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Expression of Four Glutamine Synthetase Genes in the Early Stages of Development of Rainbow Trout (*Oncorhynchus mykiss*) in Relationship to Nitrogen Excretion*

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The incorporation of ammonia into glutamine, catalyzed by glutamine synthetase, is thought to be important in the detoxification of ammonia in animals. During early fish development, ammonia is continuously formed as yolk proteins and amino acids are catabolized. We followed the changes in ammonia and urea-nitrogen content, ammonia and urea-nitrogen excretion, glutamine synthetase activity, and mRNA expression of four genes coding for glutamine synthetase (*Onmy-GS01–GS04*) over 3–80 days post fertilization and in adult liver and skeletal muscle of the rainbow trout (*Oncorhynchus mykiss*). Both ammonia and urea-nitrogen accumulate before hatching, although the rate of ammonia excretion is considerably higher relative to urea-nitrogen excretion. All four genes were expressed during early development, but only *Onmy-GS01* and *-GS02* were expressed at appreciable levels in adult liver, and expression was very low in muscle tissue. The high level of expression of *Onmy-GS01* and *-GS03* prior to hatching corresponded to a linear increase in glutamine synthetase activity. We propose that the induction of glutamine synthetase genes early in development and the subsequent formation of the active protein are preparatory for the increased capacity of the embryo to convert the toxic nitrogen end product, ammonia, into glutamine, which may then be utilized in the ornithine-urea cycle or other pathways.

Glutamine synthetase (L-glutamate:ammonia ligase (ADP forming), EC 6.3.1.2) catalyzes the ATP-dependent conversion of glutamate and ammonium to glutamine. Glutamine formation plays a key role in nitrogen metabolism, including nucleotide, amino acid, and urea biosynthesis. Glutamine synthetase is critical in the detoxification process of the highly mobile and toxic ammonia (for reviews, see Refs. 1–3). During early fish development, endogenously feeding embryos rely on the catabolism of yolk protein and amino acids for fuel, resulting in a high rate of ammonia production (for review see Ref. 4). Indeed, in both the rainbow trout, *Oncorhynchus mykiss* (5), and African catfish, *Clarias gariepinus* (6), ammonia levels steadily rise during embryogenesis and peak after hatch. Although the egg capsule or chorion is permeable to ammonia (7, 8), elimination is slow in the absence of respiratory convection (9, 10) and direct contact with bulk water. Thus, glutamine synthetase may play a central role in maintaining low tissue levels of ammonia throughout the critical time of organ development during embryogenesis.

Glutamine may be further utilized to synthesize urea in the rainbow trout embryo. In fish, glutamine is the nitrogen-donating substrate for the first step in the ornithine urea cycle (OUC)[1] (11). Griffith (12) suggested that urea synthesis may be important during protracted teleost embryogenesis, as ammonia excretion is restricted and the rate of protein catabolism is high. Dépêche *et al.* (13) demonstrated significant urea production in rainbow trout embryos from the incorporation of $NaH^{14}CO_3$, the carbon substrate for the urea cycle. As well, exposure to elevated water ammonia levels results in a significant increase in tissue urea-nitrogen concentrations in trout embryos (14). Glutamine synthetase is induced along with the key OUC enzyme, carbamoyl-phosphate synthetase III, and other OUC enzymes during early life stages in rainbow trout (5, 14–16) and several other teleost species (17- 20). Indeed, the levels of glutamine synthetase and OUC enzyme activities are high in early stages of trout development relative to adult liver levels (5, 16).

The functional glutamine synthetase enzyme consists of eight identical subunits, with some microheterogeneity between subunits (21). Developmental expression of glutamine synthetase has been studied in several species. In rat brain, a single glutamine synthetase gene is expressed in 14-day embryos, well before enzyme activity can be detected (22). A study of the ontogeny of one glutamine synthetase gene in sea urchin embryos revealed mRNA expression in the unfertilized egg (*i.e.* of maternal origin), as well as in several embryonic stages (23). There are two glutamine synthetase isoforms in *Drosophila* with some quantitative variation in the developmental pattern of expression (24). It has been estimated that transcriptional activation of the embryonic genome in rainbow trout occurs at about 3 dpf (25). Glutamine synthetase activity was detected in "eyed up" embryos when the yolk sac was dissected away from the embryonic body (14). Hence, transcription of glutamine synthetase should occur sometime after 3 dpf but well before hatching in rainbow trout.

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[1] The abbreviations used are: OUC, ornithine urea cycle; dpf, days post fertilization; CYA, complete yolk absorption; ANOVA, analysis of variance.

TABLE I
Primer and probe sequences for detection of glutamine synthetase gene (Onmy GS01–GS04) expression in rainbow trout embryos using real-time PCR

	Forward primer 5′ → 3′	Reverse primer 5′ → 3′	Probe 5′ → 3′	Length of amplicon
				bp
Onmy-GS01[a]	CTGCAGTCTGTGTTCAGGGTAGA	CATCTGTCTGGAATTGTTAAGTCCATA	TACCTTTTGATCACTGCCAACATTGCCC	101
Onmy-GS02	GGCAGTGTCTTTAAATGGCAACA	ACGCTACAATTGGCAAGACTGA	CTGTCTCCAGATTTGACACATTCCTGGATCAT	136
Onmy-GS03	GTGTATCAATTTGCTACTCATGTTTAACAT	AAAATGGGTTCTTGATACAACTTCTACTAA	AAGGATCCAAGGTGCATCTGTGTTTTTATACATG	191
Onmy-GS04	TTAATGAAAGATGGTGGCTGACA	CTGCAGGAAACGCGAGATC	CATTGTCTTCCCCTTTTGAGTCTTCTAGTGGG	105
β-Actin	GACCCAGATCATGTTTGAGACCTT	CGTAGCCCTCGTAGATGGGTACT	ACTCCGGTGACGGCGTGACCC	152

[a] GenBank™ accession numbers: *Onmy-GS01* (AF390021), *Onmy-GS02* (AF390022), *Onmy-GS03* (AF390023), *Onmy-GS04* (AF390024), and β-actin (AJ438158).

