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PATHWAYS FOR UREA PRODUCTION DURING EARLY LIFE OF AN AIR-BREATHING TELEOST, THE AFRICAN CATFISH *CLARIAS GARIEPINUS* BURCHELL

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Summary

Embryos and larvae of the African catfish *Clarias gariepinus* excrete significant quantities of urea. The present study focused on the potential urea-generating pathways during early development of this teleost; uricolysis, argininolysis and the ornithine–urea cycle (OUC). Uricase, allantoinase, allantoicase and ureidoglycollate lyase of the uricolytic pathway were expressed in all early life stages and in adult liver of *C. gariepinus*. Uricase activity increased in starved larvae compared with yolk-sac larvae. The key regulatory enzyme of the teleost OUC, carbamoyl phosphate synthetase III (CPSase III), was expressed predominantly in muscle of developing *C. gariepinus* larvae and showed negligible activity in the absence of its allosteric effector *N*-acetyl-L-glutamate. CPSase III and ornithine carbamoyl transferase activities increased in fed larvae compared with starved larvae. In contrast to the early developmental stages, adult *C. gariepinus* expressed only low and variable levels of CPSase III, suggesting that, under the experimental conditions employed, OUC expression is influenced by developmental stage in this species. The data indicate that early *C. gariepinus* life stages express the enzymes necessary for urea production by uricolysis, argininolysis and the OUC, and this may explain why urea tissue levels and urea excretion rates are substantial during the early development of this air-breathing teleost.

Key words: *Clarias gariepinus*, African catfish, embryo, larva, urea, excretion, uricolysis, ornithine–urea cycle, carbamoyl phosphate synthetase III, arginine.

Introduction

Urea rarely constitutes more than 10–20 % of the total nitrogen excreted by teleosts (Wood, 1993), except in a few species such as the Lake Magadi tilapia *Oreochromis alcalicus grahami* (Randall et al., 1989). In early teleost life stages, however, urea may be a more important end-product of nitrogen metabolism, as shown for *Zoarces viviparous* (Korsgaard, 1994) and the Atlantic cod (*Gadus morhua*, Chadwick and Wright, 1999). During early development of the African catfish *Clarias gariepinus*, urea constitutes 62 % of the total nitrogen excreted (as ammonia-N + urea-N) in embryos, 20 % during yolk-sac and starved larval stages, and 44 % after metamorphosis in fed larvae (Terjesen et al., 1997). In contrast, immersed adult *C. gariepinus* (Eddy et al., 1980) and *C. batrachus* (Saha and Ratha, 1989) excrete less urea, accounting for 13 % and 15 % of total nitrogen excreted, respectively. The African catfish is an air-breathing teleost tolerant to wide fluctuations in water availability and temperature (Donnely, 1973). Spawning occurs shortly after heavy rainfall, and the embryos are deposited in a few centimetres of water on vegetation in temporarily flooded areas (Greenwood, 1955; Bruton, 1979).

Three pathways may be responsible for urea production in teleosts: hydrolysis of arginine (argininolysis), catabolism of uric acid (uricolysis) and the ornithine–urea cycle (OUC). Arginine from the diet or from protein turnover is hydrolyzed by the enzyme arginase (Wood, 1993). On the basis of arginine depletion rates in *C. gariepinus* yolk-sac and starved larvae *in vivo*, Terjesen et al. (Terjesen et al., 1997) showed that argininolysis could account for approximately one-third of the urea excreted, suggesting that other pathway(s) for urea synthesis were also functional.

In adult teleosts, uricolysis serves to degrade purines originating from the diet or from nucleic acid turnover and may be the dominant pathway for urea synthesis in adults of most species (Goldstein and Forster, 1965; Vellas and Serfaty, 1974; Wright, 1993). During early development, *de novo* purine synthesis can be expected to provide building blocks for larval growth. Given that nucleotides are energetically expensive to synthesize (Stryer, 1988), it is probable that a rapidly growing yolk-sac larva that relies on endogenous nutrition would not express enzymes for purine degradation. However, to our

knowledge, no studies have investigated the uricolytic pathway in teleost embryos and larvae.

In teleosts, urea may also be formed from glutamine, HCO_3^-, ATP and aspartate by the OUC (Anderson, 1995). Only a few teleost species express significant activities of OUC enzymes in the adult (e.g. Mommsen and Walsh, 1989; Randall et al., 1989; Saha and Ratha, 1989), whereas all species so far studied express OUC enzymes during early life, as reported for guppy (*Poecilia reticulata*, Dépêche et al., 1979), rainbow trout (*Oncorhynchus mykiss*, Wright et al., 1995), Atlantic cod (*Gadus morhua*, Chadwick and Wright, 1999) and Atlantic halibut (*Hippoglossus hippoglossus*, Terjesen et al., 2000). Expression of the OUC in early teleost life stages has been hypothesized to assist in detoxification of ammonia accumulating from amino acid catabolism, which may be a particular problem for species with large embryos (Griffith, 1991; Wright et al., 1995). The early life stages of *C. gariepinus* are relatively small, however (individuals weigh 1–2.5 mg), and, although ammonia levels also increase in yolk-sac larvae of *C. gariepinus*, considerably more ammonia is excreted than is accumulated (Terjesen et al., 1997).

The main objective of the present study was to describe the biochemical pathways that may be responsible for the high rate of urea excretion by *C. gariepinus* larvae (Terjesen et al., 1997). We measured the activities of the enzymes of uricolysis, argininolysis and the OUC in the early life stages of *C. gariepinus* and in adult *C. gariepinus* tissues to investigate whether developmental stage influences the expression of the urea-producing pathways. If enzymes for the OUC were expressed, another aim of this study was to describe the basic kinetics and tissue location of the key regulatory enzyme carbamoyl phosphate synthetase III (CPSase III). In addition, we measured urea and uric acid levels in early life stages, which adds to data from our previous study on urea and ammonia excretion and tissue ammonia concentrations (Terjesen et al., 1997), to provide a detailed view of nitrogen metabolism and excretion in this species.

Materials and methods

Facilities and fish

This study was approved by the Animal Experiment Committee at Wageningen University. Fertilized embryos of *Clarias gariepinus* Burchell were produced by artificial reproduction, as outlined previously (Hogendoorn and Vismans, 1980; Terjesen et al., 1997). Water temperature was maintained at 27.8±0.2 °C, pH 8.3±0.1, and electrical conductivity at 295±60 $\mu S\,cm^{-1}$ (means ± S.D.). Total ammonia levels in the aquarium outlet water did not exceed 5 $\mu mol\,l^{-1}$ (Orion 9512 electrode, Thermo Orion, MA, USA). A fed group (fed larvae) was offered live *Artemia franciscana* instar I nauplii (from RH grade cysts, INVE Aquaculture, Belgium; according to the method of Verreth and Den Bieman, 1987) from 73 h post-fertilization. An additional group (starved larvae) was included to study the effects of starvation (terminated at 194 h post-fertilization).

Sampling procedures for embryos and larvae

Sampling was conducted according to the method of Terjesen et al. (Terjesen et al., 1997); in particular, fed larvae were not collected until 12 h after the last feeding. Samples were taken in triplicate for wet and dry mass and urea determination by collecting 2–20 individuals. Because of limited tissue availability, only a few samples were collected for uric acid analysis.

Samples were taken in triplicate for both OUC and uricolytic enzyme studies by collecting 0.4–1.2 g of embryos or larvae per sample according to the protocol of Terjesen et al. (Terjesen et al., 2000), followed by freezing in liquid nitrogen. All samples except those used for dry mass measurements were stored at −139 °C during the experimental period in Wageningen. Thereafter, samples were transported to the University of Bergen and stored at −80 °C until analysis.

