

Physiological and molecular ontogeny of branchial and extra-branchial urea excretion in posthatch rainbow trout (*Oncorhynchus mykiss*)

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Zimmer AM, Wood CM. Physiological and molecular ontogeny of branchial and extra-branchial urea excretion in posthatch rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol Regul Integr Comp Physiol* 310: R305–R312, 2016. First published November 25, 2015; doi:10.1152/ajpregu.00403.2015.—All teleost fish produce ammonia as a metabolic waste product. In embryos, ammonia excretion is limited by the chorion, and fish must detoxify ammonia by synthesizing urea via the ornithine urea cycle (OUC). Although urea is produced by embryos and larvae, urea excretion (J_{urea}) is typically low until yolk sac absorption, increasing thereafter. The aim of this study was to determine the physiological and molecular characteristics of J_{urea} by posthatch rainbow trout (*Oncorhynchus mykiss*). Following hatch, whole body urea concentration decreased over time, while J_{urea} increased following yolk sac absorption. From 12 to 40 days posthatch (dph), extra-branchial routes of excretion accounted for the majority of J_{urea} , while the gills became the dominant site for J_{urea} only after 55 dph. This represents the most delayed branchial ontogeny of any process studied to date. Urea transporter (UT) gene expression in the gills and skin increased over development, consistent with increases in branchial and extra-branchial J_{urea} . Following exposure to 25 mmol/l urea, the accumulation and subsequent elimination of exogenous urea was much greater at 55 dph than 12 dph, consistent with increased UT expression. Notably, UT gene expression in the gills of 55 dph larvae increased in response to high urea. In summary, there is a clear increase in urea transport capacity over posthatch development, despite a decrease in OUC activity.

urea transporter; larval development; nitrogen excretion; urea excretion; urea excretion (J_{urea}); early life stage

IN MOST FRESHWATER TELEOST fish species, the majority of nitrogenous waste is excreted primarily as ammonia, which can easily be diffused and diluted into the surrounding environment. In embryonic stages, however, the presence of the chorion capsule, though permeable to ammonia (21), causes poor convection with the surrounding environment, which limits ammonia excretion (J_{amm}) (5). In addition, metabolism at this stage is fueled, in part, by the oxidation of yolk proteins and amino acids, which generate a metabolic ammonia load, leading to increasing whole body ammonia levels prior to hatch (1, 3, 6, 24, 37). Overall, this is problematic in embryonic fish, as the accumulation of metabolic ammonia can be toxic if ammonia levels rise substantially (23). To limit the potentially toxic accumulation of metabolic ammonia, early life stage (ELS) fish convert ammonia to urea, which is a less toxic N waste product than ammonia.

Unlike typical adult fish, ELS fish express a fully functional ornithine-urea cycle (OUC), which produces 1 mol of urea at

the expense of 5 mol of ATP and is responsible for the majority of urea synthesis at this stage (3, 6, 10, 12, 24, 29, 30, 37). Over development, however, the gene expression of key OUC enzymes is eventually silenced (11), such that adult fish do not express a fully functional OUC, although the mechanism of this silencing is unclear and does not appear to involve the methylation of the CPSIII promoter region (12). Urea excretion (J_{urea}) in rainbow trout (*Oncorhynchus mykiss*) is relatively low in embryos and posthatch larvae (6, 27, 29, 37, 38, 38) and increases following yolk sac absorption (6, 37, 38). In most fish species, urea transport across epithelia occurs via a facilitated diffusion urea transporter (UT) (15, 33, 34). There are three UT isoforms in fish: UT-A, UT-C, and UT-D (13). In zebrafish, UT-A is expressed in the gills, while both UT-A and UT-C are expressed in the kidney (13). Furthermore, using in situ hybridization, UT-A mRNA was demonstrated to be present in the yolk sac skin, gill, liver, and kidney of posthatch (4–8 days postfertilization) larval zebrafish (1). In ELS rainbow trout, the pattern of increasing whole body J_{urea} over development nearly identically matches that of increasing whole body UT-A gene expression (9). In addition, the morpholino knockdown of UT-A in zebrafish embryos caused a 90% inhibition of J_{urea} (1), clearly indicating an integral role of UT-A in J_{urea} by ELS fish. The ontogeny of J_{urea} might also be related to morphological changes over larval development. Following hatch, fish lack a functional gill and rely on cutaneous surfaces for physiological processes typically performed by the gills, such as oxygen uptake (7, 35, 42), ionoregulation (7, 42), and J_{amm} (41, 42). All of these processes eventually shift from the skin to the gills over development, though J_{amm} and Na^+ uptake shift much earlier in development (15–16 days posthatch, dph) than oxygen uptake (26–28 dph) (7, 42). Therefore, given that the majority of J_{urea} occurs branchially in adult rainbow trout (14), it is possible that J_{urea} increases as a function of branchial surface area available for transport, as previously suggested (8, 37).

