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Rhesus Glycoprotein and Urea Transporter Genes Are Expressed in Early Stages of Development of Rainbow Trout (*Oncorhynchus mykiss*)

CARRIE C. HUNG[1,2], C. MICHELE NAWATA[2], CHRIS M. WOOD[2], AND PATRICIA A. WRIGHT[1]*

[1]*Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada*

[2]*Department of Biology, McMaster University, Hamilton, Ontario, Canada*

ABSTRACT The objective of this study was to determine if the genes for the putative ammonia transporters, Rhesus glycoproteins (Rh) and the facilitated urea transporter (UT) were expressed during early development of rainbow trout, *Oncorhynchus mykiss* Walbaum. We predicted that the Rh isoforms Rhbg, Rhcg1 and Rhcg2 would be expressed shortly after fertilization but UT expression would be delayed based on the ontogenic pattern of nitrogen excretion. Embryos were collected 3, 14 and 21 days postfertilization (dpf), whereas yolk sac larvae were sampled at 31 dpf and juveniles at 60 dpf (complete yolk absorption). mRNA levels were quantified using quantitative polymerase chain reaction and expressed relative to the control gene, elongation factor 1α. All four genes (Rhbg, Rhcg1, Rhcg2, UT) were detected before hatching (25–30 dpf). As predicted, the mRNA levels of the Rh genes, especially Rhcg2, were relatively high early in embryonic development (14 and 21 dpf), but UT mRNA levels remained low until after hatching (31 and 60 dpf). These findings are consistent with the pattern of nitrogen excretion in early stages of trout development. We propose that early expression of Rh genes is critical for the elimination of potentially toxic ammonia from the encapsulated embryo, whereas retention of the comparatively benign urea molecule until after hatch is less problematic for developing tissues and organ systems. *J. Exp. Zool. 309A:262–268, 2008.*

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Nitrogen waste excretion during early life stages in fish presents three unique challenges not encountered by later stages. First, endogenous feeding involves a high rate of amino acid and protein catabolism resulting in the continuous production of potentially toxic ammonia (for a review, see Wright and Fyhn, 2001). Second, as embryos lack functional gills, nitrogen excretion to the environment is presumed to be cutaneous during early life stages. Even well after hatch, the skin accounts for at least 40% of the respiratory surface area in salmonids at the end of the larval stage (Rombough and Moroz, '90; Rombough and Ure, '91; Wells and Pinder, '96). Third, before hatch embryos are encapsulated in an acellular coat or chorion. The rainbow trout chorion is permeable to ammonia (Smith, '47; Rice and Stokes, '74; Wright et al., '95; Rahaman-Noronha et al., '96; Steele et al., 2001; Essex-Fraser et al., 2005), but the presence of the chorion adds an additional diffusion barrier that impedes oxygen diffusion (Ciuhandu et al., 2007) and likely nitrogen excretion. To ensure that ammonia wastes do not accumulate and adversely impact sensitive developmental processes, embryos must either convert ammonia to nontoxic by-products, such as urea or glutamine, or eliminate ammonia.

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*Correspondence to: Patricia A. Wright, Department of Integrative Biology, University of Guelph, Guelph, Ont., Canada N1G 2W1. E-mail: patwrigh@uoguelph.ca

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The incorporation of ammonia into glutamine, catalyzed by glutamine synthetase (GSase), is the first step in the fish urea cycle (Anderson, '95). In trout, urea synthesis in embryos was first demonstrated by Dépêche et al. ('79) and urea cycle enzymes have been detected in early development (Rice and Stokes, '74; Wright et al., '95; Korte et al., '97; Steele et al., 2001). Indeed, the levels of GSase and urea cycle enzyme activities are high in early life stages relative to adult liver levels (Wright et al., '95; Korte et al., '97). The ontogenic patterns of gene expression for GSase and the key urea cycle enzyme, carbamoyl phosphate synthetase III (CPSase III), in trout are similar. The levels of mRNA of two of the four GSase genes were first detected at 14 days postfertilization (dpf), with highest levels at 21 dpf (Essex-Fraser et al., 2005), whereas CPSase III mRNA levels were detected at 3 dpf and peaked at 10–14 dpf (Korte et al., '97). Hence, the capacity to synthesize glutamine and urea are in place well before hatching.