Gene sequences for glutamine synthetase have been reported in several fish species (26–30). Recently, four glutamine synthetase isoforms were isolated from adult trout tissues, *Onmy-GS01–GS04* (29). In addition, *Onmy-GS01*, *-GS02*, and *-GS04* were assigned to three different linkage groups of the rainbow trout map (31) based on polymorphic sites in the 3′-untranslated region.[2] Overall, these findings, along with sequence analysis (29), support the hypothesis that *Onmy-GS01*, *-GS02*, *-GS03*, and *-GS04* are separate loci. Preliminary evidence suggests that mRNA expression of *Onmy-GS01–GS04* varies between different adult tissues (29), but the functional significance of the four isoforms is not understood. Due to the importance of ammonia detoxification during early development, we decided to first investigate whether one or more of the glutamine synthetase genes are critical in trout embryogenesis. Thus, the aim of this study was to determine the developmental expression of glutamine synthetase and relate these changes to major developmental landmarks (*e.g.* hatching) and nitrogen excretion. We measured developmental changes in the level of mRNA expression of *Onmy-GS01*, *-GS02*, *-GS03*, and *-GS04* from 14 to 80 dpf, as well as in adult liver and skeletal muscle tissue. Glutamine synthetase activities, ammonia and urea-nitrogen excretion rates, and ammonia and urea-nitrogen content were also measured between 3 and 80 dpf.

EXPERIMENTAL PROCEDURES

Animals

Rainbow trout (*O. mykiss* Walbaum) embryos were purchased on the day of fertilization from Rainbow Springs Trout Farm (Thamesford, Ontario, Canada) and held in continuous-flow, mesh-bottom incubation trays supplied with local well water (10 °C, pH 8; water hardness 411 mg/liter as $CaCO_3$; Ca^{2+}, 5.24 mEq/liter; Cl^-, 1.47 mEq/liter; Mg^{2+}, 2.98 mEq/liter; K^+, 0.06 mEq/liter; Na^+, 1.05 mEq/liter) at the Hagen Aqualab, University of Guelph, Guelph, Ontario, Canada. Embryos in the incubation trays were shielded from light. The pigmented eye was clearly visible (eyed-up stage) at 14 dpf. After 100% hatching (25–30 days), yolk sac larvae were transferred to mesh-sided, well aerated, floating baskets within a 2-meter circular tank of continuous flow water. Five days after 100% hatching, feeding was administered (1.3–5.0% body weight, Martin Mills Inc., Elmira, Ontario, Canada) via a conveyer belt system. CYA occurred at 50 dpf (juveniles).

Adult rainbow trout (donated by the Alma Aquaculture Research Station, Alma, Ontario, Canada) were kept in circular tanks in recirculating, freshwater (10 °C, pH 8) in the Hagen Aqualab, University of Guelph, Guelph, Ontario, Canada. Fish were fed trout pellets (Martin Mills Inc.) daily *ad libitum*.

Experimental Protocol

Nitrogen excretion rates were measured as previously described (5). For analysis of total RNA, enzyme activities, and concentrations of ammonia and urea-nitrogen, fish were collected at appropriate stages, quickly blotted dry, immediately frozen in liquid nitrogen, and stored at −80 °C until later analysis (1–3 months). For measurements in adult tissues, fish were killed by a sharp cranial blow, and tissues were immediately removed and frozen in liquid nitrogen, followed by storage at −80 °C until later analysis (1–3 months).

Analyses

Ammonia and Urea-nitrogen Concentration—Water samples were analyzed for ammonia concentration using the indophenol blue method (32). Urea-nitrogen content was measured using a colorimetric assay as described by Rahmatullah and Boyde (33). Ammonia and urea-nitrogen excretion rates were expressed per gram of whole wet embryo (micromoles/g/h). Urea-nitrogen was calculated by accounting for the two nitrogens per urea molecule. Percent urea-nitrogen excretion was calculated as urea-nitrogen excretion rate/(urea-nitrogen excretion rate + ammonia excretion rate) × 100.

Whole embryo, tissue, and yolk ammonia and urea-nitrogen concentrations were measured as described by Wright *et al.* (5). The final supernatant was analyzed for ammonia concentration using a Sigma diagnostic kit (171 UV, Sigma-Aldrich Inc., Oakville, Ontario, Canada). Urea-nitrogen concentration was measured using the method described by Rahmatullah and Boyde (32). Ammonia and urea-nitrogen content were expressed as micromoles/g. The turnover time is the amount of time required for an organism to clear the total content of a substance from its system, where turnover time (h) = ammonia or urea-nitrogen tissue content (micromoles/g)/ammonia or urea-nitrogen excretion rate (micromoles/g/h).