The overall goal of the present study was to identify the temporal patterns, sites, and mechanisms of urea excretion in posthatch larval rainbow trout. As described above, J_{urea} is generally low following hatch and increases following complete yolk sac absorption (CYA). We hypothesized that J_{urea} would initially occur cutaneously following hatch, similar to the case of all physiological exchanges (O_2 , ammonia, Na^+) studied to date and that over development, the gills would eventually become the dominant site for excretion. We predicted that the transition to branchial J_{urea} (where 50% of J_{urea} first occurs across the gills) would occur at ~13–15 dph in conjunction with the skin-to-gill shift for the excretion of the primary nitrogenous waste, ammonia (41, 42). We further hypothesized that increases in J_{urea} by the gill and/or skin

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would occur in conjunction with tissue-specific increases in the gene expression of UT-A, the isoform that appears to be responsible for 90% of J_{urea} in zebrafish larvae (1). Moreover, we predicted that an increase in UT-A gene expression would be associated with an overall increase in urea permeability and transport capacity. To test these latter predictions, trout were exposed to exogenous urea in the water and, thereafter, whole body urea concentration, J_{urea} , and tissue-specific UT gene expression were assessed.

MATERIALS AND METHODS

Fish. Eyed-up rainbow trout (*Oncorhynchus mykiss*) embryos were purchased from Rainbow Springs Hatchery (Thamesford, ON, Canada) and held at 12°C in hatching trays with flow-through Hamilton dechlorinated tap water (moderately hard: $[\text{Na}^+] = 0.6$ meq/l, $[\text{Cl}^-] = 0.8$ meq/l, $[\text{Ca}^{2+}] = 1.8$ meq/l, $[\text{Mg}^{2+}] = 0.3$ meq/l, $[\text{K}^+] = 0.05$ meq/l; titration alkalinity 2.1 meq/l; pH ~8.0; hardness ~140 mg/l as CaCO_3 equivalents). Hatching took place ~1 wk after purchase, and this marked the beginning of the experimental period, or 0 dph. CYA occurred at ~30 dph, at which point exogenous feeding began with daily meals of commercial trout pellets (Martin Profishent Aquaculture Nutrition, Tavistock, ON, Canada; 45% crude protein, 9% crude fat, and 3.5% crude fiber) at ~5% fish body mass. All experiments performed after CYA (40 and 55 dph) were conducted following a 48-h fast; experimental temperature was 12°C throughout. All experimental procedures were approved by the animal care committee of McMaster University.

Experimental design. The study consisted of two experimental series. In *series 1*, a divided chamber approach similar to that used previously (7, 42) was employed to follow the ontogeny of branchial (gill) and extra-branchial (skin, kidney, and/or gut) urea excretion (J_{urea}) in developing posthatch larval rainbow trout (range of individual wet fish mass = 0.0707–0.272 g). In *series 2*, 12 dph larvae (mean wet mass = 0.094 ± 0.004 g) and 55 dph juveniles (mean wet mass = 0.197 ± 0.016 g) were exposed to exogenous urea (25 mmol/l) for 12 h to determine how fish at these stages handle a urea load.