Ammonia and urea concentrations continuously rise in trout during embryonic development (Wright et al., '95; Essex-Fraser et al., 2005), indicating that elimination of nitrogenous waste products does not keep pace with the rate of synthesis. In trout, ammonia excretion dominates over urea excretion during early life stages (Smith, '47; Rice and Stokes, '74; Wright et al., '95; Wright and Land, '98; Essex-Fraser et al., 2005). In fact, the turnover time (time it would take to clear the concentration of ammonia or urea–nitrogen calculated as tissue content divided by excretion rate) of ammonia is very low compared with urea until well after hatch (Essex-Fraser et al., 2005). Rainbow trout embryos excrete ammonia primarily in the nonionic form NH_3 (Rahaman-Noronha et al., '96), a process previously assumed to be via simple diffusion through the lipid bilayer membrane. The NH_3 partial pressure (P_{NH_3}) gradient from the embryo to the environment is maintained by an acidic unstirred layer of water next to the chorion (Rahaman-Noronha et al., '96). As NH_3 passes through pores in the chorion into the unstirred layer, NH_3 combines with H^+ to form NH_4^+, thereby maintaining the P_{NH_3} gradient.

Urea excretion in trout embryos is dependent, in part, on a phloretin-sensitive facilitated urea transporter (UT; Pilley and Wright, 2000), with properties similar to that reported in other fish (e.g. Walsh et al., 2000, 2001; Mistry et al., 2001; Walsh and Smith, 2001; Morgan et al., 2003; McDonald and Wood, 2004). Recently, Rhesus glycoproteins (Rh genes) have been associated with facilitation of NH_3/NH_4^+ movement across microbial, plant and animal cells (Huang and Peng, 2005). A controversy exists in the literature about whether some Rh genes code for proteins that transport the NH_3 gas or the NH_4^+ ion and whether transport is passive or active (Bakouh et al., 2004; Khademi et al., 2004; Nakhoul et al., 2005). In aquatic species, an Rh isoform (Rh-CM) was first identified in the gills of the crab *Carcinus maenas* (Weihrauch et al., 2004). In rainbow trout (Nawata et al., 2007), as well as the puffer fish *Takifugu rubripes* (Nakada et al., 2007a) and mangrove killifish *Kryptolebias marmoratus* (Hung et al., 2007), several isoforms of Rh genes are expressed. In trout gill, Rhbg, Rhcg1 and Rhcg2 are constitutively expressed and Rhcg2 mRNA levels are upregulated under conditions of high external ammonia, possibly to facilitate ammonia excretion to the environment (Nawata et al., 2007). Recently, Rhcgl expression has been detected in zebra fish *Danio rerio* larvae (Nakada et al., 2007b). Given the expression of Rh isoforms in excretory tissues of adult fish and the implication of Rh proteins in NH_3/NH_4^+ transport, it is highly likely that Rh genes are expressed during early salmonid development when elimination of ammonia is crucial.

The aim of this study was to determine if the genes coding for the Rh and UT proteins were expressed during early life stages in rainbow trout (*Oncorhynchus mykiss*). We hypothesized that the ontogenic pattern of gene expression would correlate with the established rates of ammonia and urea excretion during early life stages. If true, then the Rh isoforms (Rhbg, Rhcg1 and Rhcg2) would be expressed shortly after fertilization but UT expression would be delayed. Using quantitative polymerase chain reaction (Q-PCR), we measured relative mRNA levels of three Rh isoforms (Rhbg, Rhcg1, Rhcg2) and UT in trout embryos, yolk sac larvae and juveniles. The pattern of expression was compared with ammonia and urea excretion rates and turnover times measured previously in the same batch of individuals (Essex-Fraser et al., 2005).

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*O. mykiss* Walbaum) embryos were purchased on the day of fertilization from Rainbow Springs Trout Farm (Thamesford,

Ontario, Canada). The embryos were maintained as described in Essex-Fraser et al. (2005). The same batch of fish was used in both studies.

cDNA synthesis and Q-PCR analysis

Total RNA was extracted from rainbow trout embryos and larvae of different developmental stages including 3, 14, 21, 31 and 60 dpf using Trizol (Invitrogen Canada Inc., Burlington, Ontario, Canada). RNA samples obtained were checked by Bioanalyzer (Agilent Technologies, Mississauga, Ontario, Canada) to ensure that integrity was intact. Two micrograms of RNA was used per sample for cDNA synthesis. Before cDNA synthesis, RNA was treated with DNase I (Invitrogen) to prevent any genomic DNA contamination. First-strand cDNA was synthesized using superscript reverse transcriptase II (Invitrogen) with an adaptor oligodT primer ($GACTCGAGTCGACATCGAT_{17}$).