RNA Extraction and cDNA Synthesis—Total RNA was extracted from 14- and 21-dpf embryos, 31-dpf yolk sac larvae, 60- and 80-dpf juveniles, and adult liver and muscle samples using TRIzol reagent (Invitrogen). An extra phenol:chloroform:isoamyl alcohol (25:24:1) step was added to samples that contained large amounts of yolk (14, 21, and 31 dpf). RNA was stored at −80 °C for up to 6 months. To eliminate possible genomic DNA, total RNA (3 μg) was treated with deoxyribonuclease I, amplification grade (Invitrogen). The DNase-treated total RNA samples were reverse transcribed using the enzyme SuperScript II Reverse Transcriptase (Invitrogen) and primer, Poly-T. Non-reverse transcribed controls were synthesized using the same reaction but substituting diethyl pyrocarbonate-treated water for the SuperScript enzyme.

Real-time PCR—mRNA expression of *Onmy-GS01–GS04* was quantified from the above cDNA products using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers and dual-labeled probes (Table I) were designed for each gene using PrimerExpress software (version 2.0.0, Applied Biosystems). All probes were dual-labeled with 6-carboxyfluorescein fluorescent reporter at the 3′-end and 6-carboxytetramethylrhodamine quencher at the 5′-end. Each PCR reaction contained a 5-μl template, 12.5 μl of Taqman Universal PCR Master Mix (no AmpErase UNG, Applied Biosystems), and 2.5 μl of forward and reverse primers (9 μM) and probe (2.5 μM). The following conditions were used; 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. To correct for variability in amplification efficiency between different cDNAs, a standard curve was performed (34) for each glutamine synthetase gene using serial dilutions of cDNA samples from trout brain tissue, known to have high glutamine synthetase activity. The relative dilution of a given sample was extrapolated by linear regression using the threshold cycle of each unknown. To account for differences in cDNA loading and RNA reverse transcriptase efficiency, each sample was normalized to the expression level of the control gene β-actin. Two control genes were tested for consistency of expression between developmental stages; β-actin and 18 S rRNA. The expression of both genes varied over developmental time. When expressed as a ratio with glutamine synthetase mRNA, the ontogenic changes were very similar between glutamine synthetase mRNA:β-actin and glutamine synthetase mRNA:18 S rRNA. Thus, β-actin was used as the control gene. To account for differences in β-actin expression between early stages and adult liver and muscle, the

[2] K. Gharbi, R. Danzmann, and M. Ferguson, unpublished observations.

level of expression of β-actin within each group of samples was normalized to a randomly selected "control" group (60 dpf) according to Billiau *et al.* (35) as follows: individual value within a group/(mean value within a group/mean value of control group).

Samples were assayed in triplicate with only one target gene assayed per well. One PCR reaction from each primer set was purified using a QIAquick PCR purification kit (Qiagen) and sequenced to ensure that each primer set was only amplifying the target sequence. Non-reverse transcribed RNA and water-only controls were run to ensure that no genomic DNA was being amplified and that reagents were not contaminated.

Glutamine Synthetase Activity—Enzyme activity was measured in early stages (3, 10, 21, 31, 60, and 80 dpf) and adult tissue homogenates, prepared as previously described (5). Glutamine synthetase activities were calculated from the amount of product (γ-glutamyl hydroxamate) formed via the γ-glutamyl transferase reaction (36) from 0 to 30 min at 26 °C. Activity was expressed as the number of micromoles of product formed per gram of wet weight tissue per minute (μmol/g/min).

Statistical Analyses—Changes in variables during development were analyzed using least squares linear regression. If inspection suggested a non-linear approach would be more appropriate, this was tested with an *F*-test. GraphPad Prism (version 3.00, GraphPad Software, San Diego, CA) was used to calculate linear and non-linear fits and to compare fits. Levels in the yolk were compared with those in the embryo using a paired *t* test. The *t* tests were used to test if low values were significantly different from zero. If there was no obvious linear or non-linear relation of a variable with dpf, then ANOVA and the Tukey's test were used to test for differences during development.

Statistical analysis for glutamine synthetase mRNA expression was performed using SigmaStat software version 3.00 (SPSS Inc., Chicago, IL). Expression of *Onmy-GS01–GS04* was compared using one-way ANOVA and a Dunn's post-hoc test. All values are presented as means ± S.E., and significant differences were detected at $p < 0.05$.

RESULTS

Nitrogen Excretion and Ammonia and Urea-nitrogen Content—The rate of ammonia excretion increased in a linear manner during early life stages ($r^2 = 0.94$; Fig. 1*A*). Urea-nitrogen excretion was not detectable at 3 dpf (*t* test, $t = 1.89$, $p = 0.12$); then it increased in a sigmoidal manner (a sigmoidal fit significantly better than a linear fit, $F_{2,32} = 6.748$, $p = 0.0036$; Fig. 1*B*). The percentage of nitrogen excreted as urea was much less than ammonia but increased over time (ANOVA, $F_{4,28} = 26.53$, $p = 0.000$; Fig. 1*C*). About 8% of nitrogen was excreted as urea up to day 31 and about 22% at 60 and 80 dpf (Tukey, $p < 0.001$); it was not different between days 10 and 31 (Tukey, $p > 0.3$) or between days 60 and 80 (Tukey, $p > 0.8$).

