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Dogmas and controversies in the handling of nitrogenous wastes: Expression of arginase Type I and II genes in rainbow trout: influence of fasting on liver enzyme activity and mRNA levels in juveniles

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Summary

Through analysis of a cDNA library and third-party annotation of available database sequences, we characterized the full-length coding regions of rainbow trout (*Oncorhynchus mykiss*) Type I, *Onmy*-ARG01, and Type II, *Onmy*-ARG02, arginase genes. Two partial related arginase sequences, *Onmy*-ARG01b and *Onmy*-ARG02b, and a full-length zebrafish arginase coding region (*Danio rerio*), *Dare*-ARG02, are also reported. Comparison of vertebrate arginase sequences shows that both Type I and Type II genes in bony fishes contain a mitochondrial targeting N-terminal domain. This suggests that the cytosolic Type I arginase found in ureotelic vertebrates arose in the common ancestor of amphibia and mammals. *Onmy*-ARG01 and *Onmy*-ARG02 mRNA was detected in liver, kidney, gill, intestine, red muscle and heart tissues. *Onmy*-ARG01 was expressed at a significantly higher level relative to *Onmy*-ARG02 in liver and red muscle tissue. To investigate whether there was differential regulation of *Onmy*-ARG01 and *Onmy*-ARG02, juvenile trout were fasted for 6 weeks and hepatic enzyme activities and mRNA levels were compared with those of fed control fish. There was a 3-fold increase in liver arginase activity and a 2-fold increase in *Onmy*-ARG02 mRNA levels but no change in *Onmy*-ARG01 mRNA levels in fasted fish relative to fed fish. These findings indicate that both types of arginase genes are present and expressed in rainbow trout and that the pattern of expression varies between tissues. The increase in liver arginase activity after a 6-week fast is due, in part, to an increase in the expression of *Onmy*-ARG02 mRNA levels.

Key words: ornithine–urea cycle, arginine, urea, nitrogen metabolism, mitochondrial targeting, *Oncorhynchus mykiss*.

Introduction

Arginase (EC 3.5.3.1), found in all five kingdoms of organisms, catalyses the conversion of arginine to urea and ornithine (Hird, 1986). Most microorganisms and the two invertebrates studied to date (*Caenorhabditis elegans* and *Drosophila*) have only one type of arginase, whose function is unrelated to the ornithine–urea cycle (OUC; Samson, 2000). There are two forms of arginase in terrestrial ureotelic vertebrates encoded by separate genes (Spector et al., 1985; Grody et al., 1989). Arginase Type I (ARG01) is a cytosolic enzyme (Ikemoto et al., 1990) that anchors the hepatic OUC [carbamoyl phosphate synthetase (CPSase), ornithine carbamyl transferase (OTCase), argininosuccinate synthetase, argininosuccinate lyase and arginase]. Arginase Type II (ARG02) is mitochondrial, is more highly expressed in nonhepatic tissues and its function is less well understood (eg. Gotoh et al., 1996; Morris et al., 1997; Miyanaka et al., 1998; Iyer et al., 1998). It has been suggested that Type II arginase could be most important for the synthesis of ornithine, a precursor for polyamines, glutamate and proline (for review, see Jenkinson et al., 1996), although it may also regulate the availability of arginine for nitric oxide synthase and, in turn, regulate nitric oxide-dependent apoptosis (Gotoh and Masataka, 1999). The appearance of two distinct arginase genes in *Xenopus* and mammals is thought to be due to a gene duplication event that occurred before the divergence of mammals and amphibians from their most recent common ancestor (Patterton and Shi, 1994; Jenkinson et al., 1996; Perozich et al., 1998). To our knowledge, there is no information in the literature on whether one or two arginase genes exist in fish, although the availability of zebrafish (*Danio rerio*) expressed sequence tag (EST) and pufferfish (*Takifugu rubripes*) genome sequences should facilitate the search for answers.

Arginase is ubiquitous in fish tissues, with highest activities found in liver and kidney tissue (Cvancara, 1969; Portugal and Aksnes, 1983; Singh and Singh, 1988; Korte et al., 1997;

Felskie et al., 1998). Despite this, only a handful of adult teleost species is known to have a functional OUC (Randall et al., 1989; Saha and Ratha, 1998; Walsh, 1997; reviewed by Anderson, 2001), although the OUC is present in the more ancient fish lineages (i.e. elasmobranchs and the coelacanth; Griffith, 1991; Anderson, 1991; Walsh and Mommsen, 2001). Urea synthesis occurs early in teleost development (Kaushik et al., 1982; Wright et al., 1995; Pilley and Wright, 2000), and radiolabeled HCO_3^-, a substrate for the OUC, is incorporated into urea in rainbow trout (*Oncorhynchus mykiss*) embryos (Dépêche et al., 1979). The key piscine OUC enzyme, CPSase III, along with OTCase, arginase and the accessory enzyme glutamine synthetase, is induced early in the development of rainbow trout and other teleost species, although CPSase III is absent in adult liver tissue (Wright et al., 1995; Terjesen et al., 1998, 2001; Chadwick and Wright, 1999; Steele et al., 2001; Todgham et al., 2001). Thus, the OUC appears to be functional early in development but, of the OUC enzymes, only arginase activities remain high in later life stages.

