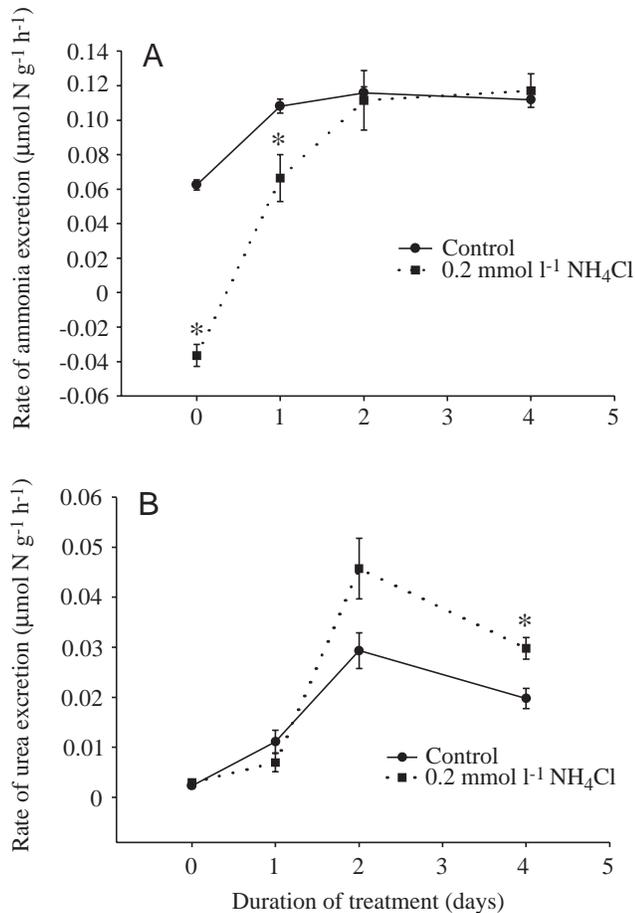


Fig. 4. Rates of ammonia (A) and urea (B) excretion ($\mu\text{mol N g}^{-1} \text{h}^{-1}$) of rainbow trout embryos over time during a 4 day treatment with $0.2 \text{ mmol l}^{-1} \text{ NH}_4\text{Cl}$ (means \pm S.E.M., $N=6$). An asterisk indicates a significant difference from controls.

were significantly elevated immediately following treatment (0h), but returned to control values by 24 h (Fig. 3B).

Ammonia excretion in series III was measured during treatment ($0.2 \text{ mmol l}^{-1} \text{ NH}_4\text{Cl}$) as opposed to after treatment as in series II. Ammonia excretion was significantly depressed on the first day of treatment (day 0) and was, in fact, negative, indicating an uptake of ammonia into the embryos (Fig. 4A). Ammonia excretion recovered by day 2. Urea excretion in ammonia-exposed embryos (series III) was significantly higher than in controls only on day 4 (Fig. 4B).

In series II and III, ammonia levels in the yolk were significantly higher (+102% and +304%, respectively) than control yolk levels following either acute or chronic ammonia exposure (Fig. 5A). In the embryonic tissues, however, the ammonia concentration was not altered significantly by these treatments. Urea levels in the yolk were unchanged by acute or chronic ammonia exposure, but they were significantly elevated in the embryonic tissues (Fig. 5B).

There were distinct differences in ammonia and urea levels in different tissue compartments. Particularly in ammonia-exposed embryos, ammonia concentration in the yolk fraction