Ammonia concentration in the whole embryo was increased in a linear fashion during early development (Fig. 2*A*). Ammonia concentration in the yolk was more than twice that of the larvae at 31 dpf (paired *t* test, $t = 8.15$, $p = 0.000$; Fig. 2*A*). Urea-nitrogen concentration was low before hatching, increased during hatching, and then decreased after hatching (non-linear fit better than linear fit, $F_{1,33} = 158.6$, $p < 0.001$; Fig. 2*B*). Urea-nitrogen concentration was ~10% greater in the larvae than in the yolk at 31 dpf (paired *t* test, $t = 2.97$, $p = 0.031$; Fig. 2*B*).

The ammonia turnover time (time required to clear the embryo of its ammonia content) was initially very long at 3 dpf, but then rapidly decreased by 10 dpf (non-linear sigmoidal fit better than a linear fit, $F_{2,32} = 149.2$, $p < 0.001$; Fig. 2*C*). At 10 dpf ammonia turnover was ~1 day, at 21 dpf it was about one-half day, and at 60 and 80 dpf total ammonia content was turning over every 3 h. Urea-nitrogen turnover was high before hatching and decreased after hatching; it could not be calculated at 3 dpf, because urea-nitrogen excretion was not detectable (Fig. 2*C*). Urea-nitrogen turnover times at 10 and 21 dpf were ~1 week and significantly greater than those at 31–80 dpf (ANOVA, Tukey, $p < 0.004$). Urea-nitrogen turnover times at 10 and 21 dpf were not different (Tukey, $p > 0.4$) and also did not differ between 31, 60, and 80 dpf (Tukey, $p > 0.2$). Similar to ammonia turnover times, urea-nitrogen was turning over about every 3 h at 60 and 80 dpf.

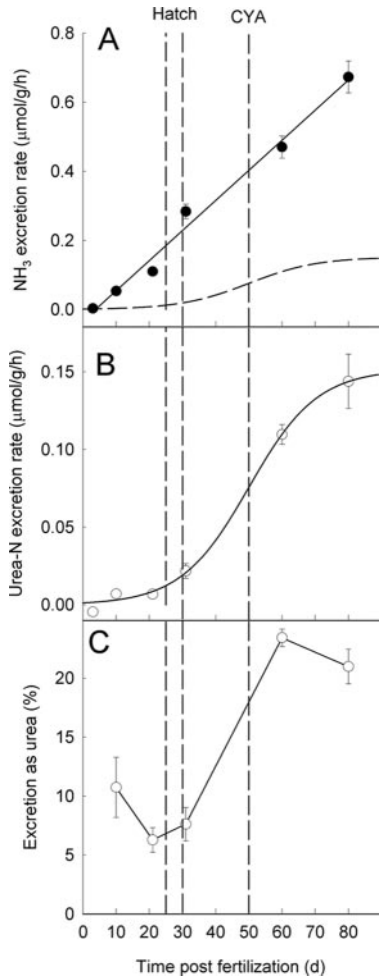

FIG. 1. **Changes in the rates of ammonia and urea-nitrogen excretion during early development in rainbow trout embryos.** Values are means ± S.E. ($n = 6$), *vertical lines* demarcate the time of hatching between 25 and 30 dpf, and *CYA* = complete yolk absorption. *A*, rate of ammonia excretion; *solid line* is least squares linear regression; *dashed line* shows urea excretion for comparison. *B*, rate of urea-nitrogen excretion, *solid line* is least squares non-linear sigmoidal regression. *C*, relative percentage of total nitrogen excretion (ammonia plus urea-nitrogen) excreted as urea. Values were not calculated for 3 dpf, because urea-nitrogen excretion was not different from zero at that time.

Glutamine Synthetase mRNA Expression and Enzyme Activity—All four glutamine synthetase genes were expressed during early development (Fig. 3). At 14 dpf, the level of expression of *Onmy-GS01–GS04* was relatively low, but *-GS01* and *-GS03* were clearly the first genes expressed. By 21 dpf, *Onmy-GS01* and *-GS03* mRNA levels were significantly higher than at 14 dpf (11- and 18-fold, respectively) and the level of *Onmy-GS02* and *-GS04* mRNA remained low (ANOVA, Dunn's, $p < 0.05$; Table II, Fig. 3). By 31 dpf, just after hatch, all four glutamine synthetase genes were expressed, with *Onmy-GS03* significantly higher than *Onmy-GS01*, *-GS02*, and *-GS04*. At several developmental stages, glutamine synthetase mRNA levels were higher relative to expression in adult liver and muscle tissue (Table II). For example, *Onmy-GS01* mRNA levels were ~10-fold higher at 21 and 80 dpf relative to adult muscle tissue. *Onmy-GS03* expression was 71- and 107-fold higher at 21 dpf relative to levels in muscle and liver, respectively. *Onmy-GS02* and *-GS04* expression were relatively low throughout early development.