mRNA expression levels of *Rhbg* (GenBank EF051113/EF051114), *Rhcg1* (GenBank DQ431244/EF051115), *Rhcg2* (GenBank AY619986) and *UT* (GenBank EF688013) were quantified from the above cDNA products. In this study, two control genes, elongation factor—1α (*EF1α* GenBank AF498320) and β-*actin* (GenBank AF157514) were tested for consistency of expression between developmental stages. For each Q-PCR reaction, 5 μL of 1:4 dilution cDNA sample was used for the target (*Rh* and *UT* genes) and control genes (*EF1α* and β-*actin* genes). Primers used in this study are listed in Table 1 (*Rhbg*, *Rhcg1*, *Rhcg2*, *UT*, *EF1α* and β-*actin*).

The quantification of gene expression was performed using RT^2 Real-Time^{TM} SYBR Green/ROX (Applied Biosystems, Warrington, UK) with thermal cycle: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Samples were assayed in triplicate with only one target gene assayed per well. For each gene, a standard curve was performed for each gene to ensure that the Q-PCR amplification efficiency was above 95% with their respective primer pair. Specificity of each primer pair was verified by obtaining one single dissociation curve. This is further confirmed with gel electrophoresis to ensure that only a single PCR product of the desired size was obtained for each reaction. Q-PCR products of all five genes were gel purified (Montage PCR filter unit, Millipore, Mississauga, Ontario, Canada), sequenced and confirmed that each primer set only amplified its target sequence. Nonreverse transcribed RNA and water-only controls Q-PCR were also conducted to ensure that no genomic DNA was being amplified and that reagents were not contaminated.

Statistical analysis for mRNA expression was performed using Statistix software (Analytical Software, Tallahassee, FL). One-way analysis of variance was followed by the least significant difference test to determine where significant differences were present ($P < 0.05$). All values have been presented as means ± SE ($N = 8$).

TABLE 1. Primers used in this study[1]

Primer	Sequence 5′→3′
Rhbg forward	CGA CAA CGA CTT TTA CTA CCG C
Rhbg reverse	GAC GAA GCC CTG CAT GAG AG
Rhcg1 forward	CAT CCT CAG CCT CAT ACA TGC
Rhcg1 reverse	TGA ATG ACA GAC GGA GCC AAT C
Rhcg2 forward	CCT CTT CGG AGT CTT CAT C
Rhcg2 reverse	CTA TGT CGC TGG TGA TGT TG
UT forward	GTA TAG GCC AGG TGT ATG GG
UT reverse	GAT CGC CTC AAA TGG AGC TG
EF1α forward	GGA AAG TCA ACC ACC ACA G
EF1α reverse	GAT ACC ACG CTC CCT CTC AG
β-Actin forward	ACT GGG ACG ACA TGG AGA AG
β-Actin reverse	AGG CGT ATA GGG ACA ACA CG

UT, urea transporter; EF1α, elongation factor 1α.
[1]Primers were previously published by Nawata et al. (2007) with the exception of UT primers.

RESULTS

mRNA levels of control genes, EF1α and β-actin

The pattern of expression of two control genes was similar during early development in trout (Table 2). At 3 dpf, EF1α and β-actin were below the level of detection. EF1α mRNA levels did not vary significantly between 14 and 60 dpf, whereas β-actin levels were significantly lower at 14 dpf vs. later stages (21–60 dpf). Hence, EF1α was selected as the most consistent control gene.

mRNA levels of Rh genes

All three Rh genes, Rhbg, Rhcg1 and Rhcg2, were expressed in rainbow trout embryos starting at 14 dpf (Fig. 1). Although Rhbg was detected, mRNA levels remained generally low between 14 and 60 dpf. In contrast, Rhcg1 mRNA levels increased ~2-fold between 14 and 60 dpf. The Rh gene with the highest mRNA levels was Rhcg2. For example, at 60 dpf Rhcg2 mRNA levels were 145- and 18-fold greater compared with Rhbg and Rhcg1 mRNA levels, respectively. Rhcg2 mRNA

TABLE 2. Relative mRNA expression levels of two control genes: (a) EF1α and (b) β-actin

dpf	Mean EF1α mRNA expression[1]	SEM
(a)		
3	Below detection limit	NA
14	0.691	0.261
21	1.178	0.417
31	1.294	0.457
60	1.109	0.392
	Mean β-actin mRNA expression	SEM
(b)		
3	Below detection limit	NA
14	0.317[2]	0.120
21	1.192	0.422
31	1.798	0.559
60	1.798	0.636

EF1α, elongation factor 1α; ANOVA, analysis of variance; dpf, days postfertilization.
[1]No significant difference ($P > 0.05$) was observed among EF1α mRNA expression levels at different developmental stages; $N = 8$, (one-way ANOVA).
[2]Indicates that mRNA expression level of β-actin at day 14 dpf was significantly lower ($P < 0.05$) than at days 21, 31 and 60 dpf; $N = 8$.