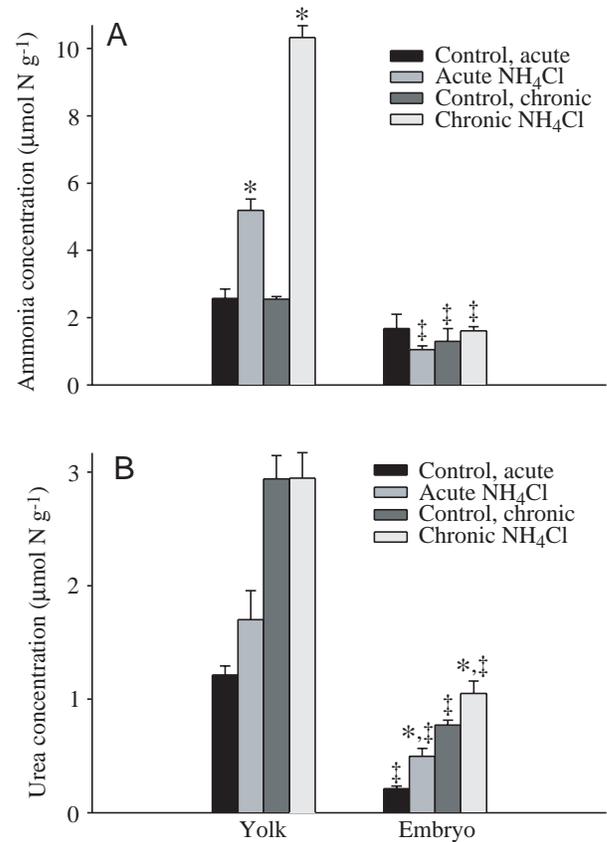


Fig. 5. Ammonia (A) and urea (B) concentrations ($\mu\text{mol N g}^{-1}$) in the yolk and embryonic tissues after either acute (2h) exposure to $10 \text{ mmol l}^{-1} \text{ NH}_4\text{Cl}$ or chronic (4 days) exposure to $0.2 \text{ mmol l}^{-1} \text{ NH}_4\text{Cl}$ (means \pm S.E.M., $N=6$). An asterisk indicates significant difference between control and treated animals; a double dagger indicates significant difference between yolk and embryo.

Table 1. Enzyme activities in embryos immediately following a 4 day treatment with $0.2 \text{ mmol l}^{-1} \text{ NH}_4\text{Cl}$

Enzyme	Control	NH_4Cl
CPSase III	0.780 ± 0.105	0.744 ± 0.059
CPSase II	1.514 ± 0.080	1.646 ± 0.087
OTCase	0.614 ± 0.065	0.643 ± 0.076
GSase	0.285 ± 0.013	0.294 ± 0.011
Arginase	0.260 ± 0.015	0.270 ± 0.015

Values are means \pm S.E.M., $N=6$.

CPSase II, III, carbamoyl synthetases II and III; OTCase, ornithine transcarbamoylase; GSase, glutamine synthetase.

CPSase II and III activities are reported as $\text{nmol min}^{-1} \text{g}^{-1}$ embryonic body; other enzyme activities are reported as $\mu\text{mol min}^{-1} \text{g}^{-1}$ embryonic body.

was several-fold higher than in the embryo fraction (Fig. 5A). In all groups, urea levels in the yolk fraction were three- to fivefold higher than in the embryo fraction (Fig. 5B).

There was no effect of chronic exposure (4 days) to $0.2 \text{ mmol l}^{-1} \text{ NH}_4\text{Cl}$ on urea cycle enzyme activities in series III embryos (Table 1).

Table 2. Individual amino acid concentrations in embryos and yolks of rainbow trout immediately following a 4 day treatment with 0.2 mmol l⁻¹ NH₄Cl

Amino acid	Embryo (nmol g ⁻¹)		Yolk (nmol g ⁻¹)	
	Control	NH ₄ Cl	Control	NH ₄ Cl
Essential				
Histidine	90±24	97±5	1398±190	1060±70
Threonine	594±129	519±59	3299±465	2443±303
Valine	175±36	152±9	2919±234	1459±125*
Methionine	187±23	185±13	2434±381	1538±155*
Arginine	155±41	130±15	2283±269	957±99*
Phenylalanine	403±53	442±32	1967±259	1241±124*
Isoleucine	116±21	99±6	1546±106	709±64*
Leucine	162±25	146±9	1757±104	887±60*
Lysine	167±49	130±19	1973±124	1083±86*
Total essential	2035±276	1929±121	18361±1844	11119±987*
Nonessential				
Aspartate	337±33	446±54	3680±708	3064±259
Glutamate	678±125	697±28	5875±555	3865±432*
Asparagine	95±10	95±13	717±80	327±25*
Serine	110±16	114±14	1123±130	550±59*
Glutamine	308±64	303±19	2980±183	1652±144*
Glycine	99±15	95±13	890±65	536±39*
Alanine	264±43	236±24	3611±427	1528±88*
Taurine	747±123	830±110	3089±295	1837±166*
Tyrosine	331±33	345±27	1637±270	825±120*
Tryptophan	142±18	157±15	1068±141	697±65*
Hydroxyproline	2138±634	3864±1381	8095±1539	4629±779
Proline	472±338	396±94	862±152	476±149
Total nonessential	5735±787	7553±1396	34843±3260	20250±2021*
Total amino acids	7770±957	9482±1436	53205±5074	31369±2851*

Values are means ± S.E.M. (N=6).