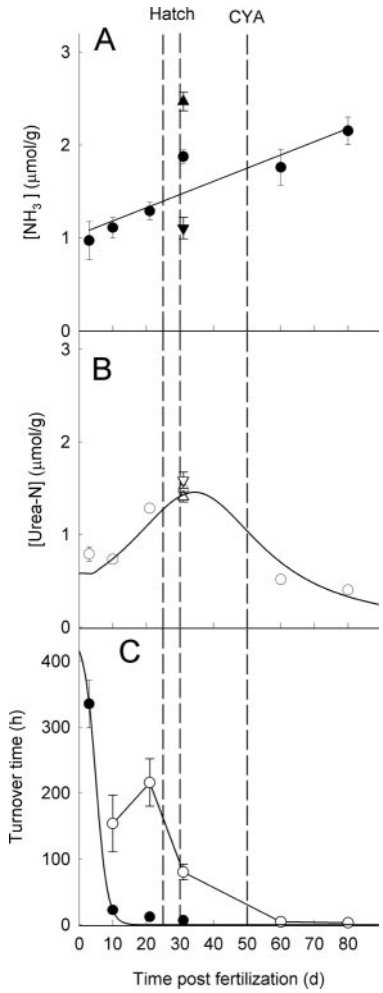

FIG. 2. **Changes in the concentrations of ammonia and urea-nitrogen concentrations during early development in rainbow trout embryos.** Values are means ± S.E. ($n = 6$), *vertical lines* demarcate the time of hatching between 25 and 30 dpf, and *CYA* = time of complete yolk absorption. *A*, increase in the concentration of ammonia; *solid line* is least squares regression for the total (larvae plus yolk). Ammonia concentration in the yolk (*upward triangle*) was significantly greater than that in the isolated larvae (*downward triangle*) at 31 dpf. *B*, change in the concentration of urea-nitrogen; *solid line* is least squares non-linear regression (Lorentzian, $r^2 = 0.87$) for the total (larvae plus yolk). Concentration in the yolk (*upward triangle*) was significantly less than that in the isolated larvae (*downward triangle*) at 31 dpf. *C*, turnover time (time it would take to clear the concentration of ammonia or urea-nitrogen calculated from the excretion rates in Fig. 1 and the concentrations in *A* and *B* for ammonia (*filled symbols*), *solid line* is nonlinear least squares sigmoidal regression and for urea-nitrogen (*open symbols*). Urea-nitrogen turnover time is not plotted at 3 dpf, because urea-nitrogen excretion was not different from zero at that time ($t = 1.89$, $p = 0.12$).

Glutamine synthetase activity in the whole embryo increased in a linear fashion during early life stages ($r^2 = 0.98$; Fig. 4). Glutamine synthetase activity was relatively low in all adult tissues examined, except brain, where activity was observed to be ~200-fold greater than that found in the next highest tissue (liver) (Tukey, $p < 0.05$; Fig. 5).

DISCUSSION

Four glutamine synthetase loci are found in rainbow trout. Two distinct evolutionary lineages, *Onmy-GS01/03* and *Onmy-GS02/04*, were identified (29) indicating at least two rounds of gene duplications. The gene pairs in each lineage most likely represent the results of the ancestral salmonid tetraploidization event estimated to have occurred 25–100 million years ago (37). The coding sequences of these isoforms are very similar, whereas the 3′-untranslated regions are more diverged. The nucleotide sequences of the 3′-untranslated region for *Onmy-GS01* and *-GS03* were 81% homologous, whereas *Onmy-GS02* and *-GS04* were 79% homologous. The low level of variation in the coding sequence of the isoforms made probe and primer design for mRNA analysis rather tricky. The approach we selected was real-time PCR using a gene-specific probe and set of primers that matched unique 3′-untranslated region sequences in each isoform. In preliminary trials, a subsample of each reaction was sequenced, and we confirmed that only one glutamine synthetase isoform was amplified for each set of primers.

The results of this study support our previous evidence (29) that the four isoforms of glutamine synthetase in trout (*Onmy-GS01–GS04*) are derived from separate genes. First, all four glutamine synthetase isoforms were expressed during early development and second, the relative mRNA levels varied between isoforms depending on the developmental age or tissue type. The pattern of mRNA expression observed suggests that *Onmy-GS01* and *-GS03* play a more important role during very early embryonic stages (14–31 dpf) compared with *Onmy-GS02* and *-GS04*. In other words, we presume that the functional octomeric enzyme would have a proportionally higher ratio of *Onmy-GS01* and *-GS03* subunits relative to *Onmy-GS02* and *-GS04* at these early stages. Interestingly, these gene pairings correspond to the two distinct evolutionary lineages identified above. It may be that the duplicated genes (*e.g. Onmy-GS01* and *-GS03*) have developed specialized regulatory subfunctions within the embryo. For example, at the time when we observed a large induction of *Onmy-GS01* and *-GS03* (14–21 dpf), the cerebral hemispheres thicken and become prominent (38). Further studies are necessary to determine if *Onmy-GS01* and *-GS03* mRNA in embryos levels are higher in brain tissue relative to other tissues and if these isoforms play a role in neural development.

The timing of the induction of glutamine synthetase during early life stages may have particular significance for ammonia detoxification. As stated in the Introduction, the encapsulated salmonid embryo catabolizes endogenous yolk proteins and amino acids resulting in ammonia generation at a time when ammonia elimination is not efficient. Indeed, ammonia concentrations rose by about 2-fold between 3 and 31 dpf, despite a steep linear increase in the rate of ammonia excretion. It should be noted that the higher yolk ammonia content relative to the embryonic body at 31 dpf probably is related to pH differences between these compartments (8). The elevation of ammonia in the embryo, however, may have been contained by the induction of glutamine synthetase. In fact, glutamine synthetase activity in young trout (*e.g.* 21 dpf) was comparable to activities in adult liver and other tissues. This is surprising given that the glutamine synthetase assay was performed on whole embryos (*i.e.* yolk plus embryonic body: 3, 10, and 21 dpf) where the presence of the relatively large yolk mass would dilute the embryonic tissue enzyme activity. Glutamine synthetase activities in the current study are consistent with those reported for adult trout (16, 39) and embryos (14). Our embryo data suggest that the early induction of *Onmy-GS01* and *-GS03* is preparatory for the increased capacity of the embryo to synthesize glutamine from excess ammonia before and just after hatching.