Larval dissection

For wet mass, dry mass and urea determinations, 10–20 yolk-sac larvae aged 30, 55 or 82 h post-fertilization were dissected into yolk and body compartments (as described by Terjesen et al., 1997). The body compartment (i.e. yolk-free animal) is termed ‘larval body’. An attempt was made to establish the distribution of CPSase III in fed larvae aged 217 h post-fertilization (30±2 mg wet mass per individual). Livers (constituting less than 10 % of body mass) were dissected from 100 individuals while they were held in ice-water. Samples of posterior body segments were collected to demonstrate possible expression of CPSase III in skeletal muscle tissue. Approximately 60 larvae were cut quickly in two (1–2 mm behind the anus to avoid obtaining kidney tissue) while held in ice-water. Except for a minor contribution from bone, connective and neural tissue, the posterior segment should constitute mostly larval muscle tissue.

Sampling procedures for adult tissues

Tissue samples of adult *C. gariepinus* were collected for enzyme activity determinations, relative water content and urea measurements. One female (1.0 kg) and two males (2.8 and 3.0 kg), which had been fasted for 48 h, were quickly killed by a sharp blow to the head, and blood samples were collected from posterior haemal arches. Thereafter, liver, kidney and posterior dorsal muscle tissues were excised, rinsed in 0.9 % NaCl, blotted on tissue paper, weighed and then frozen in liquid nitrogen. Blood samples were centrifuged at 2240 ***g*** for 10 min, and plasma was frozen in liquid nitrogen. Samples were subsequently stored as described above.

Analytical procedures

Preparation of samples for enzyme activity measurements in crude extracts

All samples were processed at the University of Bergen in a cold-room at 4 °C. Since uricolytic enzymes have not been

measured in embryonic teleosts, variations in extraction and assay procedures were tested. The optimal extraction procedure was as follows. Frozen samples (0.2–1.1 g) were homogenized in 2.5–6.0 ml of fresh extraction buffer (50 mmol l^{-1} Hepes, pH 7.5, 50 mmol l^{-1} KCl, 0.5 mmol l^{-1} EDTA, 5 % glycerol, w/w, and 0.015 mg ml^{-1} trypsin inhibitor) using an IKA T25 Basic Ultra-Turrax homogenizor with an S25N-10G knife at 8000 revs min^{-1} for 12 s and 24 000 revs min^{-1} for 12 s. While incubating the tubes in ice-water, samples were sonicated for 1 min, followed by a pause of approximately 4 min, and then sonicated for 1 min at an amplitude setting of 40 (Soncis and Materials Vibra-Cell, CV18 probe). Allantoicase and ureidoglycollate lyase (UGL) assays did not produce reliable results if shorter sonication times were employed. For uricase and allantoinase, however, no consistent significant differences between procedures were observed, so all the data were pooled. OUC enzymes were extracted according to the method of Terjesen et al. (Terjesen et al., 2000). After centrifugation for 10 min at 14 500 ***g*** and 4 °C, supernatants were passed through Sephadex G-25 columns (2 cm×16 cm; Pharmacia Biotech, Uppsala, Sweden) equilibrated with extraction buffer (as described by Terjesen et al., 2000).

CPSase gel filtration chromatography

As outlined by Anderson (Anderson, 1995), useful criteria for the detection of CPSase III in tissues also containing CPSase II (pyrimidine synthesis) are (i) activation by *N*-acetyl-L-glutamate (AGA), (ii) higher activity with glutamine than with ammonia as a substrate, (iii) lack of inhibition by uridine triphosphate (UTP) and (iv) separation of CPSase II and III by gel filtration chromatography and elution of a potential CPSase III peak at a position corresponding to a molecular mass of 160 kDa. Thus, gel filtration chromatography of whole larvae (315 h post-fertilization) and dissected liver and muscle extracts (217 h post-fertilization) was conducted at 4 °C (as described by Anderson and Walsh, 1995; Terjesen et al., 2000) on a Sephacryl S300HR column (1.6 cm×60 cm; Pharmacia Biotech) equilibrated with freshly prepared buffer containing 100 mmol l^{-1} KCl, 50 mmol l^{-1} Hepes (pH 7.5), 0.5 mmol l^{-1} EDTA, 15 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} ATP, 10 mmol l^{-1} $NaHCO_3$, 2 mmol l^{-1} dithiothreitol, 0.015 mg ml^{-1} trypsin inhibitor and 10 % glycerol (w/w). CPSase elution profiles were constructed from assays with both glutamine and AGA present in the reaction mixture. The molecular masses of the eluted proteins were estimated using the method of Terjesen et al. (Terjesen et al., 2000). Protein concentration was determined after Bradford (Bradford, 1976).

Uricolytic enzyme assays

Because they are markedly unstable, substrates, cofactors and standards were prepared shortly before use and incubated on ice. Each sample was run in duplicate, and controls containing no substrate or no extract were included. For each assay, standards were run with known concentrations of products or substrates dissolved in the same solution as the samples. Activity was calculated from the linear part of the curve (product formation or substrate depletion *versus* time) after subtracting the control rate. All reactions were performed at 26 °C and were linear with respect to extract concentration.

Uricase (EC 1.7.3.3) was assayed essentially as described by Brown et al. (Brown et al., 1966). The final reaction mixture contained 60 μmol l^{-1} uric acid, 367 mmol l^{-1} glycine (pH 9.7), 8.3 mmol l^{-1} Hepes (pH 7.5), 8.3 mmol l^{-1} KCl, 0.1 mmol l^{-1} EDTA, 0.8 % glycerol (w/w) and 0.0025 mg ml^{-1} trypsin inhibitor in a final volume (i.e. including extract) of 1200 μl. Before each assay, the reaction mixture was bubbled with O_2 at room temperature (20–25 °C) for 60 min. The reaction was initiated by the addition of 200 μl of extract (10 μl for adult liver) and followed by the decline in absorbance at 293 nm (A_{293}) as uric acid was converted into the non-absorbing allantoin.

Allantoinase (EC 3.5.2.5.) was assayed essentially as described by Takada and Noguchi (Takada and Noguchi, 1983) employing differential analysis of glyoxylate derivatives (Vogels and Van der Drift, 1970). The final reaction mixture contained, in a volume of 500 μl: 50 mmol l^{-1} Tris (pH 8.2), 25 mmol l^{-1} allantoin, 12 mmol l^{-1} Hepes (pH 7.5), 12 mmol l^{-1} KCl, 0.1 mmol l^{-1} EDTA, 1.2 % glycerol (w/w) and 0.004 mg ml^{-1} trypsin inhibitor. Reactions were initiated by addition of 120 μl of extract (5 μl for adult liver) and terminated at 0 and 60 min by addition of 500 μl of 0.5 mol l^{-1} HCl, followed by boiling for 5 min. Subsequently, the tubes were cooled on ice, and the contents were neutralized with 500 μl of 0.5 mol l^{-1} KOH in 0.1 mol l^{-1} Hepes/0.1 mol l^{-1} KCl. After centrifugation at 9600 ***g***, glyoxylate in the supernatant was determined by adding NADH and lactate dehydrogenase (LDH, Sigma L 2625) at 340 nm. Assuming a NADH extinction coefficient of 6.22 mmol l^{-1} cm^{-1}, standards gave a recovery of 74±18 % (mean ± S.D., *N*=48). The incomplete recovery reduced the sensitivity of the assay but did not affect calculations since standards were included at each run.