*Significantly different from the control value.

Total amino acid levels in the embryonic tissues were not significantly different between control and ammonia-exposed (chronic) embryos (Table 2). Levels of sixteen individual essential and non-essential amino acids in the yolk, however, were significantly lower (-34% to -58%) after 4 days of exposure to 0.2 mmol l⁻¹ NH₄Cl. There were no differences between individual amino acids in embryonic tissues with ammonia exposure.

Discussion

Localization of OUC enzymes

This study provides evidence of urea cycle expression in non-hepatic tissues of rainbow trout just after hatching because CPSase III, GSase, OTCase and arginase are co-localized to the embryonic body (yolk sac and liver removed). These findings are consistent with previous reports of CPSase III expression in skeletal muscle tissue of juvenile and adult rainbow trout, but not in liver tissue (Felskie, 1996; Korte et al., 1997). In addition, the data support our hypothesis that enzyme analysis on whole-animal homogenates will underestimate

enzyme activity in the embryonic tissues because of the presence of the relatively large yolk containing low or undetectable activities of all four measured enzymes. The enzyme activities measured in the embryonic body and liver in the present study are consistently higher than in the whole-animal homogenates of approximately the same developmental stage (just hatched) used by Wright et al. (Wright et al., 1995). Indeed, OTCase activity is 200-fold higher in the liver (present study) than in the whole animal (Wright et al., 1995). It is also interesting that the level of CPSase III activity in the embryonic body (consisting largely of skeletal muscle) is relatively high (two- to sevenfold higher) compared with juvenile (Felskie, 1996) and adult (Korte et al., 1997) trout muscle tissue.

The presence of relatively high levels of OTCase activity in liver tissue of newly hatched trout is difficult to reconcile with the absence of CPSase III activity (CPSase III provides the substrate for OTCase), indicating the lack of a functional hepatic urea cycle in hatched trout. OTCase is not thought to be involved in other metabolic reactions. Felskie et al. (Felskie et al., 1998) suggested that urea cycle intermediates may be

shuttled between tissues (e.g. from muscle to liver) in some fishes, but this has not been demonstrated. In the alkaline-lake-adapted tilapia *Oreochromis alcalicus grahami*, the activities of all the urea cycle enzymes are present in the skeletal muscle tissue at levels that surpass those measured in the liver (Lindley et al., 1999). At least in the ureotelic tilapia, urea synthesis probably occurs primarily in the muscle tissue. Further studies in trout embryos using a radiolabelled substrate for the urea cycle would be valuable in sorting out which tissues are involved in urea-cycle-related urea synthesis.

Acute and chronic exposure to external ammonia

Hatching is not affected by treatment with either 0.2 mmol l^{-1} (4 day) or 10 mmol l^{-1} (2 h) NH_4Cl , consistent with a study on Atlantic salmon (*Oncorhynchus gorbushka*) in which embryos were exposed to $7\text{--}11 \text{ mmol l}^{-1}$ $(\text{NH}_4)_2\text{SO}_4$ (24 h) (Rice and Bailey, 1980). It does appear, therefore, that external ammonia, even at quite high levels, does not directly initiate the hatching process. In our preliminary analysis of the ammonia content of intact (whole) embryos, there was a significant elevation in ammonia levels after 2 h of exposure to 10 mmol l^{-1} NH_4Cl (control $1.83 \pm 0.08 \mu\text{mol g}^{-1}$, $N=6$, versus treated $3.87 \pm 0.13 \mu\text{mol g}^{-1}$, $N=6$). Subsequent separation of the yolk and tissue fractions, however, reveals that neither acute nor chronic ammonia treatment results in a significant elevation of ammonia concentration in the embryonic tissues. It is still possible that sustained elevation of internal ammonia levels during embryogenesis may influence the time to hatching in trout. Moreover, although environmental factors influence hatching (see Introduction), the exact developmental stage of exposure may be critical and is a consideration for future study.