Glutamine produced via the glutamine synthetase reaction during embryogenesis may be stored or shuttled to other pathways, such as the OUC (5, 13, 14, 16). In the present study, urea was clearly synthesized before hatch. There was an increase in urea-nitrogen concentration when the rate of urea-

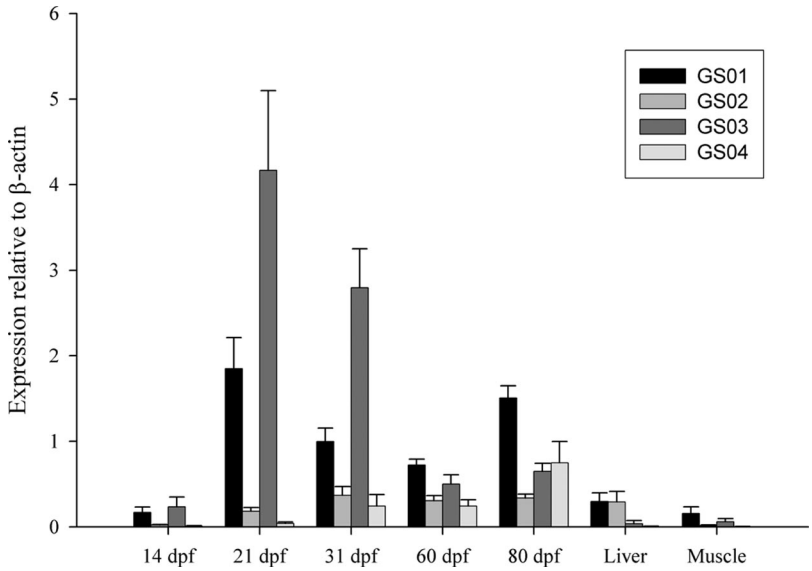

FIG. 3. **Changes in mRNA levels of glutamine synthetase genes (*Onmy-GS01–GS04*) in early life stages of rainbow trout relative to the expression of a control gene β-actin.** For comparative purposes, adult liver and skeletal muscle tissue are included. Statistical comparisons are provided in Table II. Values are means ± S. E. (n = 5 or 6 for embryo, n = 4 for liver and muscle).

TABLE II
Statistical comparisons in relative mRNA expression of glutamine synthetase genes (Onmy GS01–GS04) between early developmental and adult tissues of rainbow trout

dpf		14	Embryo												Liver		Skeletal muscle			
			21				31			60		80								
		GS04	*GS01*	*GS02*	*GS03*	*GS04*	*GS02*	*GS03*	*GS04*	*GS02*	*GS04*	*GS01*	*GS02*	*GS04*	*GS03*	*GS04*	*GS01*	*GS02*	*GS03*	*GS04*
14	*GS01*	—[a]	—[b]									—[b]								
	GS02						—[b]						—[b]							
	GS03	—[a]			—[b]															
	GS04													—[b]						
21	*GS01*					—[a]											—[b]			
	GS03		—[a]			—[a]									—[b]				—[b]	
31	*GS01*						—[a]													
	GS02						—[a]											—[b]		
	GS03							—[a]											—[b]	
60	*GS01*									—[a]	—[a]									
	GS04															—[b]				
80	*GS01*												—[a]				—[b]			
	GS02																	—[b]		
	GS04															—[b]				—[b]

[a] Significantly different from a different gene at the same developmental stage ($p < 0.05$).
[b] Significantly different from the same gene at a different developmental stage ($p < 0.05$).

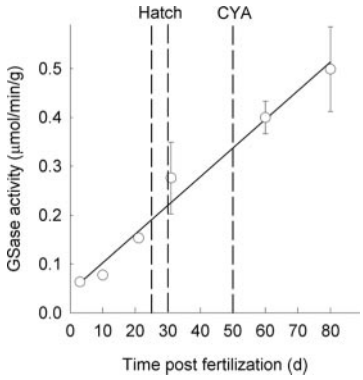

FIG. 4. **Changes in the activity of glutamine synthetase during early development in trout embryos.** Values are means ± S.E. (n = 6), *vertical lines* demarcate the time of hatching between 25 and 30 dpf, and *CYA* = time of complete yolk absorption. The *solid line* is least squares linear regression.

