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Ontogeny of urea and ammonia transporters in mahi-mahi (*Coryphaena hippurus*) early life stages

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ABSTRACT

The mechanism(s) of ammonia and urea excretion in freshwater fish have received considerable attention; however, parallel investigations of seawater fish, specifically in the early life stages are scarce. The first objective of this study was to evaluate the patterns of ammonia and urea excretion in mahi-mahi (*Coryphaena hippurus*) up to 102 hours post fertilization (hpf). Similar to other teleosts, mahi embryos are ureotelic before hatch and gradually switch to being ammoniotelic around the time of hatch. The second objective was to characterize mRNA levels of ammonia transporters (Rhag, Rhbg, Rhcg1 and Rhcg2), as well as urea transporter (UT) and sodium hydrogen exchangers (NHE3 and NHE2) during mahi development. As predicted, the mRNA levels of the Rhesus glycoprotein (Rh) genes, especially Rhag, Rhbg and the UT gene were highly consistent with the ontogeny of ammonia and urea excretion rates. Further, the localization of each transporter was examined in larvae collected at 60 and 102 hpf using in situ hybridization. Rhag was expressed in the gills, yolk sac, and operculum. Rhbg was expressed in the gills and upper mouth. Rhcg1 and NHE3 were co-localized in the sub-operculum, and Rhcg2 was expressed in the skin. Together, these results indicate that urea excretion is critical for ammonia detoxification during embryonic development and that Rh proteins are involved in ammonia excretion via gills and yolk sac, possibly facilitated by NHE3.

1. Introduction

Most teleost fish rely on yolk amino acids and proteins as their main energy source before exogenous feeding starts (Finn et al., 1991; Finn et al., 1995; Fyhn and Serigstad, 1987). Catabolism of proteins and amino acids continuously produce ammonia, a waste product that is neurotoxic when present at elevated levels in blood and tissues (Felipo and Butterworth, 2002). To avoid ammonia toxicity, early life stages (ELS) of fish either excrete ammonia or convert ammonia into less-toxic urea via the ornithine-urea cycle (OUC). The latter mechanism was proposed by Griffith (Griffith, 1991) and supported by the detection of OUC enzyme activities in ELS of rainbow trout (*Oncorhynchus mykiss*) (Wright et al., 1995) as well as other ammoniotelic species (see review (Zimmer et al., 2017b)). In the following, "ammonia" refers to total ammonia as the sum of gaseous NH_3 and ionic NH_4^+, while "ammonium" specifically refers to ionic NH_4^+.

Traditionally, it was believed that small molecules such as NH_3 and urea were excreted by passive diffusion down the partial pressure or concentration gradients across the gill epithelium. However, with the recent discovery of urea transporters and Rhesus glycoprotein (Rh) proteins for ammonia transport, the views of nitrogenous waste excretion in fish have been modified. The urea transporter (UT) was first cloned from rats in 1993 (You et al., 1993), and has since been found in a variety of fishes (McDonald et al., 2006). During the ELS, UT gene expression has been detected in the gills and skin of rainbow trout, yet the subcellular localization remains to be further examined (Zimmer and Wood, 2016).

The role of Rh proteins in ammonia transport was first suggested in 1997 (Marini et al., 1997) based on their sequence similarity to the Mep/Amt family of ammonia transporters in yeast *Saccharomyces cerevisiae* (ScMep1) (Marini et al., 1994) and the plant *Arabidopsis thaliana* (AtAmt1) (Ninnemann et al., 1994). The substrate specificity was later demonstrated by expression of human RhAG in an ammonium-uptake-deficient yeast mutant, providing direct evidence of RhAG in ammonia transport (Marini et al., 2000). In fish, the first study of Rh proteins was in the marine pufferfish (*Takifugu rubripes*) where the function and location of four Rh proteins: fRhag, fRhbg, fRhcg1, and fRhcg2 were examined (Nakada et al., 2007b). Since then, one or more members of Rh proteins have been found in rainbow trout (Nawata et al., 2007), mangrove killifish (*Krytobelias marmoratus*) (Hung et al., 2007) and

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zebrafish (Nakada et al., 2007a; Shih et al., 2008). Rh proteins are strongly expressed in gills, but are also widely distributed in erythrocytes, brain, liver, kidney, muscle, spleen, yolk sac membrane, and skin (Braun et al., 2009; Hung et al., 2007; Nakada et al., 2007a; Nawata et al., 2007). Although there is controversy over the cellular location, Rhag, Rhbg and Rhcg proteins are generally found in the apical and basolateral gill membranes, respectively (Braun et al., 2009; Nakada et al., 2007a; Nakada et al., 2007b).

A recent model for ammonia excretion in freshwater fish incorporated Rh transporters, sodium hydrogen exchangers (NHEs) or H^+-ATPase into a 'Na^+/NH_4^+ exchange complex' linking ammonia excretion with Na^+ uptake (Wright and Wood, 2009). In this model, NHEs exchange one Na^+ with one H^+, which combines with NH_3 transported via Rhcg across the apical membrane to form NH_4^+, maintaining a favorable P_{NH3} across gill epithelia (Wright and Wood, 2009). H^+-ATPase also facilitates Na^+/NH_4^+ exchange by simultaneously driving Na^+ uptake via putative Na^+ channels and promoting boundary layer acidification that traps NH_3 as NH_4^+ (Dymowska et al., 2014; Wright and Wood, 2012). In ELS of fish, the role for Rh glycoproteins in ammonia uptake was directly demonstrated by morpholino gene knockdown in larval zebrafish (Braun et al., 2009). Additionally, studies on ELS have provided strong evidence for a role of NHEs in ammonia excretion and Na^+ uptake by forming a Rh-NHEs metabolon (Kumai and Perry, 2011; Shih et al., 2012).