The developmental pattern of OUC enzyme activity raises the question of whether one or two arginase genes are expressed in rainbow trout. Adult trout arginase activity is localized to the mitochondria (Mommsen and Walsh, 1989; Korte et al., 1997), as are other fish arginases (Casey and Anderson, 1985; Carvajal et al., 1987; Mommsen and Walsh, 1989; Anderson and Walsh, 1995; Felskie et al., 1998). In the present study, we hypothesize that the arginase gene or genes in trout share more homology with the mammalian mitochondrial arginase Type II rather than the cytosolic Type I gene. Very little was known about fish arginase genes at the onset of this project. The only sequences available were those from the zebrafish EST library, indicating the presence of one arginase gene with sequence similarity to the mammalian mitochondrial Type II arginase. The question is further complicated by the fact that salmonids are tetraploid, and recent studies of trout glutamine synthetase genes revealed four distinct genes (Murray et al., 2003). In the present study, we used PCR cloning techniques and database searches to determine the complete coding regions of one arginase Type I gene (*Onmy*-ARG01) and one arginase Type II gene (*Onmy*-ARG02). In addition, the partial coding regions of two similar genes were also determined (*Onmy*-ARG02b and *Onmy*-ARG01b, respectively). Using northern blot analysis, we determined the pattern of expression of *Onmy*-ARG01 and *Onmy*-ARG02 in several adult trout tissues.

Our second aim was to investigate whether the two trout arginase genes were differentially regulated in response to dietary manipulation. In various teleost fishes, liver arginase activity is induced following several weeks of fasting (Chiu et al., 1986; Jürss et al., 1987; Singh and Singh, 1988; Wright, 1993). Increased arginase activity may be important in amino acid catabolism in fasting fish (Ballantyne, 2001; Wood, 2001). Alternatively, arginase may be induced during starvation to enhance ornithine synthesis, modulate nitric oxide production or modulate related pathways (see above). Based on the similarity between *Onmy*-ARG01 and mammalian Type I arginase (hepatic OUC-related) and between *Onmy*-ARG02 and mammalian Type II arginase (nonhepatic), we hypothesized that only *Onmy*-ARG02 expression would be enhanced in the livers of fasted juvenile trout where the OUC is non-functional. We reproduced the protocol used by Chiu et al. (1986), where juvenile trout were either fed or fasted for a 6-week period. Livers were collected at 0 and 6 weeks, and arginase activities, as well as *Onmy*-ARG01 and *Onmy*-ARG02 mRNA levels, were compared between fasted and fed fish.

Materials and methods

Animals

Juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum; 20–30 g) of both sexes were obtained from the Alma Aquaculture Station (Alma, ON, Canada). The fish were held in the Aqualab facility at the University of Guelph (photoperiod 12 h:12 h L:D, pH 7.9; water hardness 411 mg l^{-1} as $CaCO_3$; Ca^{2+}, 5.24 mequiv l^{-1}; Cl^-, 1.47 mequiv l^{-1}; Mg^{2+}, 2.98 mequiv l^{-1}; K^+, 0.06 mequiv l^{-1}; Na^+, 1.05 mequiv l^{-1}) for 2 weeks prior to experimentation. Fish were randomly divided into four groups [two control tanks, two experimental (fasted) tanks] and placed in 125-litre tanks supplied with continuous flow-through water (total ammonia concentration in control or experimental tanks was <1 μmol l^{-1}). Fish were fed trout pellets (Martin's Feed Mills, Elmira, ON, Canada) at 1.8% of their body mass twice a day for 5 days each week. Water temperature was 10–12°C.

Cloning

The polymerase chain reaction (PCR) was used to amplify the arginase gene from a primary rainbow trout cDNA library constructed from mRNA isolated from the combined gill and kidney tissues of 12 trout (1×10^9 PFU ml^{-1}; S. F. Perry, personal communication). The initial degenerate primer combinations used, based on known vertebrate arginase sequences, were ARG*e5f1 (5′-TTGGG CTTAG AGAYG TGGAY C) and ARG*e7r1 (5′-GGGTC CAGNC CATCA ATGTC AAA) or ARG*e7r2 (5′-CCTTC TCTGT ARGTN AGTCC TCC). To obtain further sequence information of the *Onmy*-ARG02 transcript, we employed anchored PCR using the vector-specific primers pBKCMV*r1 and pBKCMV*f1 (Murray et al., 2003) in combination with either *Onmy*-ARG02 specific reverse primers, ARG*e7r1 or ARG-Onmy*e6r2 (5′-TGGTC GAAAG AGACT TCCAT G), or the forward primer ARG-Onmy*e5f2 (5′-TGAAG GACTT GGGTG TCCAG). PCR reactions were conducted on a PTC-150 MiniCycler™ thermal cycler (MJ Research Inc., Incline Village, NV, USA). The thermal profile consisted of an initial cycle of 95°C for 5 min, 55–60°C for 30 s, and 72°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55–60°C for 30 s, 72°C for 1.5 min, and finished with an elongation of the 72°C step for 7.5 min. Reaction volumes were 25 μl, consisting of 1.5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} dNTPs, 1× reaction buffer, 0.2 μmol l^{-1} of each primer and 1 unit of HotStar *Taq* polymerase (Qiagen

Inc., Mississauga, ON, Canada). The PCR products were separated by gel electrophoresis, isolated using a QIAGEN QIAquick gel extraction kit (Qiagen Inc.) and cloned into pGEM-T Easy Vector system (Promega Corp., Madison, WI, USA). Plasmid inserts were sequenced using an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Foster City, CA, USA).

Analysis of EST and pufferfish genomic databases

The GenBank EST database (http://ncbi.nlm.nih.gov/) was searched for all rainbow trout, zebrafish and pufferfish entries with similarity to the deduced amino acid sequences of the identified trout arginase genes through tBlastn searches. Resulting EST sequences were then analysed as described below to establish sets of contiguous sequence information. In a similar manner, the draft sequence of the pufferfish genome (release #3; http://fugu.hgmp.mrc.ac.uk/) was searched for scaffolds containing arginase genes. Putative coding regions were extracted from the genomic sequence scaffolds.