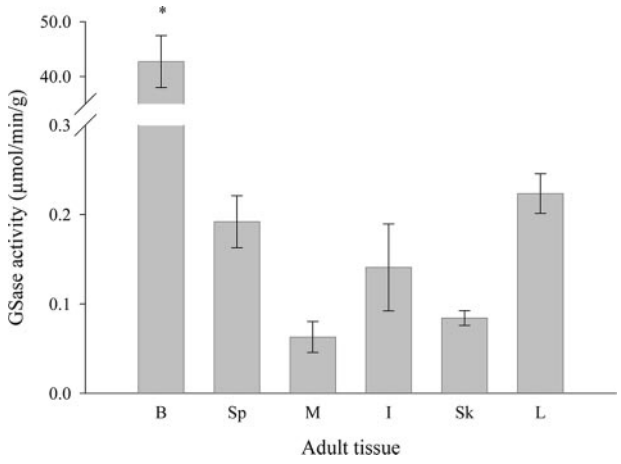

FIG. 5. **Activity of glutamine synthetase in six tissues of adult rainbow trout.** Values are means ± S.E. (n = 6). An *asterisk* denotes a significant difference in glutamine synthetase activity relative to other tissues. *B*, brain; *Sp*, spleen; *M*, white muscle; *I*, large intestine; *Sk*, skin; *L*, liver.

nitrogen excretion remained very low. The key OUC enzyme, carbamoyl-phosphate synthetase III, along with other OUC enzymes is induced before hatching in rainbow trout (5, 14, 16). Indeed, carbamoyl-phosphate synthetase III mRNA was detected as early as 3 dpf but peaked at 10–14 dpf in trout embryos raised under similar conditions to the present study (16). Thus, part of the glutamine synthesized in the embryo is undoubtedly converted to urea via the OUC.

Urea excretion in young trout constitutes a relatively small

component of total nitrogen excretion (8–22%), similar to adult trout (reviewed in Ref. 40). The rate of urea elimination was initially slow and urea accumulated in the embryo prior to hatching, resulting in relatively long turnover times (~1 week) compared with ammonia (0.5–1 day). The observed differences in turnover time may relate to the lower permeability of urea relative to ammonia (adult rainbow trout gill: urea 2.6×10^{-6} cm/s (41) *versus* NH_3, 1.5×10^{-4} cm/s − 2.3×10^{-3} cm/s (42)). Urea excretion is dependent, in part, on a phloretin-sensitive, saturable (K_m = 2 mM) urea transporter in rainbow trout embryos (43), as has been documented in other teleost tissues (44–46). If the full expression of this transporter does not occur until after the gills are completely functional (at hatch gills account for only ~4% of the potential respiratory surface area (9)), then this might explain the longer urea turnover times. The developmental timing of the rainbow trout urea transporter is unknown, but warrants further study.

We compared *Onmy-GS01–GS04* mRNA levels in young trout with two adult tissues, liver and skeletal muscle. Overall, expression of the four glutamine synthetase isoforms in these two adult tissues was very low, with only trace levels of *Onmy-GS04*. The lack of *Onmy-GS04* expression in liver and muscle tissue agrees with Northern analysis of trout tissues (29). Taken together, one might suspect that *Onmy-GS01*, *-GS02*, *-GS03*, and *-GS04* are not co-expressed in adult tissues, but this is not the case. In a separate study, we have detected significant levels of mRNA for all four glutamine synthetase isoforms in three regions of the rainbow trout brain.[3] The glutamine synthetase activity in the brain is three orders of magnitude higher compared with other adult tissues. Brain glutamine synthetase has an important role in regulating neurotransmitter metabolism, as well as detoxifying ammonia (47). Hence, there is differential expression of *Onmy-GS01–GS04* in adult trout tissues, and the pattern of expression in adult tissue does not follow the *Onmy-GS01/03* and *-GS02/04* pairings observed during early life stages.