Substantial work has been done in freshwater fish, however, our knowledge of the Rh proteins in seawater fish remains limited to the initial work on pufferfish, longhorn sculpin (*Myoxocephalus octodecemspinosus*) and a study on seawater-acclimated steelhead trout (Claiborne et al., 2008; Nakada et al., 2007b; Nawata et al., 2010; Wood and Nawata, 2011). In addition, there is a lack of information regarding nitrogenous waste excretion during the early developmental stages of pelagic marine fish. Therefore, the mahi-mahi (*Coryphaena hippurus*; hereafter referred to as "mahi"), a representative of marine pelagic teleosts was used in this research, to investigate the ontogeny of ammonia and urea excretion rates during the ELS of seawater fish. Furthermore, we examined the expression and localization pattern of the genes coding for Rh and UT proteins by using quantitative PCR (qPCR) and in situ hybridization. As hypothesized, ontogeny of gene expression correlated with ammonia and urea excretion rates. Expression of NHEs, possibly involved in ammonia excretion, were also measured.

2. Material and methods

2.1. Experimental animals

Mahi broodstock were captured off the coast of Miami, Florida, USA using hook and line angling techniques. The fish were then transferred to University of Miami Experimental Hatchery (UMEH) and acclimated in $80m^3$ fiberglass maturation tanks equipped with recirculating aquaculture systems for water and temperature control (IACUC protocol # 12–064). All embryos used in the experiments described herein were collected within 2 h to 6 h following a volitional (non-induced) spawn using standard UMEH methods (Stieglitz et al., 2017). A prophylactic formalin treatment (37% formaldehyde solution) was administered to freshly fertilized embryos, followed by a 0.5 h rinse with a minimum of 300% filtered, UV-sterilized seawater exchange by volume. A small sample of eggs was collected from each spawn to microscopically assess fertilization rate and embryo quality. Embryos from captive mahi at the UMEH show constant and high quality over time as assessed by hatch and larval survival (Kloeblen et al., 2018). Rare spawns demonstrating low fertilization rate (< 85%) or frequent developmental abnormalities (> 5%) were not used. Embryos were subsequently kept in rearing tanks in a temperature controlled environmental chamber (26.5 °C) with a 16:8-h light:dark cycle. Mahi embryos usually hatch between 42 and 46 h post fertilization (hpf), depending mainly on ambient temperature.

2.2. Determining ammonia and urea excretion rates

The defined starting point for the current experiment was approximately 6 hpf based on embryonic developmental stage and average observed spawning times (2–6 AM). Either 40 embryos or 10 yolk sac larvae were placed in individual wells with 2 ml of UV-sterilized seawater each, on a 12-well plate (Greiner bio-one, Austria). Four wells filled with seawater but no organisms were used to control and correct for background levels of ammonia or urea. The plate was then put into a temperature-controlled incubator. To measure urea and ammonia excretion rates, water samples (1.5 ml) were collected after 6 h of incubation and immediately frozen (−20 °C) for later determination of urea and ammonia concentrations.

Water samples were analyzed for ammonia and urea levels using Micromodified Indophenol Blue (Ivančič and Degobbis, 1984) and Diacetyl Monoxime (Boyde and Rahmatullah, 1980) assays, respectively. Excretion rate (picomoles N individual^{-1} min^{-1}) were corrected for background ammonia and urea levels and calculated from the change in concentration in pmol l^{-1} multiplied by the volume of the seawater, divided by the number of individuals per well and the elapsed time. Urea excretion rates were multiplied by 2 to account for the two nitrogen atoms removed with every molecule of urea.

2.3. cDNA synthesis, cloning and quantitative PCR (qPCR)

At least 20 mahi embryos and larvae were collected in triplicates from different developmental stages. Total RNA was extracted from frozen samples using RNA STAT-60 solution (Tel-Test, Friendsworth, TX) according to the manufacture's protocol. Total RNA was quantified by Spectra-Drop (SpectraMax Plus 384, Molecular Devices). For each isolate, 10 μg of total RNA was treated with DNase I (Turbo DNA-free Kit; Thermo Fisher Scientific, Waltham, MA) to remove genomic DNA contamination followed by gel electrophoresis to confirm RNA integrity. cDNA was synthesized from 1 μg DNase I-treated total RNA using the SuperScript IV First-Strand Synthesis System (Invitrogen, Waltham, MA). Final cDNA products were diluted tenfold in sterile water. An aliquot of each cDNA sample was taken and pooled for later cloning.

Fragments of EF1α, Rhag, Rhbg, Rhcg1, Rhcg2, UT, NHE2 and NHE3 were cloned from pooled cDNA using degenerate primers (Table 1). The primers were designed using an alignment made with genes from other teleost fish, such as large yellow croaker (*Larimichthys crocea*), bicolor damselfish (*Stegastes partitus*), Japanese pufferfish (*Takifugu rubripes*), Red drum (*Sciaenops ocellatus*) and black rockcod (*Notothenia coriiceps*). PCR reactions were performed using Taq DNA polymerase (Qiagen, Germantown, MD) and the following cycling

Table 1
Degenerate primer sets for initial cloning of gene fragment.