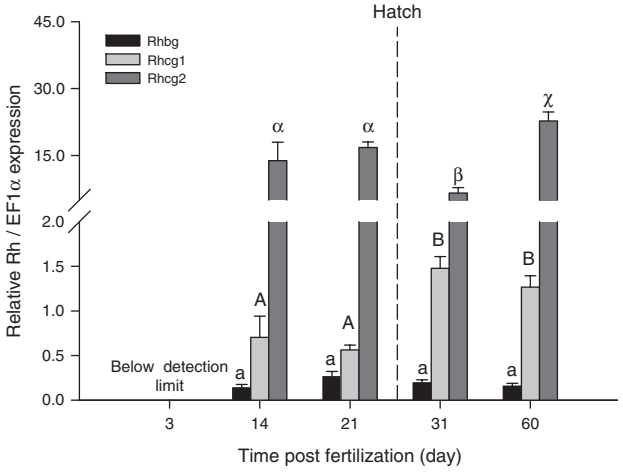


Fig. 1. Changes in mRNA levels of Rhesus glycoprotein genes (Rhbg, Rhcg1, Rhcg2) in early life stages of rainbow trout (*O. mykiss*) relative to the expression of a control gene elongation factor 1α (EF1α). At 3 days postfertilization (dpf) mRNA levels were below the detection limit. Lowercase letters that are not shared among bars indicate significant differences in Rhbg mRNA levels between different developmental stages ($P < 0.05$). Uppercase letters that differ indicate significant differences in Rhcg1 mRNA levels between different developmental stages ($P < 0.05$). Greek letters that differ indicate significant difference in Rhcg2 mRNA levels between different developmental stages ($P < 0.05$). Vertical dashed line indicates hatch time between 25 and 30 dpf. Values are means ± SEM; $N = 8$ (one-way ANOVA, $P < 0.05$). ANOVA, analysis of variance.

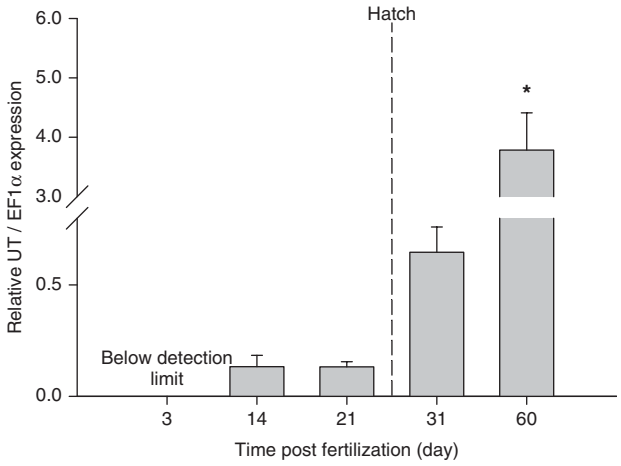


Fig. 2. Changes in mRNA levels of a urea transporter gene (UT) in early life stages of rainbow trout (*O. mykiss*) relative to the elongation factor 1α (EF1α). At 3 days postfertilization (dpf) mRNA levels were below the detection limit. Asterisk indicates significant difference between 60 dpf and all other stages ($P < 0.05$). Vertical dashed line indicates hatch time between 25 and 30 dpf. Values are means ± SEM; $N = 8$ (one-way ANOVA, $P < 0.05$). ANOVA, analysis of variance.

levels remained relatively high between 14 and 60 dpf; however, there was a significant (2.5-fold) decline between 21 and 31 dpf and an increase (3.5-fold) between 31 and 60 dpf.