Sequence analysis

DNA sequence was edited and contiguous sequences were assembled using the Sequencher 4.1.2 program (Gene Codes Corp., Ann Arbor, MI, USA). Alignments were constructed using ClustalW (Thompson et al., 1994) contained within the MacVector 7.0 program (Oxford Molecular Group). Gene phylogenies were estimated from a DNA alignment of the coding sequence using the maximum likelihood method of the computer program Paup 4.0b10 (Sinauer Associates Inc., Sunderland, MA, USA). Three hundred bootstrap trees were constructed to estimate confidence in the branch topology.

Experimental protocol

To determine the tissue distribution of *Onmy*-ARG01 and *Onmy*-ARG02, adult rainbow trout were killed by a sharp blow to the head. Tissues were removed immediately and frozen in liquid nitrogen, followed by storage at –80°C until isolation of RNA (<1 month).

To investigate liver arginase expression in response to a dietary manipulation, fish were divided into two groups: fed or fasted. The fed group were distributed equally between two 50-litre tanks and, likewise, the fasted group were distributed equally between two 50-litre tanks. Prior to the start of the experiment, five fish were killed from each tank (control, 0 week). Fish were weighed and the liver was removed. Whole livers were frozen immediately in liquid nitrogen and stored at –80°C for later isolation of RNA and analysis of enzyme activity (<1 month). Subsequently, only the fed group were given a ration of 1.8% daily (fed twice a day). The fasted group did not receive food for the remainder of the experiment. At the end of the 6th week, 10 fish from the fed group (fed control, 6 weeks) and 10 fish from the fasted group (fasted, 6 weeks) were killed, and tissues were collected as described above.

Northern analysis

To determine the tissue distribution of arginase mRNA or whether arginase mRNA levels changed with fasting, total RNA was isolated from trout liver by the guanidine isothiocyanate method using Trizol (Gibco-BRL, Burlington, ON, Canada). RNA (10 μg lane^{-1}) was separated on a 1.5% agarose gel in the presence of 1.0 mol l^{-1} formaldehyde and transferred to a nylon membrane. Filters were hybridized with ^{32}P-labelled probes. Probes were generated by PCR using a cloned fragment of cDNA as a template and primers specific for either *Onmy*-ARG01 (240 bp), *Onmy*-ARG02 (655 bp) or β-actin (286 bp; control gene). [α-^{32}P]-dCTP (3000 Ci mmol l^{-1}) was used to label probes using a random priming kit (Invitrogen/Gibco-BRL). Following hybridization with *Onmy*-ARG01 probe (65°C, overnight), high-stringency washes were conducted (65°C). Membranes were placed in a PhosphorImager cassette and relative RNA concentrations were estimated from the density of bands using the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA, USA). Membranes were stripped, re-hydridized with the *Onmy*-ARG02 probe and analysed as described above. Finally, membranes were stripped, re-hybridized with the β-actin probe and analyzed as before. The ratio of the density of the arginase:β-actin band was calculated. Separate membranes containing RNA isolated from different individuals were analyzed and presented as means ± S.E.M. (N=3–4).

Enzyme activity

Liver tissue was homogenized in buffer as described by Felskie et al. (1998), except tissue was diluted 200-fold in buffer. Arginase activities were measured after the tissue homogenate was passed through a Sephadex G25 column, as described previously (Felskie et al., 1998). Total protein measurements were taken before and after the column chromatography step, and final enzyme activities corrected accordingly. The assay was linear with time and tissue amount. The assay was performed with 50 μl of homogenized tissue extract and the reaction was stopped after 10 min. Activities are presented as μmol min^{-1} g^{-1} wet mass liver tissue or μmol min^{-1} mg^{-1} protein. Protein was measured by the dye-binding method of Bradford (1976) using Bio-Rad Laboratories reagents and bovine serum albumin as a standard.

Statistics

A single factor analysis of variance (ANOVA) was conducted on the arginase activities between fed control (0 week), fed control (6 week) and fasted (6 week). Where significance was detected, the analysis was followed up with a Tukey's test. Comparisons between tissues or fed and fasted liver arginase:β-actin mRNA levels were assessed with an unpaired Student's t-test. Significant difference was detected at P<0.05.

Results

Identification of arginase genes in bony fishes

Two PCR fragments were amplified from a cDNA library, constructed from the combined gill and kidney tissues of 12 adult rainbow trout, using the degenerate primers described.