Gene	Product length, bp	Accession number	Sequence
ef1α	1505	MH550809	FWD 5′-CCACATCAACATCGTGGTCA-3′ REV 5′-TAATCAGCATAAGCCAGTCC-3′
Rhag	1203	MH500278	FWD 5′-CSTAYGCCACCAACATGAGG-3′ REV 5′-AGTTCTGGTCTGGAGGYTGT-3′
Rhbg	970	MH500279	FWD 5′-GCTTCATCCTGGAGATCATC-3′ REV 5′-GCCAGAACATCCACAGGTAG-3′
Rhcg1	904	MH500280	FWD 5′-AAGCGSTACAGCTTTGGTGC-3′ REV 5′-KATAGCCACACAGAGGCCTG-3′
Rhcg2	926	MH545690	FWD 5′-CTGCTTTGTCTGGCAGATTGC-3′ REV 5′-CCAAARGTGGAGATGATGCC-3′
UT	979	MH545692	FWD 5′-TGCTGATGGCYGTGTTCTCC-3′ REV 5′-AGGTGCACCTGCCAGGTGAG-3′
NHE2	875	MH545691	FWD 5′-AAGATCACCATCTGGGTGCC-3′ REV 5′-ATGTAGCCCCAGTTCCACTC-3′
NHE3	989	MH550810	FWD 5′-CTGCAAACTCATCATCGAGG-3′ REV 5′-GAACTTGTTCAGGATCCAGG-3′

Table 2
Primer sets used for qPCR.

Gene	Product Length, bp	Sequence
ef1α	144	FWD 5′-GCATCGACAAGAGAACCATC-3′ REV 5′-CAAACTTCCACAGAGCAATG-3′
Rhag	140	FWD 5′-CGTATGCCACCAACATGAGG-3′ REV 5′-GTCAGTCTCATTGCCATGGTG-3′
Rhbg	154	FWD 5′-CTACCCAAGTTTCCAGGATG-3′ REV 5′-GAAGAAGCCCTGCATGAGAG-3′
Rhcg1	162	FWD 5′-GTTCCACTCCCTGGACTACAG-3′ REV 5′-AGTGTGATCCCAAACAGTGTC-3′
Rhcg2	153	FWD 5′-ATATCCCAGCTTCCAGGATG-3′ REV 5′-AACCAGCCTTGCATGAGAAG-3′
UT	139	FWD 5′-ACTGGTACTGGTGGCTATTG-3′ REV 5′-TGGAGACACACTAGGATGTTG-3′
NHE2	153	FWD 5′-TCAGCATTGGTCTGATTGTGG-3′ REV 5′-TGTTCTCGAAGAAAGGTCTGG-3′
NHE3	144	FWD 5′-GTGACCAACATCATCCCAGAG-3′ REV 5′-GAGGATGACTTGAGGCAGCAG-3′

conditions: 94 °C for 3 min followed by 35 cycles at 94 °C for 30s, a temperature gradient of 55–60 °C for 1 min, and 72 °C for 1 min. PCR products were gel-purified using QIAquick Gel Extraction Kit (Qiagen, Germantown, MD), cloned using TOPO-TA vector (Thermo Fisher Scientific, Waltham, MA) and sequenced (Genewiz, Inc., South Plainfield, NJ, USA).

Primers (Table 2) used for qPCR were designed based on the sequence information obtained from the previous step. Products were amplified, TA-cloned, and sequenced to validate the primers were indeed amplifying the targeted sequences.

All qPCR experiments were performed in the Stratagene Mx3005P instrument using the Power SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA) in 96-well plates. Reaction volume was 25 µl and reactions consisted of 2 µl of diluted cDNA as template and 400 nM of each primer of the genes of interest. Cycling was as follows: 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s. Specificity of each primer pair was verified by melt curve analysis and gel electrophoresis. Each sample and no-template controls were run in triplicate. Raw data analysis of the primer pair efficiency was performed with the PCR Miner software (Zhao and Fernald, 2005) and the efficiencies were between 80%- 86%. mRNA expression of all genes was normalized to EF1α mRNA expression. All values were presented relative to the values at 6 hpf.

2.4. Whole mount in situ hybridization (ISH)

The fragments of Rhag (936 bp), Rhbg (970 bp), Rhcg1 (904 bp), Rhcg2 (926 bp), a UT (962 bp), NHE2 (926 bp) and NHE3 (989 bp) were cloned by PCR and inserted into the pGEM-T Easy vector (Promega, Madison, WI). Specific DIG-labeled RNA probes were synthesized (Roche, Basel, Switherland) according to the manufacturer's instructions using PCR products amplified from inserted fragments as templates. GFP (green fluorescence protein) and Rhag sense probes were also synthesized and used as negative controls. The procedure was done at room temperature (18–25 °C) (RT) unless otherwise stated. The ISH protocol used here was based on the work of Thisse and co-workers (Thisse and Thisse, 2008) with some modifications. Embryos were fixed with 4% PFA (pH 7.2) at 4 °C overnight and dehydrated in graded series of methanol (MeOH)/4% PFA washes: 1 × 5 min 30% methanol-70% 4%PFA, 1 × 5 min 50% methanol- 50% 4%PFA followed by 100% methanol at −20 °C overnight. Afterward, embryos were rehydrated in graded series of MeOH/PBS washes: 1 × 5 min in 50%MeOH- 50%PBS, 1 × 5 min in 30% MeOH-70% PBS, and 2 × 5 min in 100% PBST (1 × PBS, 0.1% Tween 20). Subsequently, embryos were refixed with 4% PFA for 20 mins and washed 2 × 5 min in PBST. Embryos were rinsed in 10 µg/ml proteinase K (Invitrogen, Waltham, MA) for 10 min, washed 2 × 5 min in PBST and refixed for 20 min in 4% PFA. The