Allantoicase (EC 3.5.3.4) protocols described in the literature showed considerable differences. Initially, the assay described by Takada and Noguchi (Takada and Noguchi, 1986) (procedure 1) was employed. This gave acceptable results when using adult mackerel (*Scomber scombrus*) liver as a positive control, but allantoicase activity in larval *C. gariepinus* extracts was not detectable using this assay. Thereafter, an assay employing urease (Sigma U 4002) with subsequent ammonia determination was developed, but reproducible results were not obtained using this method either. A continuous assay (after Streamer, 1980) based on that of Brown et al. (Brown et al., 1966) gave acceptable results, however. Variations on this protocol have been employed previously (Wright, 1993). The assay makes use of the fact that LDH will utilize glyoxylate as a substrate and concurrently oxidize NADH. The final reaction mixture contained 50 mmol l^{-1} Hepes (pH 7.5), 50 mmol l^{-1} allantoate, 50 mmol l^{-1} KCl, 0.1 mmol l^{-1} EDTA, 0.7 % glycerol (w/w), 0.002 mg ml^{-1} trypsin inhibitor, 0.15 mmol l^{-1} NADH and

22 units ml^{-1} LDH (Boehringer-Mannheim 127 221) in a volume of 720 µl. Reactions were initiated by addition of 95 µl of extract (30 µl for adult liver). Glyoxylate standards gave a recovery of 99±12 % (mean ± S.D., *N*=54). Activity was observed as a decline in A_{340}. This assay will not give correct results unless a higher activity of ureidoglycollate lyase (UGL) than of allantoicase is present, since ureidoglycollate and one urea molecule are formed in the allantoicase reaction while glyoxylate is formed only by UGL (Takada and Noguchi, 1986). However, in the present study, UGL showed considerably higher activity than allantoicase in all samples, and the use of this assay is therefore justified.

Ureidoglycollate lyase (UGL, EC 4.3.2.3) was assayed essentially as described by Pineda et al. (Pineda et al., 1994). The final reaction mixture contained 94 mmol l^{-1} Tes (pH 7.8), 5 mmol l^{-1} Hepes (pH 7.5), 5 mmol l^{-1} KCl, 2.7 mmol l^{-1} ureidoglycollate, 0.04 mmol l^{-1} EDTA, 0.4 % glycerol (w/w), 0.002 mg ml^{-1} trypsin inhibitor, 0.5 mmol l^{-1} $MnCl_2$, 0.25 mmol l^{-1} NADH and 26 units ml^{-1} LDH (Boehringer-Mannheim 127 221) in a final volume of 799 µl. Reactions were initiated by addition of 60 µl of extract (10 µl for adult liver) and followed as the decline in A_{340}. Glyoxylate standards gave a recovery of 99±8 % (mean ± S.D., *N*=57).

OUC enzyme assays

Preliminary tests showed that product formation was linear with respect to time and extract concentrations for all OUC enzyme assays, and reactions were thereafter terminated at 0 and 60 min, except for OCTase (0, 40 min). Argininosuccinate synthase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1) were not measured because of insufficient tissue quantities. However, these enzymes are generally present in teleosts (Saha and Ratha, 1989; Felskie et al., 1998).

Carbamoyl phosphate synthetase (EC 6.3.5.5., glutamine): assays for CPSase II and III activity were conducted as described by Korte et al. (Korte et al., 1997) and Terjesen et al. (Terjesen et al., 2000). The standard reaction mixture contained, in a final volume of 300 µl: 20 mmol l^{-1} ATP, 35 mmol l^{-1} $MgCl_2$ (found to be optimal for *C. gariepinus*), 21 mmol l^{-1} phosphoenolpyruvate sodium salt, 2 units of pyruvate kinase, 5 mmol l^{-1} [^{14}C]bicarbonate (4×10^6 to 8×10^6 cts min^{-1}), 55 mmol l^{-1} Hepes (pH 7.5), 55 mmol l^{-1} KCl, 0.5 mmol l^{-1} dithiothreitol (DTT), 0.5 mmol l^{-1} EDTA and, where indicated, 20 mmol l^{-1} glutamine, 1.7 mmol l^{-1} AGA, 133 mmol l^{-1} NH_4Cl and/or 1.7 mmol l^{-1} UTP. Assays were initiated by adding 100 µl of extract.

Glutamine synthetase (GSase, E.C. 6.3.1.2) was assayed by measuring the γ-glutamyl transferase reaction (Webb and Brown, 1980) with the following modifications. Reaction mixtures contained, in a final volume of 800 µl: 40 mmol l^{-1} imidazole (pH 6.8), 28 mmol l^{-1} KCl, 0.6 mmol l^{-1} DTT, 0.3 mmol l^{-1} EDTA, 28 mmol l^{-1} Hepes (pH 7.5), 19 mmol l^{-1} potassium arsenate, 15 mmol l^{-1} hydroxylamine, 60 mmol l^{-1} glutamine, 5 mmol l^{-1} $MnCl_2$ and 0.4 mmol l^{-1} ADP. Reactions were initiated with 200 µl of extract.

Arginase (EC 3.5.3.1) reaction mixtures contained 57 mmol l^{-1} glycine (pH 9.7), 10 mmol l^{-1} Hepes (pH 7.5), 10 mmol l^{-1} KCl, 0.1 mmol l^{-1} EDTA, 0.2 mmol l^{-1} DTT, 17 mmol l^{-1} arginine and 4 mmol l^{-1} $MnCl_2$ in a total volume of 250 µl. Reactions were initiated by adding 50 µl of extract (5 µl for adult liver). After terminating the reaction with 70 µl of 2 mol l^{-1} $HClO_4$ and centrifugation at 9600 ***g*** for 10 min, urea was determined according to the method of Rahmatullah and Boyde (Rahmatullah and Boyde, 1980).

Ornithine carbamoyl transferase (OCTase, EC 2.1.3.3) was assayed essentially as described by Xiong and Anderson (Xiong and Anderson, 1989). Final reaction mixtures contained, in a total volume of 500 µl: 50 mmol l^{-1} Hepes (pH 7.5), 50 mmol l^{-1} KCl, 0.5 mmol l^{-1} EDTA, 1 mmol l^{-1} DTT, 5 mmol l^{-1} carbamoyl phosphate and 10 mmol l^{-1} ornithine. Reactions were initiated by addition of 50 µl of extract.

Gravimetry and metabolite determinations

Embryonic and larval dry mass was determined, proteins precipitated and solutes extracted as described by Terjesen et al. (Terjesen et al., 1997). Samples were centrifuged at 9600 ***g*** for 10 min, and urea in the supernatant was assayed according to the method of Rahmatullah and Boyde (Rahmatullah and Boyde, 1980). The value of moles of urea-N is equivalent to 2×moles of urea. Uric acid was determined with uricase (Sigma 292-8) (as described by Di Stefano et al., 1992) on samples homogenized in 5 % ice-cold trichloroacetic acid (TCA) (final concentration). Preliminary assays resulted in low and variable uric acid levels. To improve reproducibility, a background of 10 µmol l^{-1} uric acid was subsequently added. This modification was justified since the decline in A_{293} after addition of uricase was consistently greater in samples with added uric acid than in 10 µmol l^{-1} standards. Furthermore, the added uric acid gave the same decline in A_{293} whether dissolved in larval extracts or in pure 5 % TCA (94 % recovery), as also noted for rat liver TCA extracts (94–96 % recovery; Di Stefano et al., 1992). Uric acid levels were therefore calculated by correcting for added uric acid, the change in A_{293} of samples without uricase and the self-extinction of uricase.