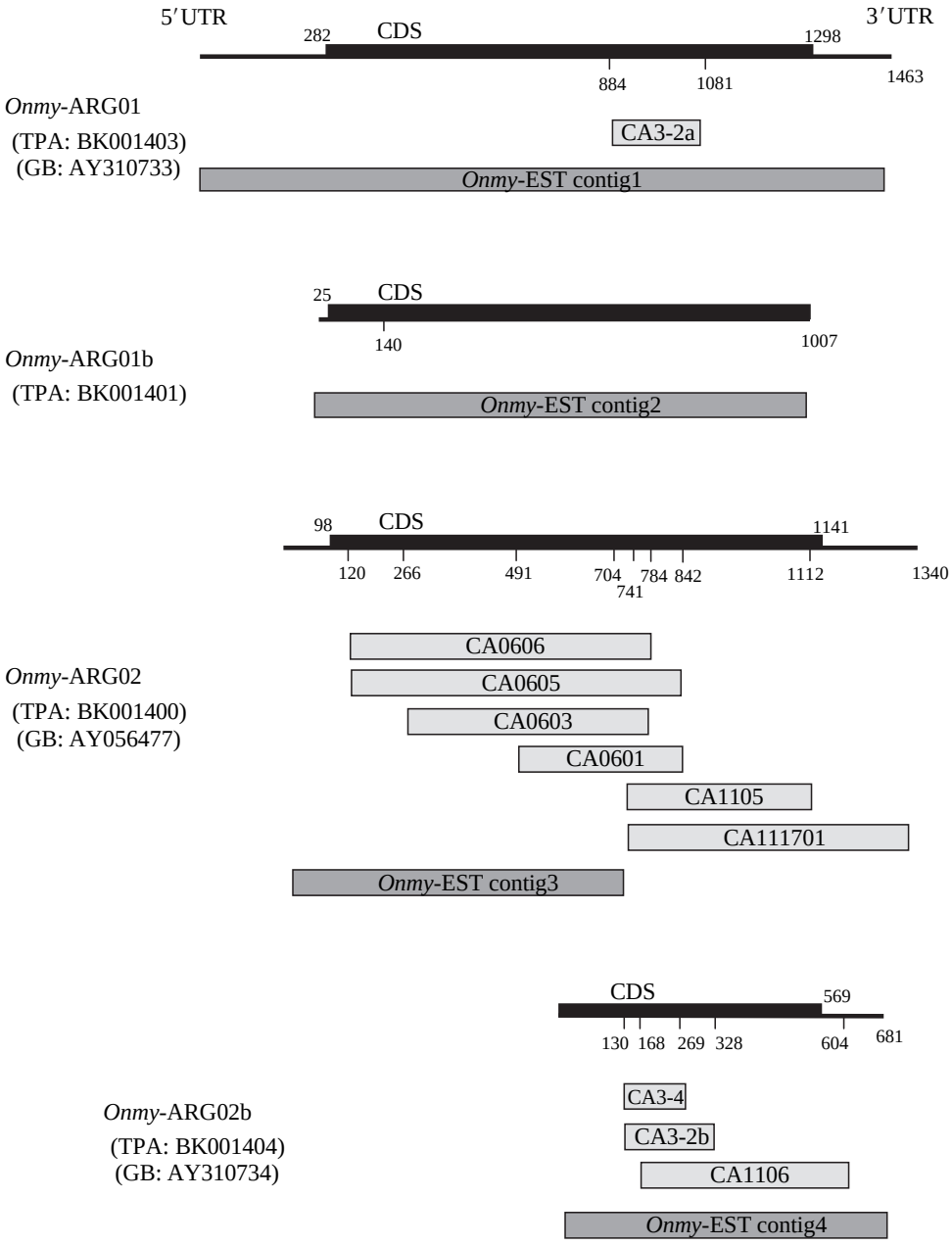

Fig. 1. Rainbow trout (*Oncorhynchus mykiss*; *Onmy*) arginase cDNA and expressed sequence tag (EST) sequences. A summary of the sequence information for each of the four contiguous sequences, *Onmy*-ARG01–*Onmy*-ARG02b, is shown above the supporting clones and EST contigs. Corresponding Third Party Annotation (TPA) and GenBank (GB) accession numbers are shown in parentheses. Coding sequence (CDS) information is indicated by a black rectangle, while the 5′ and 3′ untranslated regions (UTR) are indicated by a thick line. Positions (in base pairs) of the start and stop codons are shown above the black line while the location of the supporting clones is shown below the black line. Supporting clones are shown as either grey or hatched rectangles, indicating clones isolated from the primary cDNA library (S. F. Perry, personal communication) or database-derived EST sequence contigs, respectively.

From the resulting clones of these fragments (CA3 clones) partial sequences of both arginase Type I and Type II genes were found (Fig. 1). Gene-specific primers for the Type II gene were developed and used to amplify seven additional arginase gene fragments. Clones of these fragments (CA06 and CA11 clones) were assembled into two sets of contiguous sequence (contig) information. In total, three arginase genes were isolated from the cDNA library, *Onmy*-ARG01, *Onmy*-ARG02 and *Onmy*-ARG02b (GenBank accession nos AY310733, AY056477 and AY310734, respectively). Tentative sequences for three additional arginase Type II sequences were found in single clones. However, as these sequences were found in only a single clone and were not part of a larger contig (including the trout ESTs), we refrain from reporting them here.

Additional sequence information was obtained from an

Fig. 2. Amino acid alignment of vertebrate arginase genes. Strictly conserved residues are boxed, bolded and darkly shaded while residues that maintain the physio-chemical properties of a position are boxed and lightly shaded. A previous comparison of 21 arginase enzymes in both eukaryotes and prokaryotes (Perozich et al., 1998) identified residues that are strictly conserved, highly conserved (at least 80% conserved) and display invariant similarity (i.e. D/E, S/T or V/I/L/M). These positions are indicated beneath the alignment by *, # and ^, respectively. Three periods (…) indicate incomplete or missing sequence information. The sequences are identified with a four-letter code based on their species name and followed by either their unique GenBank accession number or a unique gene indicator, i.e. ARG01. Species included are *Oncorhynchus mykiss* (*Onmy*), *Danio rerio* (*Dare*), *Takifugu rubripes* (*Taru*), *Rana catesbeiana* (*Raca*), *Xenopus laevis* (*Xela*), *Rattus norvegicus* (*Rano*), *Mus musculus* (*Mumu*), *Sus scrofa* (*Susc*) and *Homo sapiens* (*Hosa*).