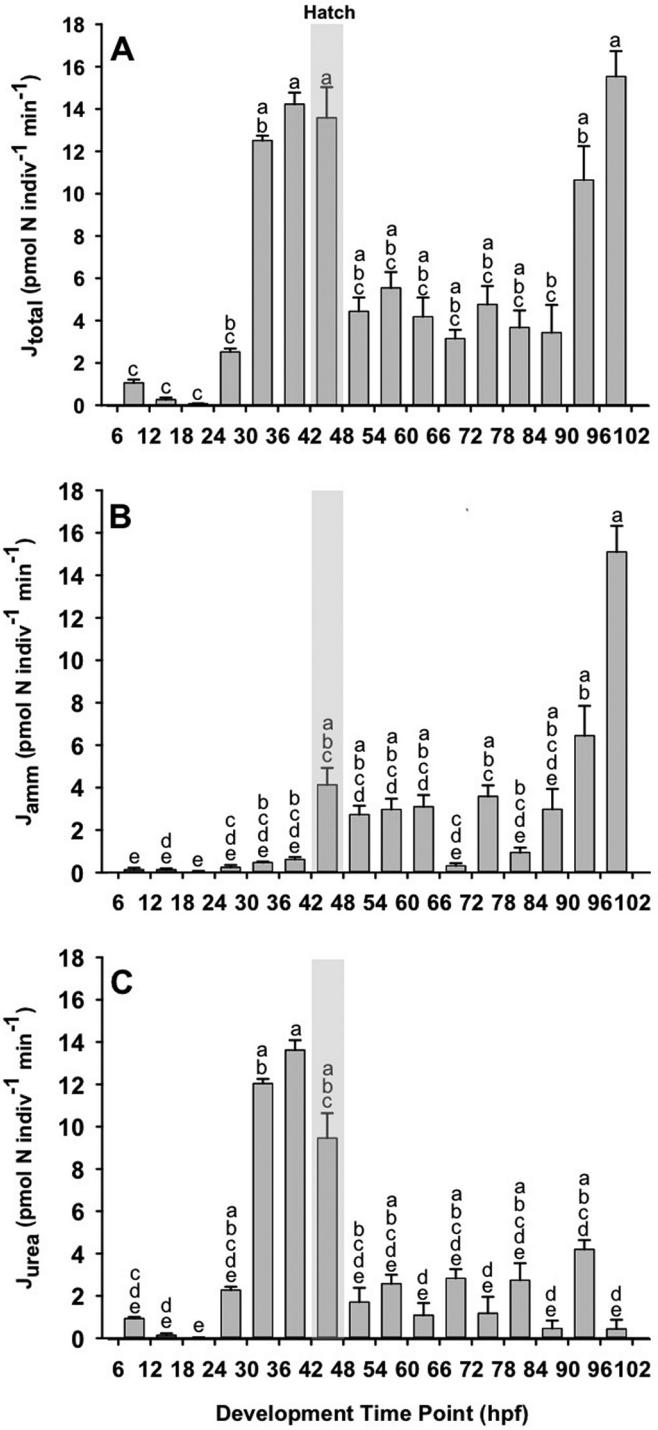

Fig. 1. Excretion rates of total nitrogenous waste (A), ammonia (B) and urea (C) in mahi-mahi during the first 102 hpf. There were 8 replicates of either 40 embryos or 10 larvae per well. The period of hatching is depicted by the shaded box. Data are presented as mean ± SEM. Different letters signify significantly different excretion rates between different developmental time points.

hybridization mix for use in prehybridization and hybridization was prepared as follows: 5.0 ml of formamide, 2.5 ml of 20 × SSC (standard sodium citrate), 50 µl of 20% Tween 20, 50 µg/ml heparin, 500 µg/ml yeast tRNA and H_2O to final volume of 10 ml. Embryos were prehybridized for 2–5 h in 300 µl of prehybridization solution at 65 °C to reduce background, the solution was discarded and replaced with hybridization solution. For hybridization, 150 ng of probe were added into 200 µl hybridization solution at 65 °C overnight. After the overnight