mRNA levels of UT

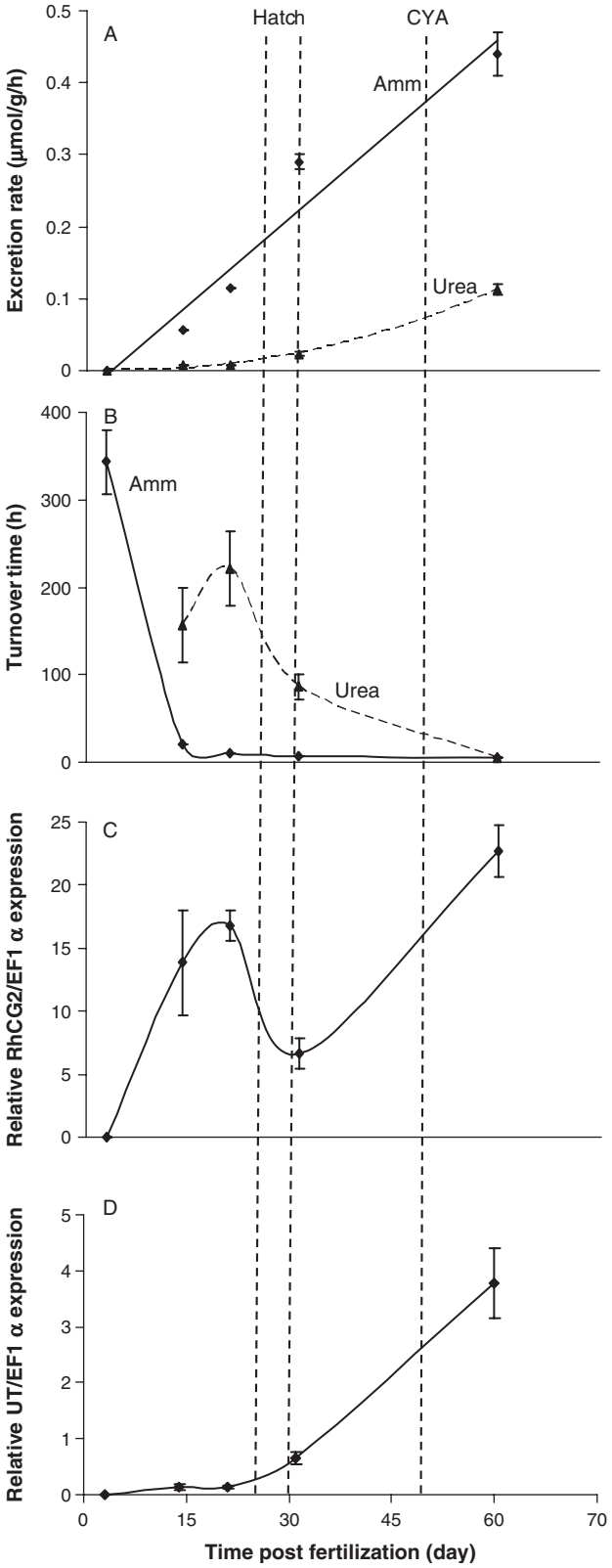
UT mRNA levels were detected at all stages except 3 dpf (Fig. 2). The expression up to 60 dpf followed a pattern of exponential increase; mRNA levels at 60 dpf were ~28-fold higher relative to levels at 14 dpf.

Ammonia and urea excretion

Ammonia excretion rates were increased linearly over developmental time and substantially higher than urea excretion rates (Fig. 3A). Turnover time or the time it would take to clear the concentration of ammonia or urea–nitrogen (N; calculated from the ammonia or urea–N content (μmol/g) over the ammonia or urea–N excretion rate (μmol/g/h), respectively; Essex-Fraser et al., 2005) was initially high during the embryonic states (Fig. 3B) for both ammonia and urea. Urea turnover time remained elevated until 60 dpf, whereas ammonia turnover times decreased to relatively low levels by 21 dpf (Fig. 3B).

DISCUSSION

The results presented here indicate that relatively high levels of mRNA for one of the Rh



genes, Rhcg2, are present in rainbow trout embryos well before hatching. This early induction of a putative ammonia transporter (Figs. 2 and 3C) correlates with the increasing rates of ammonia excretion (Fig. 3A) and decreasing ammonia turnover times (Fig. 3B) over the same time interval. In other words it takes less time for the embryo to clear ammonia from the body to the environment as hatching approaches and this may be linked, in part, to the induction of Rhcg2.