The present data demonstrate that trout embryos have a remarkable ability to tolerate environmental ammonia. This tolerance is not related to impermeability because ammonia is clearly absorbed by the embryos from the environment. For example, the significant elevation in the rate of ammonia excretion for 24 h following the acute 10 mmol l^{-1} NH_4Cl treatment indicates that excess ammonia is cleared from the embryos once they have been placed in ammonia-free water. During chronic exposure to 0.2 mmol l^{-1} NH_4Cl , ammonia excretion rates are initially reversed (day 1), indicating uptake of ammonia from the external water to the animal. Although ammonia levels in the embryonic tissues are unaltered in both groups, the yolk sac is clearly a site of ammonia storage. Yolk ammonia levels increase two- to threefold in acute and chronic exposures, respectively. Terjesen et al. (Terjesen et al., 1998) reported a twofold increase in the ammonia content of intact embryos of the marine Atlantic halibut *Hippoglossus hippoglossus* after 6 days of exposure to exceedingly high ammonia levels (27 mmol l^{-1} NH_4Cl). Both studies highlight the extreme tolerance to environmental ammonia demonstrated by some species at the embryonic stage.

We propose that ammonia tolerance is related, in part, to mechanisms that maintain low and relatively constant ammonia concentrations in the embryonic tissues. These

mechanisms may involve detoxification of ammonia by synthesis of urea and sequestration of ammonia away from the developing tissues, i.e. in the yolk sac. Urea levels in the tissues are significantly elevated in both acute and chronic NH_4Cl treatment groups. In the chronically exposed embryos, urea excretion rates are also significantly higher than in the control group. The elevation of urea concentrations in the tissues, but not the yolk, is consistent with the expression of CPSase III, OTCase, arginase and GSase in the embryonic tissues. The levels of enzyme activity are not altered by chronic ammonia exposure. The maximal activity of the rate-limiting enzyme in a pathway can be compared with the rate of product formation to determine whether the level of activity is within the expected physiological range. In this case, CPSase III activities per individual are compared with the rate of urea excretion per individual (series III), presumably a valid comparison because very little excess urea is retained in the yolk or tissue fraction (approximately 5%) during chronic ammonia exposure and most appeared to be excreted. With the above assumptions, CPSase III activity can account for approximately 70% of the urea excreted under control conditions, but only 52% during NH_4Cl exposure. Thus, approximately half the urea synthesized under hyperammonia stress may be derived from alternative sources such as *de novo* purine synthesis and subsequent uric acid degradation or arginolysis (e.g. Wright, 1993; Wright and Land, 1998).

Another possible pathway for temporary storage of ammonia is through the synthesis of the non-essential amino acids glutamine and glutamate. In embryos chronically exposed to ammonia (4 days), there are no changes in tissue glutamine or glutamate levels or in total amino acid levels, but there are significant decreases in the yolk concentrations of almost every amino acid measured. These findings are puzzling. Dilution of the yolk compartment could potentially explain these findings, but yolk water content was not significantly different between these two groups (61–63% water by mass). It is unlikely that amino acids were lost to the environment because the yolk sac membrane is rather impermeable to solutes (e.g. Potts and Rudy, 1969; Mangor-Jensen et al., 1993), and developmental decreases in yolk amino acid concentrations are not due to diffusive loss (Rønnestad, 1993). Likewise, amino acids are not transferred for storage in the embryonic tissues (Table 2). Thus, the decrease in amino acid levels in ammonia-exposed embryos may reflect catabolism (see below) or, less importantly, incorporation into yolk proteins. We propose the latter possibility to explain the disappearance of taurine, a non-metabolisable amino acid.

To understand more thoroughly the changes in nitrogen equivalents during chronic ammonia exposure, a nitrogen budget was calculated. Over the 4 days of exposure, an individual embryo absorbed $0.31 \mu\text{mol-N}$ of ammonia from the environment, and $0.05 \mu\text{mol-N}$ or approximately 16% of this amount was converted to urea. The difference in the yolk ammonia content between control and ammonia-exposed embryos is $0.43 \mu\text{mol-N}$; this level is somewhat higher than

can be accounted for simply by absorption from the environment. The difference may be due, in part, to catabolism of individual amino acids. Theoretically, approximately 0.9 $\mu\text{mol-N}$ of ammonia would be produced if the significant decreases in yolk amino acid levels observed (Table 2) were as a result of complete amino acid catabolism. The difficulty with this hypothesis is that amino acid catabolism would occur mostly in the tissues, but no excess tissue ammonia was detected, nor was the excess ammonia excreted. Thus, we propose that elevated ammonia levels in the yolk are derived primarily from the environment, with possibly a smaller proportion derived from amino acid catabolism. It should be noted, however, that stress-induced release of cortisol in adult teleosts alters protein and amino acid metabolism as well as increasing the rate of ammonia excretion to the environment (Mommensen et al., 1999). Whether cortisol is involved in hyperammonia stress in trout embryos is unknown.