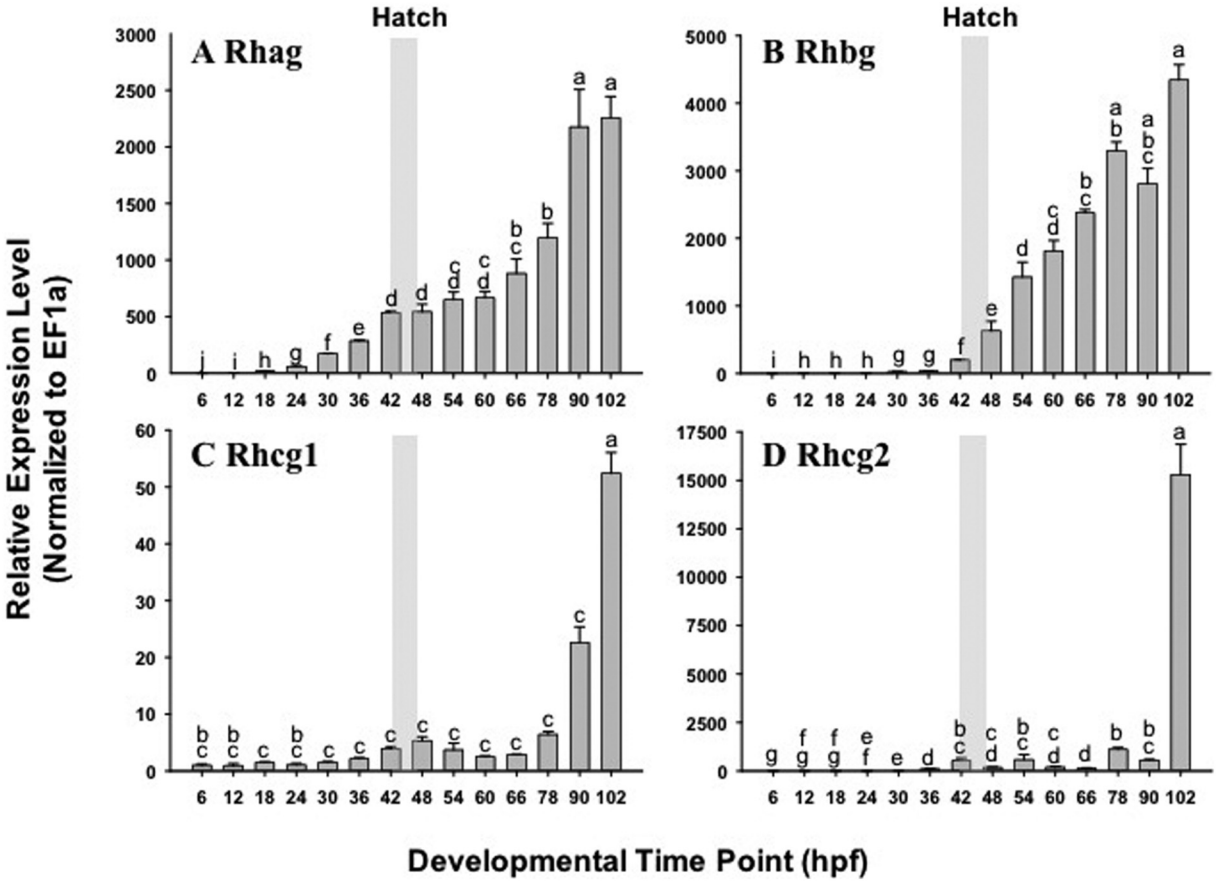

Fig. 2. Relative mRNA expression levels of Rh genes in mahi-mahi during the first 102 hpf. Values are given relative to the amount of Rhcg2 at 6 hpf (lowest relative expression). Values are means ± SEM (n = 3 with each replicate representing 40 embryos or 10 larvae). Different letters signify significantly different excretion rates between different developmental time points.

hybridization, embryos were washed to remove unbound probe as follows: 2 × 15 min in 50% fomamide-50% 2 × SSCT at 65 °C, 15 min in 100% 2 × SSCT at 65 °C, 3 × 30 min in 0.2 × SSCT at 65 °C, 2 × 5 min in MABT at RT. Afterward, the embryos were preincubated for 1 h in MABT containing 10% goat serum and 20% block reagent and then immunoreacted with 1:3000 alkaline phosphatase–coupled anti-DIG antibody (Roche) for overnight at 4 °C with gentle shaking. After incubation, embryos were serially washed as follows: 1 × 30 min in 90% MABT-10% goat serum, 3 × 30 min in MABT. Then the embryos were placed in staining buffer (100 mM Tris, pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% tween 20, and 1 mM levamisole) for 3 × 5 min. Levamisole (5 mM final) were added to BM purple substrate (Roche) and embryos were stained in a dark box for 2 h or until satisfactory coloration occurred after which embryos were washed 2 × 5 min in PBST and refixed in 4% PFA for overnight at 4 °C. Finally, the embryos were washed in 50% 4%PFA-50% Glycerol for 30 min and 100% Glycerol for overnight. Hybridization signals were evaluated by light microscopy on an inverted microscope (Leica, Germany).

2.5. Statistical analyses

Data are presented as means ± standard error of the mean (SEM). Statistical analyses were performed using SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA). All excretion rates data were compared using Kruskal Wallis test by ranks followed by Dunnett's post hoc test. For mRNA expression, Rhcg1 and UT were assessed by Kruskal Wallis test by ranks followed by Dunnett's post hoc test. The remaining five genes were assessed using one-way ANOVAs followed by Holm-Sidak post hoc tests. In some cases, non-normally distributed data were log transformed to perform one-way ANOVAs. In all cases, significant differences were set at $P < .05$.

3. Results

3.1. Ammonia and urea excretion

Nitrogenous waste flux, J_{total} (sum of J_{amm} and J_{urea}) increased significantly before hatch compared to the first 24 hpf of development (Fig. 1A). Ammonia excretion rates (J_{amm}) were comparatively very low (< 0.7 pmol N indiv^{-1} min^{-1}) before hatch (Fig. 1B), while urea excretion rates (J_{urea}) were substantially higher reaching a maximum of 13.6 pmol N indiv^{-1} min^{-1} by 42 hpf. J_{urea} decreased after hatch reaching a minimum of 0.4 pmol N indiv^{-1} min^{-1} by 102 hpf (Fig. 1C). In contrast, J_{amm} increased after hatch to nearly four-fold from 4.1 pmol N indiv^{-1} min^{-1} during hatching to 15.1 pmol N indiv^{-1} min^{-1} at 102 hpf.