There is evidence that Rhcg2 is involved in NH_3/NH_4^+ transport in fish. When cRNA of puffer fish gill Rhcg2 was injected into oocytes, transport of the ammonia analog, methylammonia was accelerated over that in control water-injected oocytes (Nakada et al., 2007a,b). In both gills and skin of adult rainbow trout, the mRNA level of Rhcg2 was upregulated more than 6-fold within 12 hr of exposure to elevated external ammonia (Nawata et al., 2007). As well, similar findings were reported in the mangrove killifish *K. marmoratus* where both skin and gill Rhcg2 mRNA levels were many folds higher in response to high environmental ammonia (Hung et al., 2007). Skin Rhcg1 and Rhcg2 mRNA levels were also significantly elevated after 24 hr of air exposure in killifish (Hung et al., 2007), a fish that volatilizes NH_3 across its cutaneous surface when in air (Frick and Wright, 2002; Litwiller et al., 2006). It appears therefore that the Rhcg2 gene is upregulated during early development, during high environmental ammonia exposure and under terrestrial conditions in fish, all situations where internal ammonia concentrations are elevated. Facilitation of NH_3/NH_4^+ transport via Rh proteins under such circumstances may be a critical strategy in avoiding the toxic accumulation of ammonia.

UT genes have not been studied in any salmonid species to our knowledge. We predicted that UT

Fig. 3. A comparison between ammonia and urea excretion rates (panel A), turnover time (time it would take to clear the concentration of ammonia or urea–nitrogen = content (μmol/g)/excretion rate (μmol/g/h); panel B), with changes in relative Rhcg2 mRNA levels (panel C) and relative UT mRNA levels (panel D). The data of panels A+B are taken from Essex-Fraser et al. (2005). In panel B, urea turnover time was not plotted at 3 dpf because urea–nitrogen excretion was not different from zero at that time. Panel C+D data are replotted from Figures 1 and 2, respectively. Values are means ± SEM. It should be noted that tissues collected for the Essex-Fraser et al. (2005) study were used in this study. Vertical lines demarcate the time of hatching between 25 and 30 dpf and CYA = complete yolk absorption. UT, urea transporter; dpf, days postfertilization.

expression would be delayed relative to Rh gene expression because of the distinct pattern of urea excretion in early development in trout. In agreement with this prediction, UT mRNA levels were very low before hatch (<21 dpf) consistent with an extremely low rate of urea excretion and comparatively high turnover times (Fig. 3). Urea content is on par with ammonia content (~1.2 μmol/g) before hatch in trout embryos (Essex-Fraser et al., 2005), but urea clearance is impeded relative to ammonia and this may be owing to a low number of UT proteins. Urea solubility is quite low in lipid bilayers (Gallucci et al., '71) and in recent years UT genes have been isolated in a variety of fish tissues (see introduction). Using physiological and pharmacological methods, Pilley and Wright (2000) found evidence for the presence of a bidirectional carrier-mediated UT in trout embryos just before hatching; acute exposure to phloretin and urea analogs [acetamide, thiourea, 1, (4-nitrophenyl)-2-thiourea] reversibly inhibited urea excretion. Evidence for a facilitated UT is consistent with the low but detectable levels of UT mRNA at 21 dpf in this study.

After hatching between 31 and 60 dpf, there is a substantial decrease in the turnover time of urea (Fig. 3B) and an increase in the rate of urea excretion (Fig. 3A). Over this same time interval, UT mRNA levels were elevated by ~6-fold. Thus, the increase in UT mRNA levels may result in an increase in the number of UT proteins that, in turn, facilitate the elimination of urea from larval and juvenile tissues. By 60 dpf, the turnover times of ammonia and urea are matched, which indicates that urea elimination is far more efficient in juvenile fish compared with earlier stages.

The gills of embryos are nonfunctional until well after hatching (see introduction) and therefore we propose that Rhcg2 (also possibly Rhbg and Rhcg1) is expressed in the skin and yolk sac membrane of embryos. In the tropical freshwater zebra fish, Rhcgl was first detected at 3 dpf on the surface of the yolk sac and shortly after (4–5 dpf) in the gill of larvae (Nakada et al., 2007b). Rhcgl expression was associated with mitochondrion-rich cells in zebra fish larvae (Nakada et al., 2007b). It is unknown if other Rh isoforms are expressed during these early stages in this species. UT proteins may also be first expressed on the cutaneous surface of trout embryos; but once the gills are developed in later stages, we predict that UT expression is primarily branchial. In conclusion, our study is the first to demonstrate the time course of Rh and UT gene expression in salmonid early life stages. The ontogenic pattern of mRNA levels for the Rh and UT genes correlates with the ammonia and urea excretion rates in developing rainbow trout.

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