Chemicals

Chemicals were of analytical grade and were purchased from Sigma Chemical Co., St Louis, MO, USA, from Merck KGa, Darmstadt, Germany, or from Boehringer-Mannheim GmbH, Mannheim, Germany. Radiochemicals were purchased from NEN Life Science Products, Belgium.

Data handling and statistical analyses

Activity is reported as µmoles of product formed or substrate consumed per minute at 26 °C, except for CPSase and uricase (nmoles of product formed or substrate consumed per minute). Michaelis–Menten constants (K_m) were calculated using non-linear curve fits to the Michaelis–Menten equation. Differences between means were evaluated by analyses of variance (ANOVAs) at a significance level of

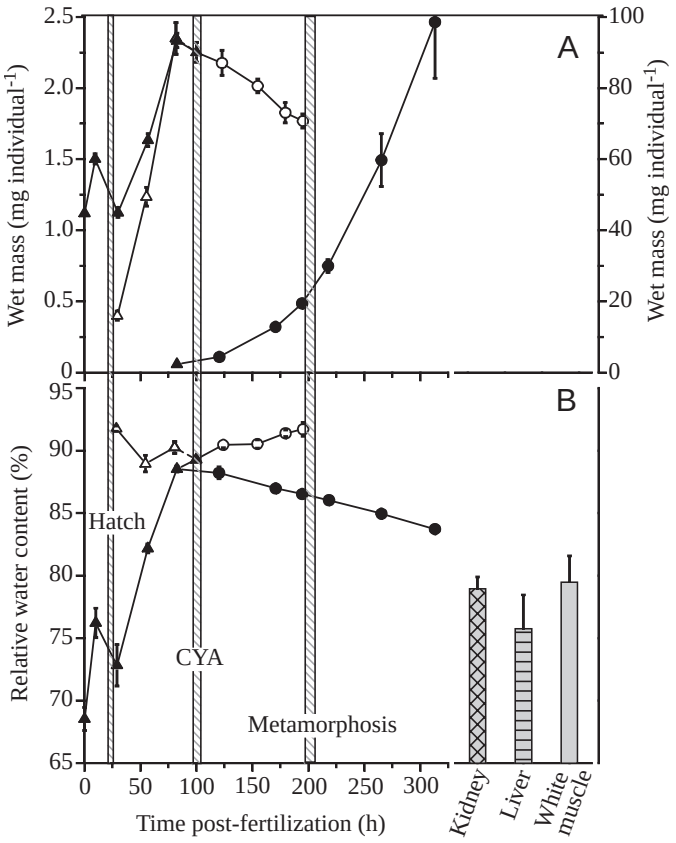

Fig. 1. Individual wet mass (A) and relative water content (B) of whole embryos and yolk-sac larvae (filled triangles), dissected larval bodies (yolk-free animal) (open triangles), starved larvae (open circles), fed larvae (filled circles) and selected adult tissues (columns) of *Clarias gariepinus*. Note that wet mass of fed larvae refers to the right-hand axis in A. Values are means ± S.D. of 8–9 samples (A) or three samples (B), except at 0 h post-fertilization (N=2, ± range). CYA, complete yolk absorption.

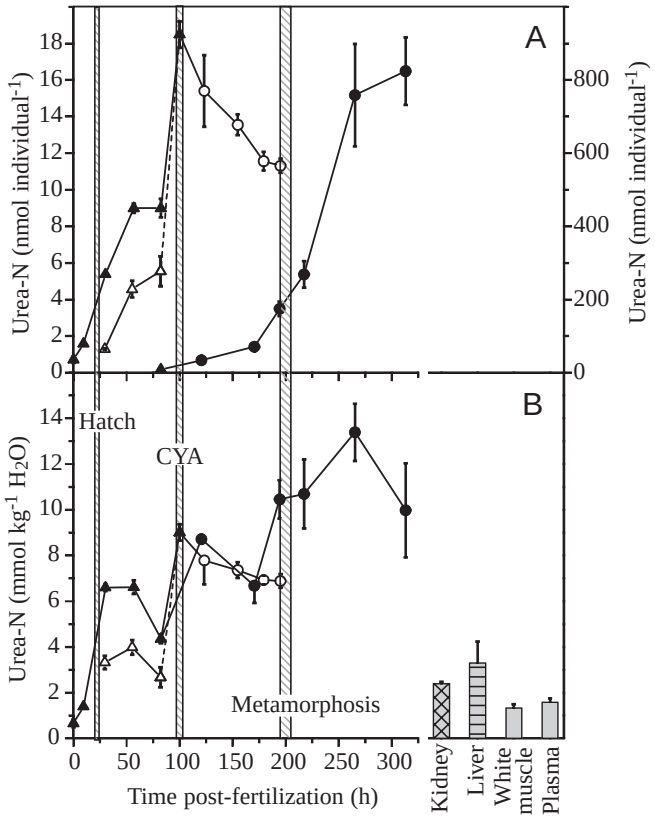

Fig. 2. Individual urea-N content (A) and concentration (B) of whole embryos and yolk-sac larvae (filled triangles), dissected larval bodies (yolk-free animal) (open triangles), starved larvae (open circles), fed larvae (filled circles) and selected adult tissues (columns) of *Clarias gariepinus*. Note that urea-N content of fed larvae refers to the right-hand axis in A. Values are means ± S.D. of three samples. CYA, complete yolk absorption.

P<0.05. When appropriate, Tukey's multiple-comparison test (Zar, 1984) was subsequently employed. Results are reported as means ± S.D., except where noted.

Results

Gravimetry and metabolite contents

The larvae hatched between 20 and 24 h post-fertilization, and complete yolk absorption (CYA) occurred around 100 h post-fertilization. Wet mass and relative water content increased up to CYA, while the fed larvae gained weight exponentially until the experiment was terminated at 314 h post-fertilization (Fig. 1). Air-breathing behavior commenced at approximately 200 h post-fertilization. Urea-N levels increased during development up to CYA (P<0.001, N=33), but declined during starvation (Fig. 2). An especially rapid increase was found close to CYA when the urea-N content doubled over a period of only 18 h (Fig. 2A). This increase was not due to a change in wet mass, which was stable during this period (Fig. 1A). Urea-N was located mainly in the yolk at the first sampling point after hatching at a concentration of 9.1 mmol kg^{-1} H$_2$O (calculated from data in Fig. 1 and Fig. 2). At the next two sampling points, the larval body contained more urea-N (51 % and 62 % of total, respectively) than did the yolk. Urea-N was calculated to be present in yolk at a level of 19 mmol kg^{-1} H$_2$O at 55 h post-fertilization. In fed larvae, tissue concentrations of urea-N fluctuated between 10 and 13 mmol kg^{-1} H$_2$O, considerably higher than in adults in which, depending on the tissue, concentrations of 1.3–3.3 mmol kg^{-1} H$_2$O were measured. Uric acid was detected at variable levels in extracts of *C. gariepinus* larvae. Starved larvae (100–155 h post-fertilization) had a uric acid concentration of 31±16 mmol kg^{-1} H$_2$O (N=3), while fed larvae (170–313 h post-fertilization) had a concentration of 59±7 mmol kg^{-1} H$_2$O (N=4).