3.2. mRNA levels of Rh genes

Four mahi Rh-related genes (Rhag, Rhbg, Rhcg1 and Rhcg2) were identified in this study. The mRNA levels of all genes were expressed relative to the amount of the gene at 6 hpf (lowest relative expression). The mRNA levels of both Rhag and Rhbg increased significantly during development and peaked at 102 hpf (Fig. 2A and B). While Rhag showed progressive increase from 18 hpf, Rhbg exhibited a sudden increase in mRNA abundance immediately before hatch. Rhcg1 expression was significantly elevated at 102 hpf and showed a trend for increased mRNA levels around hatch (Fig. 2C). Rhcg2 mRNA levels

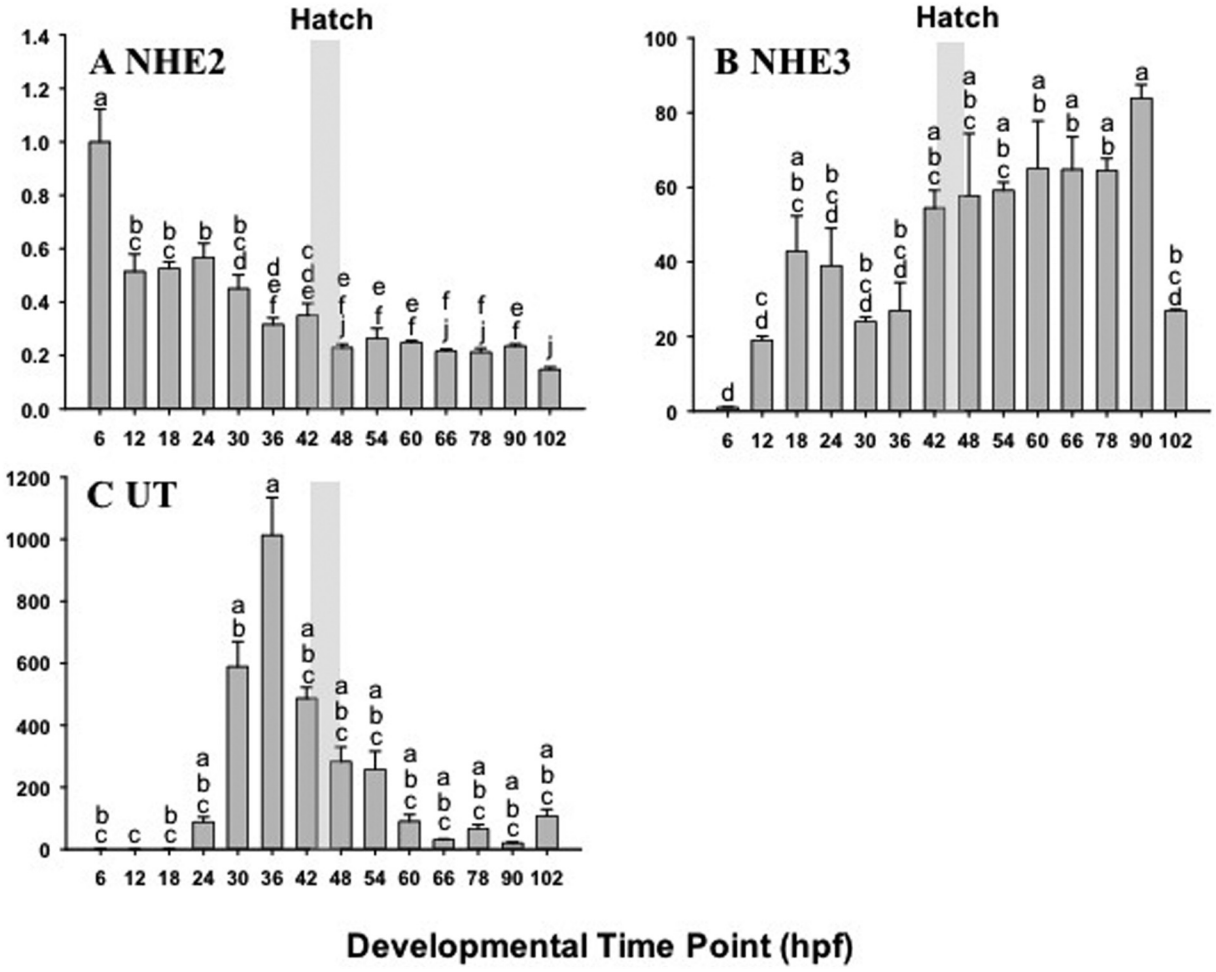

Fig. 3. Relative mRNA expression levels of NHE2 (A), NHE3 (B) and UT (C) genes in mahi-mahi during the first 102 hpf. Values are given relative to the amount of Rhcg2 at 6 hpf (Fig. 2D; lowest relative expression). The inset is an expanded view of NHE3 mRNA level. Values are means ± SEM (n = 3 with each replicate representing 40 embryos or 10 larvae). Different letters signify significantly different excretion rates between different developmental time points.

showed a 28-fold increase at 102 hpf compared with 90 hpf, but was generally low during early development (Fig. 2D).

3.3. mRNA levels of NHEs and UT genes

The mRNA level of NHE2 at 6 hpf was significantly higher than at the other stages and this gene exhibited a decreasing expression pattern during development (Fig. 3A). The mRNA levels of NHE3 showed no significant differences during development but tended to be high after hatch (Fig. 3B).