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REFERENCES

1. Korsgaard, B., Mommsen, T. P., and Wright, P. A. (1995) in *Nitrogen Metabolism and Excretion* (Walsh, P. J., and Wright, P. A., eds) pp. 259–287, CRC Press, Boca Raton
2. Ip, Y. K., Chew, S. F., and Randall, D. J. (2001) in *Fish Physiology, Vol. 20: Nitrogen Excretion* (Wright, P. A., and Anderson, P. M., eds) pp. 109–148, Academic Press, San Diego
3. Randall, D. J., and Tsui, T. K. N. (2002) *Mar. Pollut. Bull.* **45,** 17–23
4. Wright, P. A., and Fyhn, H. J. (2001) in *Fish Physiology, Vol. 20: Nitrogen Excretion* (Wright, P. A., and Anderson, P. M., eds) pp. 149–186, Academic Press, San Diego
5. Wright, P. A., Felskie, A., and Anderson, P. M. (1995) *J. Exp. Biol.* **198,** 127–135
6. Terjesen, B. F., Verreth, J., and Fyhn, H. J. (1997) *Fish Physiol. Biochem.* **16,** 311–321
7. Smith S. (1957) in *Metabolism* (Brown, M. E., ed) pp. 323–359, Academic Press, New York
8. Rahaman-Noronha, E., O'Donnell, M. J., Pilley, C. M., and Wright, P. A. (1996) *J. Exp. Biol.* **199,** 2713–2723
9. Rombough, P. J., and Moroz, B. M. (1990) *J. Exp. Biol.* **154,** 1–12
10. Rombough, P. J., and Moroz, B. M. (1997) *J. Exp. Biol.* **200,** 2459–2468
11. Anderson, P. M., and Casey, C. A. (1984) *J. Biol. Chem.* **259,** 456–462
12. Griffith, R. W. (1991) *Env. Biol. Fishes* **32,** 199–218
13. Dépêche, J., Gilles, R., Daufresne, S., and Chiapello, H. (1979) *Comp. Biochem. Physiol.* **63A,** 51–56
14. Steele, S. L., Chadwick, T. D., and Wright, P. A. (2001) *J. Exp. Biol.* **204,** 2145–2154
15. Rice, S. D., and Stokes, R. M. (1974) in *The Early Life History of Fish* (Blaxter, J. H. S., ed) pp. 325–337, Springer-Verlag, New York
16. Korte, J. J., Salo, W. L., Cabrera, V. M., Wright, P. A., Felskie, A. K., and Anderson, P. M. (1997) *J. Biol. Chem.* **272,** 6270–6277
17. Chadwick, T., and Wright, P. A. (1999) *J. Exp. Biol.* **202,** 2653–2662
18. Terjesen, B. F., Rønnestad, I., Norberg, B., and Anderson, P. M. (2000) *Comp. Biochem. Physiol.* **126,** 521–535
19. Terjesen, B. F., Chadwick, T. D., Verreth, J. A., Rønnestad, I., and Wright, P. A. (2001) *J. Exp. Biol.* **204,** 2155–2165
20. Barimo, J. F., Steele, S. L., Wright, P. A., and Walsh, P. J. (2004) *J. Exp. Biol.* **207,** 2011–2020
21. Smirnov, A. V., Tumani, H., Henne, S., Barchfeld, S., Olgemoller, U., Wiltfang, J., Lange, P., Mader, M., and Nau, R. (2000) *Clin. Chim. Acta* **292,** 1–12
22. Mearow, K. M., Mill, J. F., and Vitkovic, L. (1989) *Mol. Brain Res.* **6,** 223–232
23. Fucci, L., Piscopo, A., Aniello, F., Branno, M., Di Gregorio, A., Calogero, R., and Geraci, G. (1995) *Gene* **152,** 205–208
24. Caggese, C., Barsanti, P., Viggiano, L., Bozzetti, M. P., and Caizzi, R. (1994) *Genetica* **94,** 275–281
25. Nagler, J. J. (2000) *Fish Physiol. Biochem.* **22,** 61–66
26. Walsh, P. J., Handel-Fernandez, M. E., and Vincek, V. (1999) *Comp. Biochem. Physiol.* **124B,** 251–259
27. Walsh, P. J., Meyer, G. D., Medina, M., Bernstein, M. L., Barimo, J. F., and Mommsen, T. P. (2003) *J. Exp. Biol.* **206,** 1523–1533
28. Mommsen, T. P., Busby, E. R., von Schalburg, K. R., Evans, J. C., Osachoff, H. L., and Elliot, M. E. (2003) *J. Comp. Physiol. B.* **173,** 419–427
29. Murray, B. W., Busby, E. R., Mommsen, T. P., and Wright, P. A. (2003) *J. Exp. Biol.* **206,** 1511–1521
30. Laud, P. R., and Campbell, J. W. (1994) *J. Mol. Evol.* **39,** 93–100
31. Sakamoto, T., Danzmann, R. G., Gharbi, K., Howard, P., Ozaki, A., Sokkean, K., Woram, R. A., Okamoto, N., Ferguson, M. M., Holm, L.-E., Guyomard, R., and Hoyheim, B. (2000) *Genetics* **155,** 1331–1345
32. Verdouw, N. E., van Echted, C. J. A., and Dekkers, E. M. J. (1978) *Water Res.* **12,** 399–402
33. Rahmatullah, M., and Boyde, T. R. C. (1980) *Clin. Chim. Acta* **107,** 3–9
34. Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., and Mathieu, C. (2001) *Methods* **25,** 386–401
35. Biliau, A. D., Sefrioui, H., Overbergh, L., Rutgeerts, O., Goebels, J., Mathieu, C., and Waer, M. (2001) *Transplantation* **71,** 292–299
36. Shankar, R. A., and Anderson, P. M. (1985) *Arch. Biochem. Biophys.* **239,** 248–259
37. Allendorf, F. W., and Thorgaard, G. H. (1984) in *Evolutionary Genetics of Fishes* (Turner, B. J. ed) pp. 1–46, Plenum Press, New York
38. Ballard, W. W. (1973) *J. Exp. Zool.* **184,** 7–26
39. Todgham, A., Anderson, P. M., and Wright, P. A. (2001) *Comp. Biochem. Physiol. A.* **129,** 527–539
40. Wood, C. M. (1993) in *The Physiology of Fishes* (Evans, D. H., ed) pp. 379–425, CRC Press, Boca Raton
41. Pärt, P., Wright, P. A., and Wood, C. M. (1998) *Comp. Biochem. Physiol.* **119A,** 117–123
42. Kelly, S., and Wood, C. (2001) *J. Exp. Biol.* **204,** 4115–4124
43. Pilley, C. M., and Wright, P. A. (2000) *J. Exp. Biol.* **203,** 3199–3207
44. Walsh, P. J., Heitz, M., Campbell, C. E., Cooper, G. J., Medina, M., Wang, Y. S., Goss, G. G., Vincek, V., Wood, C. M., and Smith, C. P. (2000) *J. Exp. Biol.* **203,** 2357–2364
45. Walsh, P. J., Grosell, M., Goss, G. G., Bergman, H. L., Bergman, A. N., Wilson, P., Laurent, P., Alper, S. L., Smith, C. P., Kamunde, C., and Wood, C. M. (2001) *J. Exp. Biol.* **204,** 509–520
46. Mistry, A. C., Honda, S., Hirata, T., Kato, A., and Hirose, S. (2001) *Am. J. Physiol.* **281,** R1594–R1604
47. Cooper, A. J. L., and Plum, F. (1987) *Physiol. Rev.* **67,** 440–519

[3] P. Wright, S. Steele, A. Huitema, and N. Bernier, unpublished observations.