Uricolytic enzymes

Whole-animal uricase activity was low, but detectable, in embryonic samples, and activities increased up to CYA (P<0.001, N=16, Fig. 3A). Starved larvae showed higher uricase activity at all sampling points compared with endogenously feeding larvae, even when adjusted for yolk mass

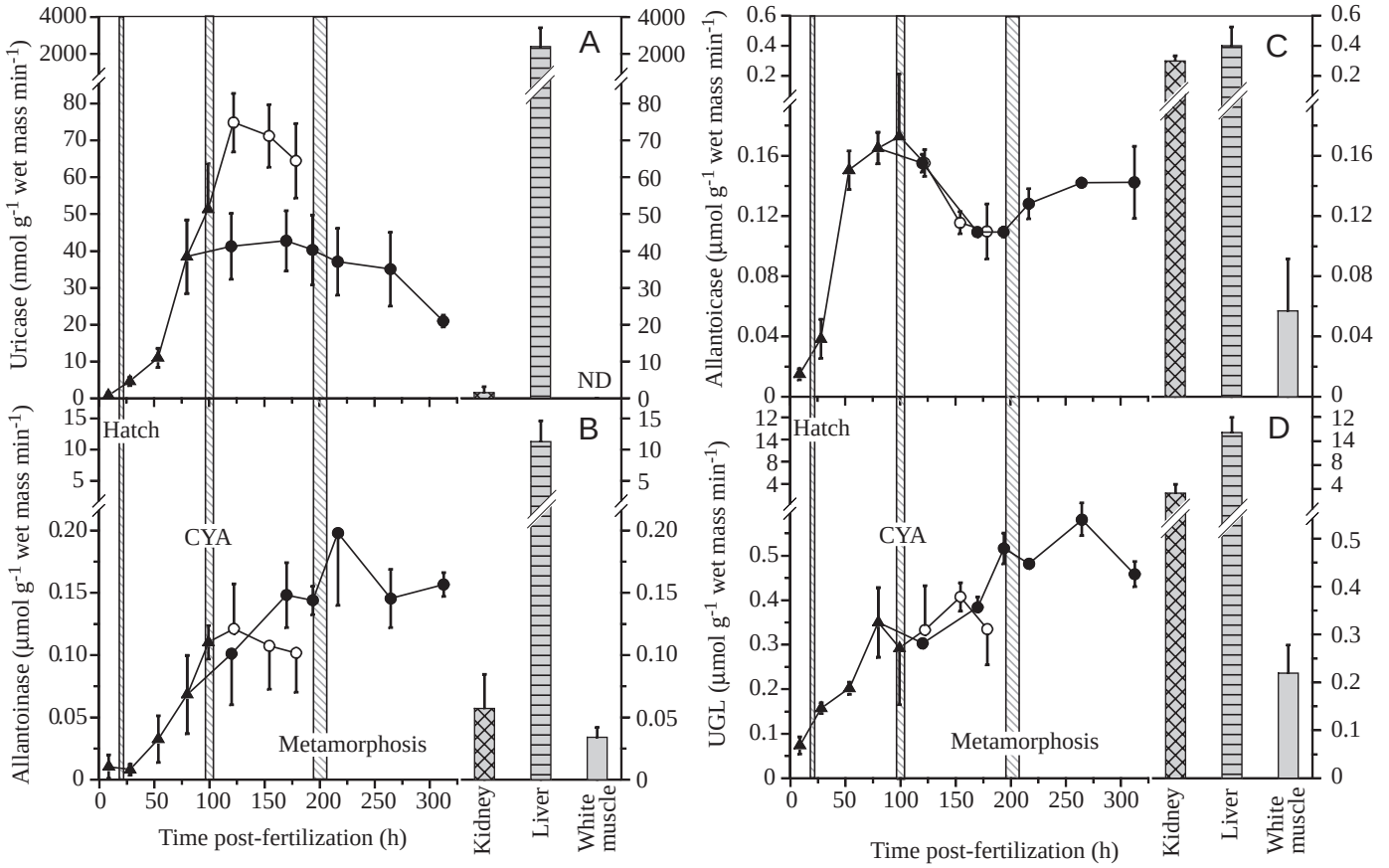

Fig. 3. Uricase (A), allantoinase (B), allantoicase (C) and ureidoglycollate lyase (UGL, D) activity in whole-animal crude extracts during early development and in selected adult tissues (columns) of *Clarias gariepinus*. Filled triangles, whole embryos and yolk-sac larvae; filled circles, larvae fed *Artemia*; open circles, starved larvae. Values are means ± S.D. of assays on 3–6 samples for uricase and allantoinase. Allantoicase and UGL activity are the means ± S.D. of three samples, except at 54, 99, 120, 170 194, 217 and 313 h post-fertilization for fed larvae (N=2, ± range). CYA, complete yolk absorption; ND, not detectable.

(P<0.001, N=30, data not shown). In fed larvae, uricase activity was stable between 119 and 264 h post-fertilization, but uricase activity was significantly lower at 313 h post-fertilization (P<0.05), except when compared with 264 h post-fertilization. Both allantoinase (Fig. 3B) and allantoicase (Fig. 3C) activity were detected at the embryonic stage. After hatching, allantoicase activity increased by a factor of 4 during the short period between 28 and 53 h post-fertilization (Fig. 3C). UGL displayed the highest *in vitro* activity of the uricolytic enzymes during early development (Fig. 3D). Allantoinase and UGL activities increased up to metamorphosis (P<0.01, N=32), when stable levels were attained. For all uricolytic enzymes, activities were generally found to follow the same pattern with age irrespective of how the activity was calculated (i.e. whether based on wet mass, dry mass or per individual), except when calculating activities per individual fed larva (because of their large individual mass). Adult *C. gariepinus* tissues, especially liver, generally showed higher uricolytic activities than whole-animal larval extracts. Liver had the highest activities of all uricolytic enzymes in adult *C. gariepinus*, except allantoicase for which kidney and liver activity did not differ (P=0.32, N=6). Uricase activity was not observed in adult muscle, in contrast to the other uricolytic enzymes which were clearly detectable in this tissue.

OUC enzymes

Both CPSase II (pyrimidine biosynthesis) and CPSase III (OUC) were detected in *C. gariepinus* larvae by subjecting extracts to gel filtration chromatography (Fig. 4; 313 h post-fertilization). Two peaks with CPSase activity were obtained, the second peak having the higher activity, corresponding to 1.1 nmol g^{-1} wet mass min^{-1}. The second activity peak showed significant activation by AGA, low inhibition by UTP, negligible activity with NH_4Cl as a substrate and a molecular mass of 150–160 kDa, all characteristics of CPSase III (Table 1). In contrast, the first activity peak was presumably a CPSase II because of its lower elution volume and contrasting characteristics compared with those of the second peak (Table 1). The effect of varying glutamine concentrations on the second activity peak (Fig. 5) shows that this CPSase III has virtually no activity (3–6 %) using standard assay conditions in the absence of its allosteric effector AGA. The apparent K_m for glutamine in the presence of 1.7 mmol l^{-1} AGA was 0.13 mmol l^{-1} but, because of the high dependence on AGA,