The mRNA levels of UT were detected at all stages (Fig. 3C). The expression increased during development, peaked around 36 hpf and then decreased thereafter. mRNA levels at 36 hpf were about 11-fold and 9-fold higher relative to levels at 24 hpf and 102 hpf, respectively.

3.4. Whole mount in situ hybridization

GFP and Rhag sense probes caused no staining. The major signals of Rhag (Fig. 4a-d, arrow head) and Rhbg (Fig. 4g-j, arrow head) were found predominantly within the developing gills. Gill arches were clearly visible at 102 hpf but not fully developed at the earlier time point (60 hpf). Rhag also occurred in the yolk sac (Fig. 4a, asterisk) at 60 hpf and operculum at 102 hpf (Fig. 4c and d, arrow). Rhbg occurred in the upper mouth region (Fig. 4g and h, arrow) at 60 hpf. At 102 hpf, Rhcg1 was found in the sub-operculum (Fig. 4e and f). Consistent with the sharp increased mRNA levels for Rhcg2 from the qPCR results, the broad and intense staining (Fig. 4k and l) suggests Rhcg2 was localized in the skin at 102hpf.

At 60 hpf, NHE3 was found in the kidney and heart (Fig. 4m). However, as shown in Fig. 4n and o, the region of NHE3 staining co-localized with the region of Rhcg1 expression (Fig. 4e and f) at 102 hpf, supporting a possible cooperation between these two transporters. NHE2 and UT mRNA exhibited indiscriminate staining at 60 (data not shown) and 102 hpf (Fig. 4p).

4. Discussion

As mahi embryos developed, they underwent a transition from initially excreting most nitrogenous waste as urea before hatch and gradually switching to being ammoniotelic around the time of hatch (Fig. 1B and C), which is in agreement with previous reports of ammonia and urea excretion patterns in the ELS of zebrafish (Braun et al., 2009) and Atlantic cod (*Gadus morhua*) (Chadwick and Wright, 1999). The significant increase in J_{total} before hatch (Fig. 1A) coincides with earlier measurements of increased oxygen consumption (Pasparakis et al., 2016), suggesting increased metabolic rate during this time of development. In addition to the high J_{total} before hatch, it should be noted that the high contribution of J_{urea} to J_{total} indicates the existence of a functional OUC, which is required to detoxify ammonia when ammonia excretion is limited by undeveloped gills and an enclosed chorion.

Excess urea from ammonia detoxification in ELS fish is believed to

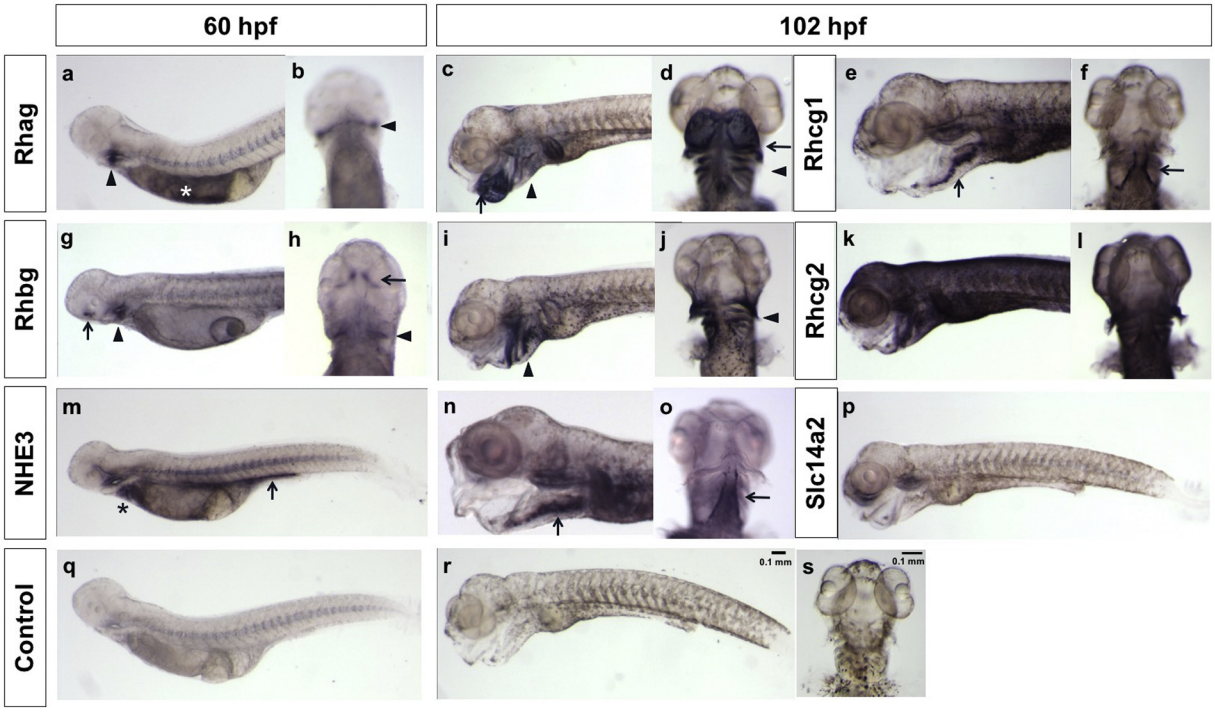

Fig. 4. In situ hybridization of Rh, UT and NHEs transcripts in mahi-mahi larvae at 60 and 102 hpf. *a-d*: Rhag (arrow head, gills; asterisk, yolk sac; arrow, operculum). *e and f*: Rhcg1 (arrow, sub-operculum). *g-j*: Rhbg (arrow head, gills; arrow, upper mouth). *k and l*: Rhcg2 (skin). *m-o*: NHE3 (arrow head, kidney; asterisk, heart; arrow, sub-operculum).