Onmy-ARG01b
Onmy-ARG01
Taru-ARG01
Raca-d38303
Raca-u26351
Xela-x69820
Mumu-u51805
Hosa-m14502
Susc-Ay039112
Onmy-ARG02b
Onmy-ARG02
Taru-ARG02
Dare-ARG02
Xela-x08406
Xela-x08407
Xela-x08408
Hosa-d86724
Mumu-u90886
Rano-u90887

Fig. 2

analysis of the growing rainbow trout EST database (May 2003). From the 15 trout arginase ESTs found in the database, four sets of contiguous sequences were determined, *Onmy*-EST contig1 (eight clones) and *Onmy*-EST contig2–4 (two clones each). One clone, CA358388, presumably an incompletely spliced mRNA, was identical to contig2 but was found to contain additional intron sequence. The EST contigs were combined with the previous sequences, confirming and extending the sequence information and revealing the presence of a second arginase Type I gene (Fig. 1). The four resulting sequence contigs, two complete *Onmy*-ARG01 and *Onmy*-ARG02 and two partial *Onmy*-ARG01b and *Onmy*-ARG02b, were reported to the Third Party Annotation database (TPA accession nos. BK001403, BK001400, BK001401 and BK001404, respectively).

In a similar fashion, the zebrafish EST database was searched for arginase sequences (May 2003). Twenty-two zebrafish ESTs were found, 20 of which form a single arginase Type II contig (TPA accession nos. BK001402). The two remaining ESTs contain unique arginase-like sequences. One (BG738104) contains stop codons in the putative reading frame, while the other (BI474173) is more similar to the arginase Type I sequences but also appears to contain frameshift mutations. In either case, this sequence information is not supported by additional ESTs, is incomplete and, therefore, was not included in further analyses.

Table 1. *Arginase activities (*N=*6) in liver tissue from rainbow trout fed control (week 0 and week 6) and fasted (week 6)*

Arginase	μmol min^{-1} g^{-1} liver	μmol min^{-1} mg^{-1} protein
Fed control		
0 weeks	77. 83±14.13	1.14±0.31
6 weeks	51.72±11.10	0.70±0.09
Fasted		
6 weeks	132.55±11.35*,†	2.17±0.24*,†

*Significantly different from fed control (0 week).
†Significantly different from fed control (6 week).

Putative arginase Type I and Type II genes were also identified from pufferfish, *Taru*-ARG01 and *Taru*-ARG02, from the genomic scaffolds FM:M000333 and FM:M002889 (release version 3), respectively. Both genes contain all the expected exons as determined through comparison with available Genscans and with the bony fish sequences reported here. Two pufferfish ESTs were identified in the database (May 2003). One is identical to the reported *Taru*-ARG01 gene (CA330269) while the other is 92% similar to the *Taru*-ARG02 gene.

Sequence comparison

Previous comparisons of 21 arginase enzymes in both eukaryotes and prokaryotes (Perozich et al., 1998) revealed 20 residues that are strictly conserved, six that display an invariant similarity (i.e. D/E, S/T or V/I/L/M) and 10 that are highly conserved (at least 80% conserved). An alignment of the putative amino acid sequences of all known vertebrate arginase genes shows that these residues are also conserved in the seven bony fish arginases reported (20/20 strictly conserved, 6/6 invariant similarity and 9/10 highly conserved; Fig. 2).