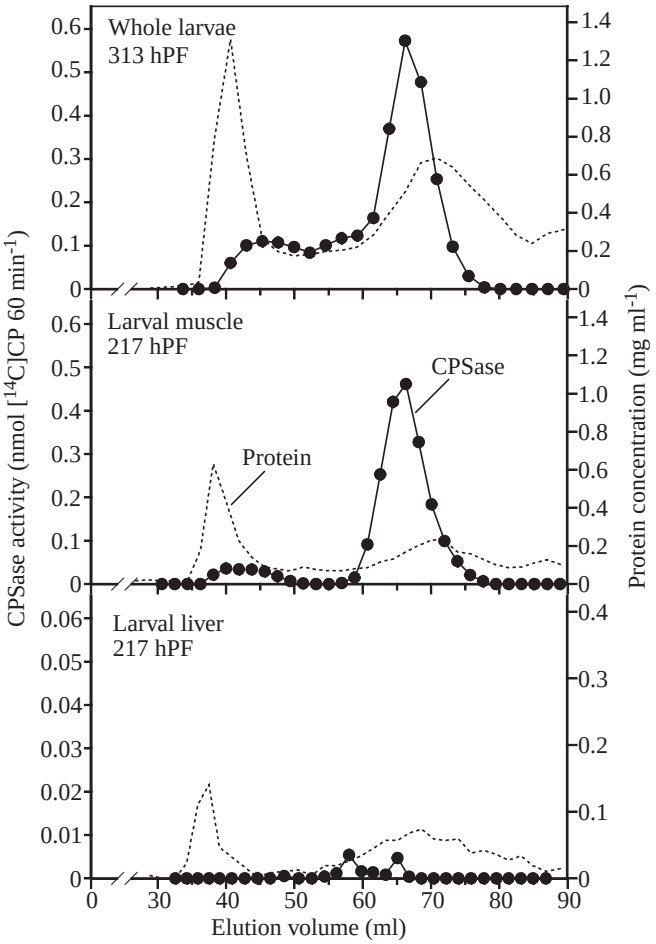

Fig. 4. Elution profile of fed *Clarias gariepinus* larval extracts subjected to gel filtration chromatography on a Sephacryl S300HR column and assayed for carbamoyl phosphate synthetase (CPSase) activity as described in the text. Note differences in scaling of the axes for the larval liver elution. Whole larvae (313 h post-fertilization) elution: flow rate 0.31 ml min^{-1}, sample volume 3.5 ml, protein concentration 7.3 mg ml^{-1}. Larval muscle tissue (217 h post-fertilization): flow rate 0.30 ml min^{-1}, sample volume 2.4 ml, protein concentration 3.9 mg ml^{-1}. Larval liver tissue (217 h post-fertilization): flow rate 0.33 ml min^{-1}, sample volume 1.9 ml, protein concentration 1.6 mg ml^{-1}. hPF, hours post-fertilization; CP, carbamoyl phosphate.

activities were too low to estimate K_m in the absence of this effector.

The tissue distribution of CPSase III in fed *C. gariepinus* larvae was determined by gel-filtration chromatography (Fig. 4; muscle and liver, 217 h post-fertilization). The muscle elution showed two activity peaks, an almost identical elution profile to that of whole larvae at 313 h post-fertilization. The second muscle activity peak eluted at 157 kDa and had a total activity of 1.8 nmol g^{-1} wet mass min^{-1}, as calculated by integration. In contrast, elution of dissected liver extracts resulted in trace levels of activity. Even if these levels represent CPSase III, the total activity calculated by integration of the curve is only 0.05 nmol g^{-1} wet mass min^{-1} or 3 % of the activity in muscle.

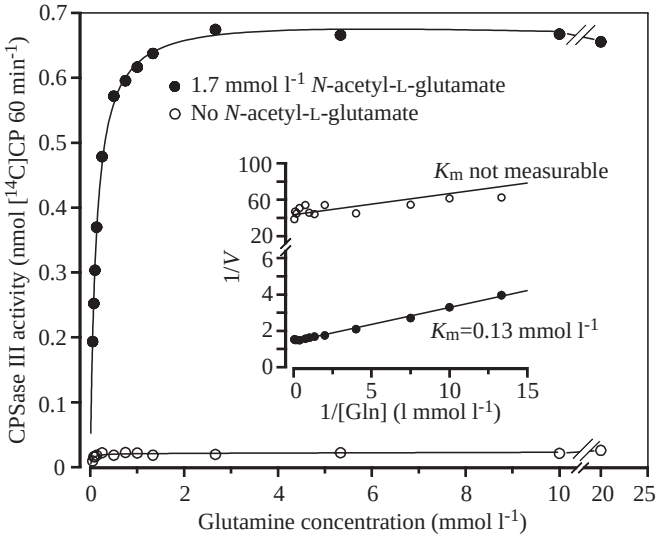

Fig. 5. Effects of varying glutamine (Gln) concentration on carbamoyl phosphate synthetase III (CPSase III) activity in *Clarias gariepinus* larval extracts (313 h post-fertilization) with or without *N*-acetyl-L-glutamate (AGA) present in the reaction mixture. The enzyme was obtained from gel chromatography (Fig. 4, whole larvae) by pooling fractions from the second peak having more than 60 % of maximum peak activity (protein concentration 0.59 mg ml^{-1}). Assays were conducted using standard conditions as described in the text, except as noted. The inset is a reciprocal plot of activity (*V*) *versus* substrate concentration. Because of the high dependence on AGA of this CPSase III, activity was too low and variable to estimate K_m in the absence of the effector. CP, carbamoyl phosphate.

Table 1. *Properties of the CPSase activities in the two peaks obtained by gel filtration chromatography of whole larval extracts (313 h post-fertilization; see Fig. 4)*

	Activity (pmol [^{14}C]CP min^{-1})	
Assay condition	Peak 1	Peak 2
No substrate	0.02	ND
Gln	1.47	0.31
Gln+AGA	1.50	9.46
% AGA activation	2	2952
Gln+AGA+UTP	0.14	8.02
% UTP inhibition	91	15
NH_4Cl+AGA	0.90	0.28
% NH_4Cl *versus* Gln	60	3

CPSase, carbamyl phosphate synthetase.

Values are the average of two replicates.

AGA (*N*-acetyl-L-glutamate) activation represents the percentage increase in activity when AGA was added to reaction mixture containing Gln (glutamine).

UTP (uridine triphosphate) inhibition was calculated as the percentage decline in activity when UTP was added to reaction mixture containing Gln+AGA.

CP, carbamoyl phosphate; ND, not detectable.