be transported via UT. In agreement with this view, an increased UT mRNA level was observed at 30 hpf (Fig. 3C) and coincided with an increased J_{urea} (Fig. 1A). In situ hybridization experiments were attempted on mahi embryos at 36 hpf when UT mRNA was highest. However, it was not possible to remove the chorion using pronase or tweezers without damaging the embryos. Earlier work on unhatched rainbow trout revealed that the urea content of the yolk was significantly higher than that of embryonic tissues (Steele et al., 2001), which indicates a need for UT located between the yolk and tissues, possibly also at the yolk sac epithelium.

The increase of J_{amm} (Fig. 1A) coincided with the upregulations of Rhag (Fig. 2A) and Rhbg (Fig. 2B) mRNA levels suggesting these two Rh proteins have a major role in ammonia excretion. Further, the in situ results suggest that Rhag and Rhbg were both expressed in the gills. However, in mammals, Rhag appears to be limited to erythrocytes (Avent et al., 2006; Huang and Peng, 2005) and thus we cannot exclude the possibility that the Rhag signal that appears to be branchial is in fact from erythrocytes trapped in the gills. It is worth noting that in pufferfish, Rhag has been detected both in erythrocytes and in the apical and basolateral membranes of pillar cells (Nakada et al., 2007b). However, in contrast to pufferfish, perfused rainbow trout gills reveals Rhag expressed only in erythrocytes (Nawata and Wood, 2008) as seen in mammals. Compared to Rhbg, which is generally restricted to the basolateral gill membrane in the species mentioned above (Claiborne et al., 2008; Nakada et al., 2007a; Nakada et al., 2007b), Rhag in mahi at 60 hpf showed darker staining in gills possibly suggesting expression in the gills as well as in the erythrocytes. Further, the localization of Rhag in the yolk sac and the operculum also supports this idea.

Given the large increase in transcription levels of Rhcg2 at 102 hpf (Fig. 2D) and cutaneous expression (Fig. 4k and l), it is tempting to speculate that skin (primarily that overlying the yolk sac) might be an important site for ammonia excretion in post-hatch larval fish as suggested previously for zebrafish (Shih et al., 2008). Rhag expressed in the yolk sac at 60 hpf (Fig. 3a) also supports this suggestion. Previous experiments observed increased Rhbg, Rhcg1 and Rhcg2 expression in the skin of adult rainbow trout after ammonia excretion was blocked by HEA or HEPES exposure (Nawata et al., 2007; Nawata and Wood, 2008). Compared to adult fish, cutaneous ammonia excretion can be expected to play a greater role in ELS fish, when branchial ammonia excretion may be limited by undeveloped gills. In post-hatch rainbow trout, the skin of larval fish accounts for the majority of both gas exchange and ion regulation following hatch, and the majority of J_{amm}. The J_{amm} also increased in parallel with the expression levels of Rhcg1 and Rhcg2 at yolk sac epithelium (Zimmer et al., 2014). The mechanism of cutaneous ammonia excretion was demonstrated in larval zebrafish and medaka, using the scanning ion electrode technique (SIET), and in these species Rhcg1 facilitates ammonia excretion in MR cells across the yolk sac surface (Shih et al., 2008; Wu et al., 2010). However, SIET is not suited for measurements in seawater experiment since any ion flux will be masked by the large amounts of ions in seawater.

The present study revealed that Rhcg1 and NHE3 were co-localized at the sub-operculum (Fig. 4e and n) and it is possible that these two transporters may cooperate in active excretion of ammonia. The linkage between $J_{Na,in}$ and J_{amm} by freshwater animals has received considerable attention for decades, since first proposed by August Krogh in 1939 (Krogh, 1939) and was recently demonstrated to occur via Rh-NHEs metabolons in rainbow trout, zebrafish and medaka (*Oryzias latipes*) (Kumai and Perry, 2011; Wu et al., 2010; Zimmer et al., 2017a). In pufferfish and longhorn sculpin, Rhcg1 is localized in chloride cells, rich in Na^+/K^+ − ATPase (NKA) (Claiborne et al., 2008; Nakada et al., 2007b). Assuming that a Rhcg1-NHE3 metabolon is at play in mahi, the Na^+ gained from NHE3 activity would be eliminated by paracellular transport across the gill (Larsen et al., 2011). In addition, the co-localization indicates that part of the opercular epithelium may play a role in ammonia excretion and possibly ion and acid-base regulation in developing mahi. Although the localization of NHE2 was not detected in this study, we cannot rule out the possibility of NHE2 involvement in ammonia excretion. However, compared to NHE2, NHE3 dominates in the Rh-NHEs metabolons in the species studied so far (Kumai and Perry, 2011; Wu et al., 2010; Zimmer et al., 2017a).

Overall, this study provided data on the ammonia and urea excretion patterns and the role of UT and Rh transporters in ELS of mahi. Expression patterns and ontogeny of nitrogenous waste excretion in this marine pelagic fish supports a role for UT and Rh proteins in urea and ammonia excretion, respectively. In addition, our findings lend support to the involvement of a Rhcg1-NHE3 metabolon in ELS of mahi.

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