Rahaman-Noronha et al. (Rahaman-Noronha et al., 1996) examined ammonia distribution in the yolk *versus* the perivitelline fluid in rainbow trout on the basis of differences in pH and electrical potential and found that compartments with a lower pH (e.g., yolk) support higher concentrations of ammonia. Likewise, the low pH of the uterine fluid surrounding the pup of the dogfish (*Squalus acanthias*) provides a sink for ammonia eliminated from both the mother and the pup (Kormanik and Evans, 1986). To predict yolk total ammonia concentration ($[T_{\text{amm}}]$) and determine whether the yolk ammonia concentration is dependent on the H^+ gradient, we used a modification of the Henderson–Hasselbalch equation (Rahaman-Noronha et al., 1996), assuming that yolk pH is 6.35 (Rahaman-Noronha et al., 1996), that extracellular pH (pHe) is 7.8 and that intracellular $[T_{\text{amm}}]$:extracellular $[T_{\text{amm}}]$ is 22 (Wilkie and Wood, 1991). The predicted control yolk $[T_{\text{amm}}]$ is 2.71 $\mu\text{mol-N g}^{-1}$, not very different from the measured value of 2.56 $\mu\text{mol-N g}^{-1}$ (Fig. 5A), suggesting that the yolk:tissue ammonia distribution was probably dependent on the H^+ gradient. When these calculations were repeated using values for NH_4Cl -exposed embryos, however, the predicted yolk T_{amm} was three- to fourfold lower than the measured values. In other words, although yolk ammonia levels are significantly elevated in NH_4Cl -treated embryos, the absolute level is not as high as expected on the basis of the measured tissue ammonia level and estimated H^+ gradient. This implies that, in the presence of external ammonia, the above assumptions are either no longer valid (e.g. the NH_4Cl treatment resulted in changes in the pHe and/or yolk pH) or the yolk $[T_{\text{amm}}]$ is influenced by other factors, such as the electrical potential (Rahaman-Noronha et al., 1996). It is reasonable to propose that an alkalization of the embryo and/or yolk as a result of NH_3 entry (and conversion to NH_4^+) would have altered the H^+ gradient between the yolk and embryo and, consequently, the yolk total ammonia concentration.

The urea content of the yolk is significantly higher than that of the embryonic tissues, with the ratio ranging from 3:1 to 6:1 (yolk:embryo; Fig. 5B). Urea, unlike NH_4^+ , is not an electrolyte and its concentration should therefore be relatively

uniform and not influenced by pH or electrochemical gradients. In fact, Raymond and DeVries (Raymond and DeVries, 1998) measured a uniform distribution of urea between muscle, serum, urine and liver in a variety of arctic teleosts. Rahaman-Noronha (Rahaman-Noronha, 1996) measured yolk urea concentration in trout embryos at the same stage of development, after manually removing yolk samples, and obtained very similar values to those in the present study. The large diffusion gradient for urea between the yolk and tissues suggests that urea transport solely by simple diffusion is unlikely. Pilley and Wright (Pilley and Wright, 2000) reported that urea excretion in rainbow trout embryos was dependent, in part, on a facilitated urea transporter, as in other fish tissues (Wood et al., 1998; Smith and Wright, 1999; Fines et al., 2001; Walsh et al., 2000). Clearly, research focused on the distribution of urea in the tissues of fish embryos is warranted.

In summary, CPSase III and other related OUC enzymes in rainbow trout embryos are expressed together in the embryonic body, with OTCase, arginase and GSase (but not CPSase III) detected in the liver tissue and, to a small extent, in the yolk. During acute or chronic hyperammonia stress, tissue ammonia levels remain constant and the time to hatching is not altered, although a considerable ammonia load is absorbed from the environment. Most of this excess ammonia is found in the yolk, whereas a small percentage is converted to the relatively non-toxic nitrogen end-product urea. These strategies may contribute to the high tolerance of early life stages compared with adult trout to excessive environmental ammonia levels. Furthermore, the data strongly indicate that analyses of enzyme activities and metabolite levels in fish embryos with a relatively large yolk mass (e.g. salmonids) requires the careful separation of the yolk from the embryonic tissues.

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