Interestingly, the three bony fish Type I arginases

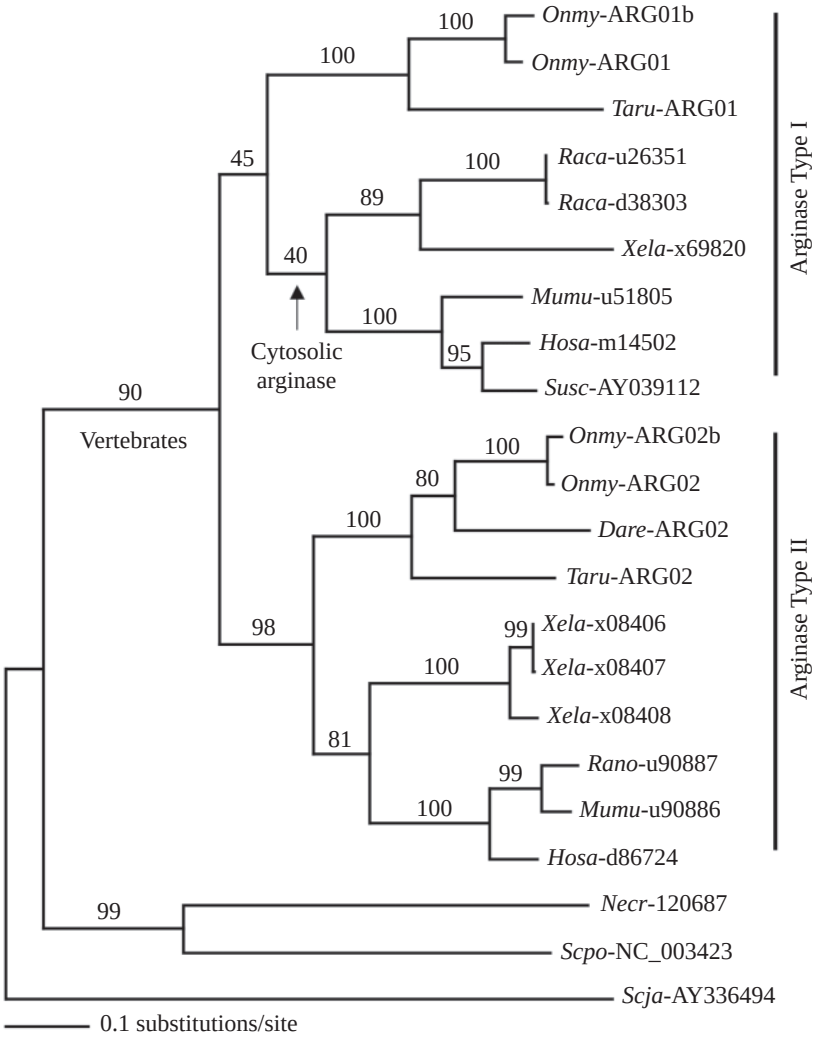

Fig. 3. Maximum likelihood phenogram (mid-point rooted) based on a DNA alignment of the coding sequence of arginase genes in vertebrates and three nonvertebrate eukaryotes. Nomenclature of genes is identical to that used in Fig. 2, with the inclusion of three nonvertebrate eukaryote arginase sequences: *Neurospora crassa* (*Necr*), *Schizosaccharomyces pombe* (*Scpo*) and *Schistosoma japonicum* (*Scja*). The location of the common ancestor of all vertebrate cytosolic arginase genes is shown by the arrow. Bootstrap values, based on 300 bootstraps replicates, are placed to the left of the appropriate nodes.

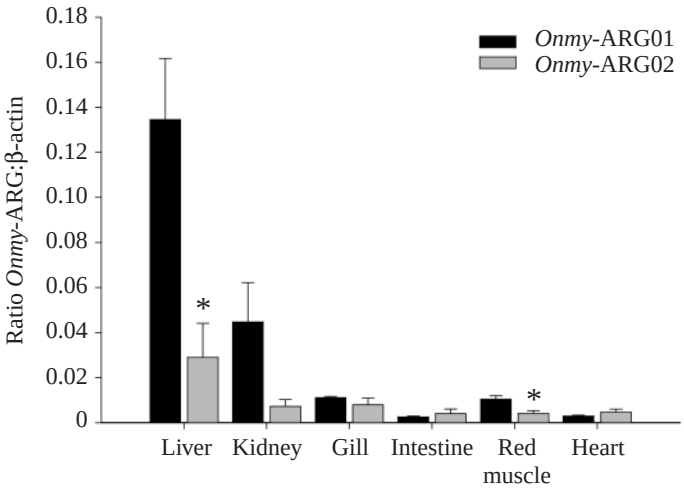

Fig. 4. The tissue distribution of arginase genes in adult rainbow trout (*Onmy*-ARG01 and *Onmy*-ARG02) is shown relative to β-actin. Total RNA (10 μg) was loaded into each lane. Tissues include liver, kidney, gill, intestine, red muscle and heart. Intensity of signal was quantified by denisitometry (see Materials and methods). Means ± S.E.M. (*N*=3). Asterisks denote significant difference from the ratio of *Onmy*-ARG01:β-actin levels ($P<0.05$).

(identified through phylogenetic analysis below) contain a much longer N-terminal domain than do the amphibian or mammalian Type I arginases. As a presumptive mitochondrial protein, Type II arginase requires an N-terminal mitochondrial targeting peptide (mTP) in order to be transported into the matrix of the mitochondria. These mTPs can be diverse in both length and sequence but are characteristic in the over-representation of positively charged residues (i.e. Arg, Ala, Ser), whereas negatively charged residues (Asp and Glu) are rare (Grivell, 1988; Emanuelsson et al., 2000). Two computer programs were used to detect the presence of mTPs in the six bony fish arginase genes for which N-terminal information is available; *TargetP* predicts the subcellular location of newly identified proteins (Emanuelsson et al., 2000), and *MitoProt* predicts the probability that a nuclear gene is exported to the mitochondria and identifies probable mTP cleavage sites (Claros and Vincens, 1996). These computer analyses indicate that it is highly probable that all six of the bony fish arginases, whether Type I or Type II, are targeted to the mitochondria. In addition, no physical restraints for the import of the proteins were found, and putative cleavage sites of the mTP were identified at position 23–25 and/or 43 (Fig. 2). Further analysis of all other vertebrate arginases (Fig. 2) reveals that only the amphibian and mammalian Type I arginases are not predicted to be imported into the mitochondria.

Phylogenetic analysis

A maximum likelihood tree (Fig. 3), including all vertebrate and three nonvertebrate eukaryotic arginase sequences for reference, was constructed from a nucleotide sequence alignment corresponding to the codons for positions 29–335 (Fig. 2). The tree topology is robust, with bootstrap values over 75% for most branching points. Two lineages of arginase genes are suggested in vertebrates, labelled Arginase Type I and Type II; however, bootstrap for the Arginase I lineage is not as well supported. Interestingly, both lineages contain the predicted branching order of vertebrate evolution; an initial split of bony fish and tetrapod genes followed by the divergence of tetrapod genes into amphibian and mammalian lineages.

Expression of arginase genes in various tissues

Onmy-ARG01 and *Onmy*-ARG02 mRNA were detected in liver, kidney, gill, intestine, red muscle and heart tissues (Fig. 4). *Onmy*-ARG01 was expressed at a significantly higher level relative to *Onmy*-ARG02 in liver and red muscle tissue (Fig. 4). Although a similar trend was observed in kidney, this difference was not significant (Fig. 4). When comparing between tissues, expression relative to β-actin is not appropriate because β-actin mRNA levels vary in different tissues (e.g. Foss et al., 1998; Murray et al., 2003). The amount of total RNA loaded appeared to be consistent between lanes, as observed from the ethidium bromide-stained gel. For each transcript, the order of the highest to the lowest level of mRNA