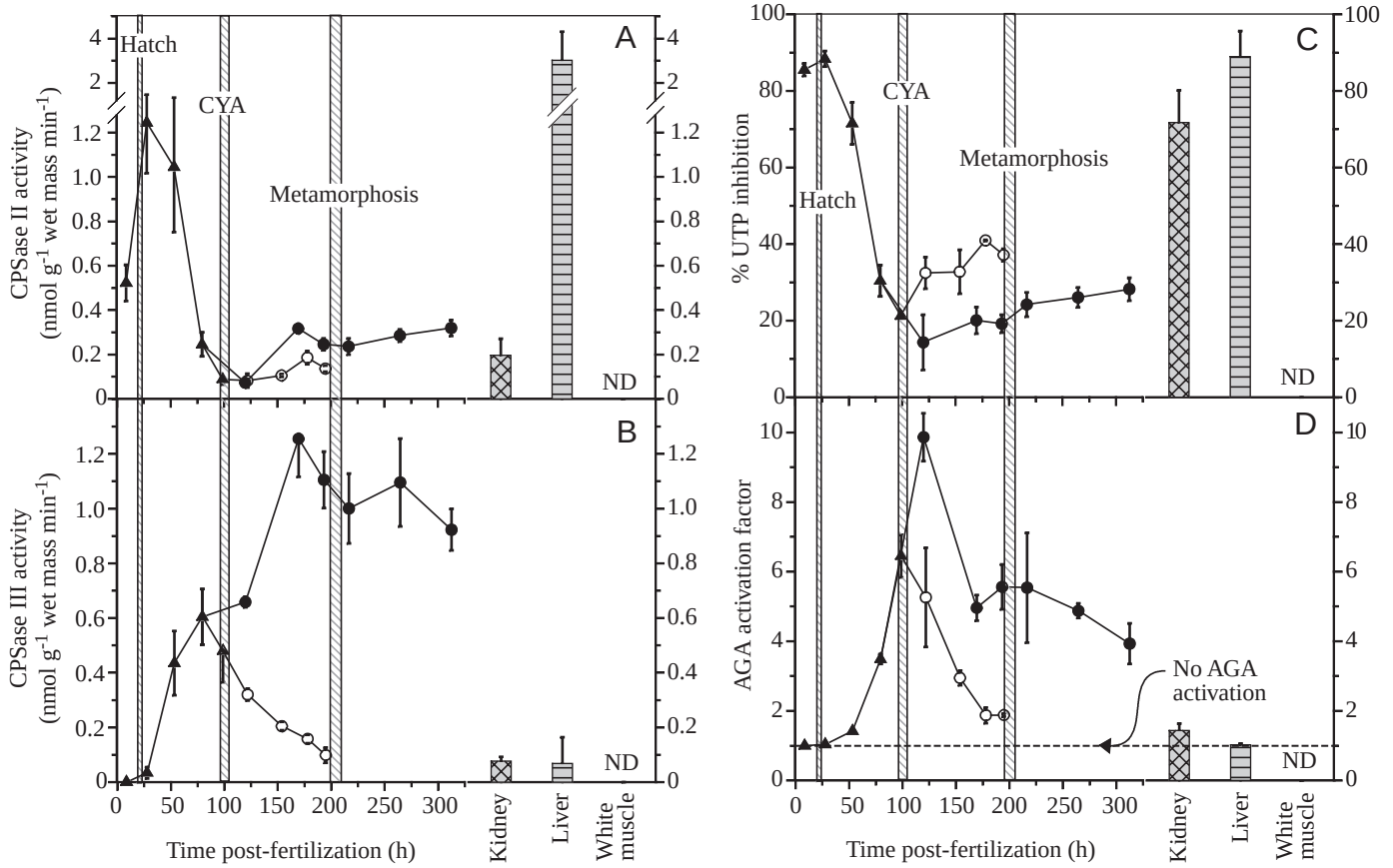

Fig. 6. Carbamoyl phosphate synthetase II (CPSase II) (A) and CPSase III (B) activity in whole-animal crude extracts during early development and in selected adult tissues (columns) of *Clarias gariepinus*. Filled triangles, whole embryos and yolk-sac larvae; filled circles, larvae fed *Artemia*; open circles, starved larvae.Values are means ± S.D. of assays on 3–4 samples, except for fed larvae at 79 and 119 h post-fertilization (*N*=2, ± range). CPSase II activity was calculated from the rate obtained with glutamine (Gln) only in the reaction mix, while CPSase III was calculated from the rate with Gln + *N*-acetyl-L-glutamate (AGA) minus the rate with Gln only. The relative capacities of the two CPSases in total carbamoyl phosphate formation *in vitro* are shown in C (% UTP inhibition calculated as described in Table 1, high values: mostly CPSase II) and D (AGA activation factor, rate with Gln + AGA/rate with Gln only, high values: mostly CPSase III). CYA, complete yolk absorption; ND, not detectable.

In whole-animal measurements, CPSase II showed higher activity during the embryonic and early yolk-sac larval stages than in the later stages (Fig. 6A). CPSase III was detectable in crude extracts throughout the experiment (Fig. 6B), but activity was far lower than for CPSase II during the embryonic and early yolk-sac stages, as illustrated by the high degree of inhibition by UTP (Fig. 6C) and lack of activation by AGA (Fig. 6D) during this period. Following hatching, CPSase III activity increased to a maximum at 79 h post-fertilization (Fig. 6B), but declined in starved larvae (P<0.001, N=16). In fed larvae, CPSase III was the dominant form and showed highest activity at 169 h post-fertilization (1.3 nmol g^{-1} wet mass min^{-1}). At 313 h post-fertilization, CPSase III activity in whole-animal crude extracts was 0.9 nmol g^{-1} wet mass min^{-1}, which is comparable with that measured using gel filtration chromatography (1.1 nmol g^{-1} wet mass min^{-1}; Fig. 4). In contrast, the activity in muscle calculated from activity eluted after gel filtration chromatography (Fig. 4; 217 h post-fertilization) was higher than in whole-animal homogenates (1.8 *versus* 1.0 nmol g^{-1} wet mass min^{-1}). With only minor changes, the reported CPSase activities followed the same trend with age irrespective of how they were calculated (except per individual fed larva). Assuming that yolk does not contain CPSase, fed larvae also had a higher CPSase III activity than yolk-sac larval bodies (data based on dry mass, P<0.001, N=25). Adult liver had a high CPSase II activity, but contained low and variable levels of CPSase III (Fig. 6A,B). Muscle tissue did not show detectable CPSase activity. Kidney extracts of adult *C. gariepinus*, however, showed a slight activation by AGA and incomplete inhibition by UTP, suggesting that a low level of CPSase III activity is present in kidney tissue.

The activities of the other assayed OUC enzymes generally increased with age when results were expressed on a whole-animal mass-specific basis (Fig. 7). It is noteworthy that, during the period of increased CPSase III expression in fed larvae (Fig. 6B), a similar increase in

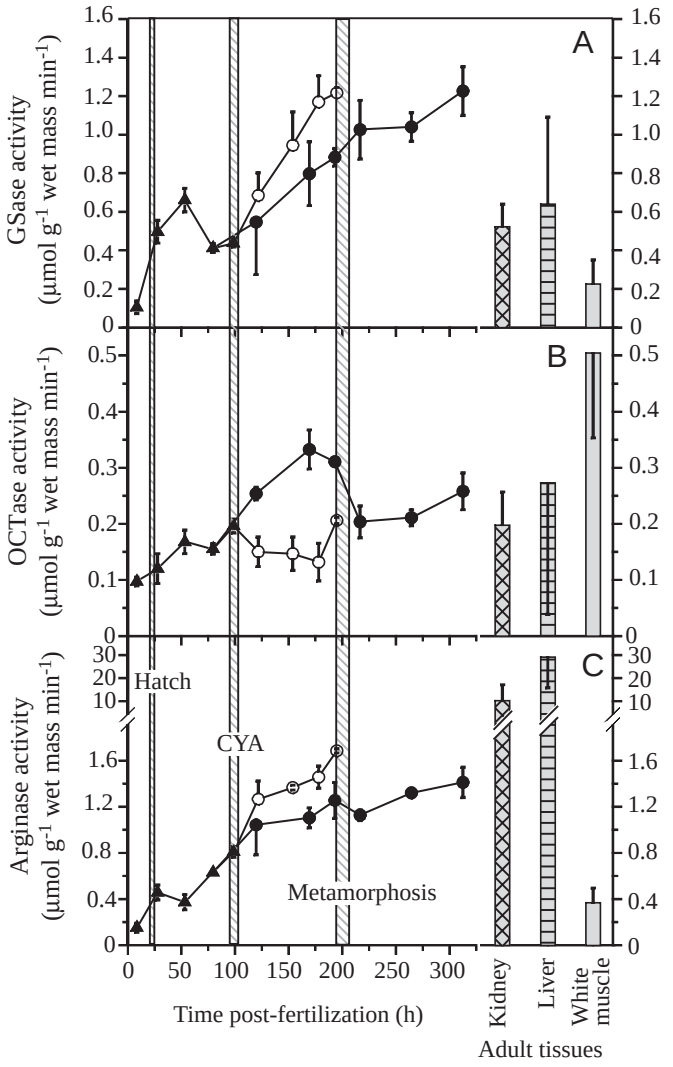

Fig. 7. Glutamine synthetase (GSase, A), ornithine carbamoyl transferase (OCTase, B) and arginase (C) activity in whole-animal crude extracts during early development and in selected adult tissues (columns) of *Clarias gariepinus*. Filled triangles, whole embryos and yolk-sac larvae; filled circles, larvae fed *Artemia*; open circles, starved larvae. Values are means ± S.D. of assays on 3–4 samples, except for fed larvae at 79 and 119 h post-fertilization (N=2, ± range). CYA, complete yolk absorption.