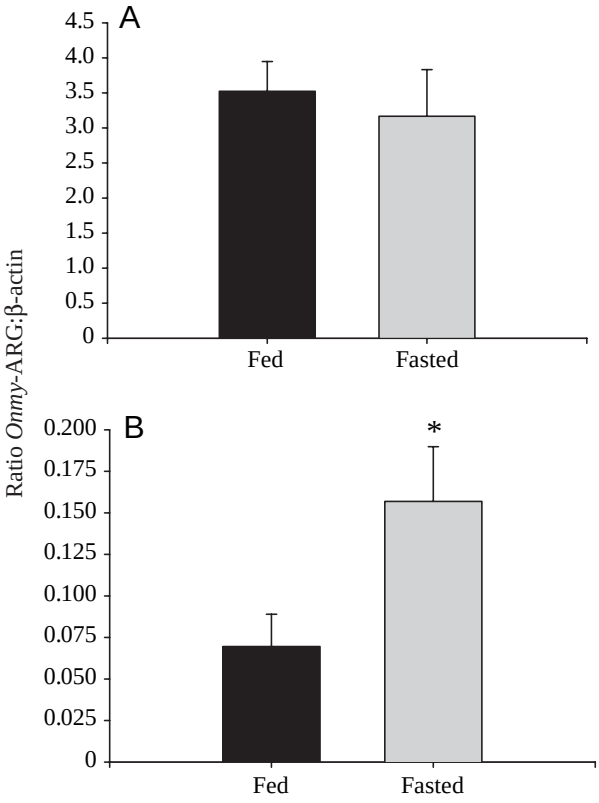

Fig. 5. The ratio of liver arginase genes, *Onmy*-ARG01 and *Onmy*-ARG02, mRNA levels relative to β-actin mRNA levels from northern blot analysis after 6 weeks of feeding (control) or fasting in juvenile *O. mykiss*. Total RNA (10 μg) was loaded into each lane. Intensity of the signal was quantified by denisitometry (see Materials and methods). Means ± S.E.M. (*N*=4). (A) *Onmy*-ARG01:β-actin levels, (B) *Onmy*-ARG02:β-actin levels. Asterisk denotes significant difference from fed group ($P<0.05$).

was: *Onmy*-ARG01: liver > kidney > red muscle ≈ gill > heart ≈ intestine; *Onmy*-ARG02: liver > kidney ≈ gill > intestine ≈ red muscle ≈ heart.

Expression of arginase genes during fasting

Following the 6-week experiment, there was a significant difference between the body mass of fasted (23±2 g; *N*=6) and fed fish (56±1 g; *N*=6). Body mass at the start of the experiment (0 week) was 26±2 g (*N*=6) and was not significantly different from the fasted fish at 6 weeks.

Hepatic arginase activity and specific activity were significantly higher in fasted relative to fed control fish, with a ~2- to 3-fold higher level in fasted compared with fed fish at 0 and 6 weeks, respectively (Table 1).

The levels of *Onmy*-ARG01 mRNA (relative to β-actin mRNA levels) were not significantly different between fasted and fed fish at 6 weeks (Fig. 5A). By contrast, there was a 2-fold elevation of *Onmy*-ARG02 mRNA levels in fasted relative to fed fish (Fig. 5B).

Discussion

Arginase genes

We hypothesized that the arginase gene or genes in trout would share more similarity with the mammalian mitochondrial Type II gene than with the cytosolic Type I gene simply because arginase activities are mostly mitochondrial in trout and the OUC is nonoperative (Korte et al., 1997; Todgham et al., 2001). Interestingly, our findings suggest that orthologues of both Type I and Type II arginase genes exist in rainbow trout (and pufferfish). The current evidence (i.e. the identification of complete coding regions and the presence of mRNA) also suggests that both of these genes are functional. For our analysis, only one zebrafish arginase gene (Type II) was presented; however, the presence of a single Type I-like EST in the database suggests the presence of Type I genes in this species. Further analysis is needed to determine the true nature of this gene and whether it is functional or an expressed pseudogene. In any case, the phylogenetic analysis clearly indicates that the Type I and Type II arginase genes arose following a gene duplication that occurred prior to the evolution of the modern bony fishes.

Recent studies of glutamine synthetase genes have revealed four separate genes in rainbow trout, also found in two evolutionarily distinct lineages (Murray et al., 2003). It was speculated that glutamine synthetase gene number rose from two to four following the tetraploidisation event that proceeded the evolution of salmonids. Given the current phylogeny of arginase genes (Fig. 3), it is tempting to invoke a similar argument here for the evolution of two Type I or Type II genes; however, preliminary sequencing evidence suggests the existence of additional Type II sequences in trout. It is interesting to note that three mRNA species hybridized to the *Onmy*-ARG02 probe (data not shown), suggesting the possibility that multiple ARG02 mRNAs arise from differential processing, the presence of alternative promoters or three distinct Type II genes. In *Xenopus*, three closely related nonhepatic arginase genes (Type II) have been isolated with distinct tissue, hormone-dependent and ontogeny-related regulation (Patterton and Shi, 1994). Interestingly, five separate bands were detected when Human Type II was used as a probe on a northern blot of human RNA, but only a single band was observed in the mouse (Morris et al., 1997). Clearly, further studies are necessary to explore the number, role and nature of multiple Type I or Type II arginase genes in rainbow trout.

Tissue distribution of arginase genes

Our results indicate that *Onmy*-ARG01 is expressed at a higher level than *Onmy*-ARG02 in trout liver tissue. This finding is in agreement with studies of Type I genes in *Xenopus* tadpole (Patterton and Shi, 1994) and mammalian liver tissue (Morris et al., 1997). Our results also show that *Onmy*-ARG01 is expressed at a higher level than *Onmy*-ARG02 in trout red muscle tissue. In other trout tissues (kidney, gill, intestine, heart), both transcripts were expressed, but there was no significant difference between *Onmy*-ARG01 and ARG02 expression (Fig. 4). This pattern of expression in nonhepatic trout tissues, regardless of the unknown expression of other possible arginase genes, is clearly different from that reported in ureotelic vertebrates. Although there are small discrepancies between the tissue distribution of arginase genes among different mammalian species, Type I arginase is generally found in liver whereas Type II arginase has a wide distribution in nonhepatic tissues (Grody et al., 1987; Gotoh et al., 1996; Morris et al., 1997; Yu et al., 2001). Thus, the wide tissue distribution of *Onmy*-ARG01 and *Onmy*-ARG02 in rainbow trout tissues suggests that the prerequisites were in place for the evolution in terrestrial ureotelic vertebrates of hepatic Type I arginase linked to the production of urea *via* the OUC and the more widely distributed Type II arginase playing other cellular roles. The evolution of a cytosolic form of arginase in ureotelic vertebrates appears to be a derived trait that occurred in the common ancestor of amphibians and mammals (Fig. 3). Indeed, Mommsen and Walsh (1989) determined the subcellular location of arginase activity in a variety of fish species and proposed that cytosolic arginase first appeared in the lungfish. They postulate that this shift in the intracellular location of liver arginase, along with the evolution of a mitochondrial ornithine transporter and ammonia-dependent CPSase I, were key events in the evolution of the OUC found in terrestrial ureotelic vertebrates. If a functioning OUC exists in bony fishes (e.g. early life stages; see Introduction), then it represents the ancestral state or has undergone a parallel (or separate) evolution.

Upregulation of arginase with fasting

Our results show that food deprivation in rainbow trout results in the induction of liver arginase activity (2–3-fold), similar to other reports (e.g. Chiu et al., 1986; Jürss et al., 1987). We hypothesized that the two trout arginase genes would be differentially regulated in response to dietary manipulation. Indeed, *Onmy*-ARG02 mRNA levels, but not *Onmy*-ARG01, were modestly elevated (2-fold) in liver tissue collected from

fasted fish. These results suggest that the increase in hepatic arginase activity is mainly due to the accumulation of mRNA because the magnitude of the changes in each were very similar. Gene expression may be turned on early and sustained during the 6-week fasting trial or the response may be delayed. Regardless, the results suggest that *Onmy*-ARG01 and *Onmy*-ARG02 play different physiological roles in trout liver. Exactly what those roles are will be an important question for future studies.

Many fish undergo significant episodes of food deprivation in nature without pronounced changes in their physical capabilities, although there are numerous metabolic adjustments. In the laboratory, metabolic rate decreased in rainbow trout fasted for 17 days (Alsop and Wood, 1997). Protein use during fasting accounts for only 14–30% of oxidative metabolic rate (for review, see Wood, 2001), although total body protein content declined 66% after a 15-day fast in juvenile (4 g) rainbow trout (Lauff and Wood, 1996). The mismatch between protein use as fuel and the fall in body protein content may be due to a redistribution of proteins into carbohydrates (i.e. gluconeogenesis) and lipids (i.e. lipogensis). Not surprisingly, intracellular amino acid levels have also been reported to decline considerably (–50%) during a long-term fast (140 days; Timoshina and Shabalina, 1970). Arginolysis, catalyzed by arginase, results in the formation of ornithine and urea. Ornithine can be transaminated to proline or oxidized to glutamate, both of which may enter the citric acid cycle after conversion to α-ketoglutarate (Nelson and Cox, 2000), providing fuel for the fasted fish. At this point, we can only speculate that *Onmy*-ARG02, but not *Onmy*-ARG01, is regulated with dietary manipulations in trout to supply ornithine, similar to one of the putative functional roles of nonhepatic Type II arginase in mammals.

In the present study, rainbow trout lost only ~12% of their body mass after a 6-week fast (not significant), which is within the range reported by others (e.g. Chiu et al., 1986; Jürss et al., 1987). Although arginase activity per gram of liver tissue and per mg of liver protein significantly increased in fasted fish, if one considers enzyme activity per gram of fish, arginase activities are similar between fasted and fed fish (6 weeks). This is simply because the fish fed for 6 weeks had more than doubled their body mass, whereas the mass of the fasted fish did not change considerably. From another perspective, one can say that the level of arginase activity was maintained in fasted fish relative to fed fish, despite a large difference in body mass. From either angle, the arginase response is not typical of other liver metabolic enzymes after a fast. Although hepatic glutamate dehydrogenase activity also increased (Jürss et al., 1987), lipogenic, glycolytic and gluconeogenic enzyme activities did not change or decreased (Lin et al., 1977; Moon and Johnston, 1980; Jürss et al., 1987).

In conclusion, our findings provide evidence for two distinct arginase genes in rainbow trout, *Onmy*-ARG01 and *Onmy*-ARG02, both with mitochondrial targeting sequences. Further, *Onmy*-ARG01 and *Onmy*-ARG02 share sequence similarities to Type I and Type II arginase genes isolated from amphibians and mammals but differ in their pattern of tissue expression. This is an important piece of information in the arginase evolutionary puzzle. Although arginase activities were previously localized to mitochondria of teleost livers (Mommsen and Walsh, 1989; Korte et al., 1997; Felskie et al., 1998), prior to our study there was little sequence information available in teleosts to determine the number and type of arginase genes present. With this sequence and expression data in hand, it will now be possible to separate the functional significance of two distinct arginase genes in the ammoniotelic rainbow trout.

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