OCTase activity was also observed (Fig. 7B). GSase and arginase activities were higher in starved larvae than in fed larvae (P<0.01, N=18), irrespective of how activities were calculated (except μmol min^{-1} individual^{-1} because of the larger mass of fed larvae). The activities of the OUC enzymes varied among individuals of adult *C. gariepinus* (Fig. 7). Pooled data did not show significant differences between tissues in OCTase and GSase expression; within the same individual, however, activities tended towards higher expression of OCTase in muscle and of GSase in liver. Arginase was clearly expressed at highest levels in liver (P<0.05, N=9).

Discussion

The present study demonstrates that all three pathways for urea production, argininolysis, uricolysis and the OUC, are expressed during early development of *C. gariepinus*. Furthermore, the present finding (Fig. 2) that urea accumulates in *C. gariepinus* larvae also suggests that the relatively high rates of urea excretion observed previously (Terjesen et al., 1997) represent metabolically produced urea. When the excretion data of Terjesen et al. (Terjesen et al., 1997) are employed, it can be estimated that urea accumulation accounts for less than 10 % of total urea production (urea-N accumulation + excretion). Nevertheless, urea-N concentrations in fed *C. gariepinus* larvae are higher than in adult *C. gariepinus* tissues (Fig. 2B), and two- to 10-fold higher than in rainbow trout and Atlantic cod larvae at first-feeding (Wright et al., 1995; Chadwick and Wright, 1999).

This is the first study to report expression of the uricolytic enzymes in early life stages of a teleost. Recently, Vigetti et al. (Vigetti et al., 2000) showed that, in *Xenopus laevis* embryos, allantoicase mRNA is present from 24 h post-fertilization. Furthermore, xanthine dehydrogenase, the enzyme preceding uricase in purine catabolism, has recently been detected in zebrafish embryos (*Danio rerio*; Ziegler et al., 2000). In endogenously feeding *C. gariepinus* embryos and yolk-sac larvae, it would appear energetically reasonable to salvage purines originating from nucleic acid turnover instead of breaking them down through uricolysis. Possibly, in the yolk-sac larvae, the uricolytic pathway is expressed in preparation for nucleotide absorption at first-feeding, when the pathway can serve to degrade purines in excess of requirements for nucleic acid synthesis, the generally accepted role for uricolysis in adult teleosts (Wood, 1993). Measurements of purine salvage capacity in endogenously feeding stages of *C. gariepinus* could serve to test this hypothesis.

Uricase activity per individual is higher in *C. gariepinus* larvae at 123 h post-fertilization (starved larvae) than in yolk-sac larvae close to CYA at both 70 and 99 h post-fertilization (P<0.01, N=14). Since the larval body compartments at these sampling points were of similar size (Fig. 1A), these results suggest that uricase is induced during starvation, possibly as a response to increased nucleotide breakdown. Interestingly, Rumsey et al. (Rumsey et al., 1991) found that liver uricase activity was correlated with dietary nucleic acid content in adult rainbow trout. However, the higher uricase activity during starvation of *C. gariepinus* larvae is not reflected in elevated urea production *in vivo* during this period (Terjesen et al., 1997), suggesting either that variations in uricase expression do not solely control uricolytic flux or that changes in other urea-producing pathways had masked any increased urea production through uricolysis. A similar relationship exists in adult tilapia (*Oreochromis niloticus*): tilapia fasted for 14 days had higher levels of liver uricase activity than fed fish even though urea excretion rates were much lower in the fasted group (Wright, 1993).

The eluted CPSase III muscle peak at 217 h post-fertilization (Fig. 4) showed a considerably higher total activity than

whole-animal extracts at 217 h post-fertilization (Fig. 6), while the larval liver elution showed only trace levels of activity (Fig. 4). Although protein levels were low in the larval liver elution, it can be calculated that a detectable activity peak would still elute if CPSase III were expressed to any significant extent in this tissue. Thus, CPSase III is predominantly expressed in muscle of larval *C. gariepinus*, as has been reported in adults of several species (Korte et al., 1997; Kong et al., 1998; Lindley et al., 1999; Terjesen et al., 2000). Larval *C. gariepinus* CPSase III showed considerable dependence on AGA in the reaction mixture for maximal activity, even at very high glutamine concentrations (Fig. 5), and this contrasts with the situation in Atlantic halibut larvae in which AGA has less influence on activity (Terjesen et al., 2000). It appears that *C. gariepinus* CPSase III has nearly as high a dependence on AGA as mammalian CPSase I, for which activity in the absence of AGA is only 3 % (Rubio et al., 1983). In view of this observation, short-term regulation of carbamoyl phosphate formation in *C. gariepinus* larvae may be influenced by changes in AGA concentrations as a result of differences in protein or amino acid absorption, as in mammals (Mejer, 1995) and as suggested for adult Gulf toadfish (*Opsanus tau*, Julsrud et al., 1998).

Several authors have suggested that expression of the OUC during early life stages of teleosts serves as a safeguard to control internal ammonia levels (Griffith, 1991; Wright et al., 1995; Korsgaard et al., 1995). Even though ammonia is predominantly (98 %) excreted, ammonia concentrations nevertheless increase during the early yolk-sac stage in *C. gariepinus*, despite the small size of these larvae (Terjesen et al., 1997). Although *in vivo* tracer studies are required to establish whether ammonia detoxification occurs in yolk-sac larvae of *C. gariepinus*, the potential for urea synthesis from ammonia does exist concurrent to this ammonia accumulation since GSase, CPSase III, OCTase and arginase were expressed during this period (Fig. 6, Fig. 7). Adult *C. gariepinus*, however, had only low and variable levels of CPSase III activity (Fig. 6), suggesting that the expression of OUC enzymes is influenced by developmental stage and, further, that any urea produced by adult *C. gariepinus* under the experimental conditions employed here is produced predominantly through uricolysis and/or argininolysis. In contrast, adults of its Asian relative *C. batrachus* had high activities of the OUC enzymes even in the absence of environmental challenges (Saha and Ratha, 1989; Saha et al., 1999). Still, *C. gariepinus* adults must also have a mechanism to detoxify or eliminate ammonia efficiently, given their high LC_{50} at 96 h of 380 μmol l^{-1} NH_3 (Britz, 1988; Oellermann, 1995) and their ability to survive for 2 months in mud (Donnely, 1973).

The present study has shown that the enzymes necessary for urea production through argininolysis (arginase), through the uricolytic pathway or through the OUC are expressed during the early life stages of *C. gariepinus*. Field studies of variables related to ammonia excretion, such as pH and ammonia levels, could further reveal whether OUC expression during the early life stages of *C. gariepinus* constitutes an adaptive mechanism to the fluctuating availability of water at the spawning sites (Greenwood, 1955).

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