Series 1: developmental ontogeny of J_{urea} . This series closely followed methods described previously for divided chamber experiments in larval trout (7, 42). Briefly, trout were randomly selected at 12, 15, 18, 21, 40, and 55 dph for the measurement of J_{urea} in a divided chamber system; note that in this system, J_{urea} could not reliably be detected earlier than 12 dph, as earlier than this, most larvae excreted no detectable amounts of urea. Larvae/juveniles were initially anesthetized to stage 3 anesthesia (16) with 0.1–0.2 g/l neutralized MS-222 (using 1 M KOH). Once stage 3 anesthesia was reached, fish were loaded individually into divided chambers. The chamber system contained a latex sheet with a small hole cut in its center, through which the head of the fish was pushed, such that the head, opercula, and pectoral fins were separated spatially from the rest of the body. The fish and latex sheet were then mounted between two 5-ml half-chambers, which were filled with aerated, dechlorinated Hamilton tap water containing 0.05 g/l neutralized MS-222 to maintain anesthesia. Following a 10-min adjustment period, ^{22}Na was added to a final concentration of 0.001 $\mu\text{Ci/ml}$ to assess leakage across the dam. After allowing 5 min of mixing of the isotope, 1-ml samples from the anterior (head) and posterior (body) chambers were taken, indicating the start of the flux period. Following 1.5 h, final 1-ml samples were taken, final chamber volumes were recorded, and fish were weighed. In all fluxes, fish recovered from anesthesia within 5 min, and isotope leak from the loaded to unloaded chamber was less than 10%. Water samples were stored at –20°C until further urea concentration and ^{22}Na gamma radioactivity analyses.

A separate group of larvae was removed on each of the days posthatch described above and were euthanized using an overdose of neutralized MS-222, immediately flash-frozen, and stored at –80°C

until later measurement of whole body urea concentration. Another group of larvae removed at 3, 12, 21, and 44 dph were euthanized; gill, yolk sac skin, and body skin tissues were collected under a dissecting microscope, which took ~4–5 min per fish. Tissues from each fish were flash frozen individually and stored at –80°C for later gene expression analysis.

Series 2: exogenous urea exposures. At 12 and 55 dph, randomly selected fish were placed into two separate 3-liter containers (aerated) with either control dechlorinated Hamilton tap water or 25 mmol/l urea (in aerated Hamilton dechlorinated tap water) at a density of 6–8 fish/l. Fish were exposed to these conditions for 12 h overnight. Following this exposure, six randomly selected fish from each treatment were removed, euthanized with an overdose of neutralized MS-222, flash-frozen in liquid nitrogen, and stored at –80°C for later measurement of whole body urea concentration. Another six fish were removed and euthanized, and the gill and body skin were collected under a dissecting microscope. These tissue samples were also flash-frozen and stored at –80°C for later gene expression analyses. Finally, another six fish were removed from each group, and J_{urea} in divided chambers was measured following the exact same protocol as that described above for *series 1*. Note that both the control and high-urea groups were placed into control (i.e., no urea) water for J_{urea} measurements. All sampling followed the same procedures outlined above.

Determination of branchial and extra-branchial J_{urea} . Urea concentration in collected water samples was measured using a protocol optimized to measure low levels of urea (22). J_{urea} ($\mu\text{mol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) in the anterior and posterior chambers was calculated using the following equation:

$$J_{\text{urea}} = \left(\left([\text{urea}]_f - [\text{urea}]_i \right) \times V \right) / (M \cdot t) \quad (1)$$

where $[\text{urea}]_f$ and $[\text{urea}]_i$ are the final and initial urea N concentrations ($\mu\text{mol N}$), respectively, measured in a given chamber. V is volume (liters) of the respective chamber, M is fish mass (g), and t is flux duration (h). To check for leakage, ^{22}Na gamma radioactivity in counts per minute (cpm) was also determined in initial and final water samples via gamma counting (Perkin Elmer Wizard 1480 3" Auto Gamma Counter; Waltham, MA). As described above, total cps in the unloaded chamber at the end of the flux were always less than 10% of those measured in the loaded chamber at the beginning of the flux.

To determine branchial and extra-branchial J_{urea} , it was necessary to correct anterior J_{urea} by subtracting the proportion of J_{urea} taking place across the skin in the anterior chamber. This was done via calculations described previously (41, 42), using skin surface area estimates reported by Zimmer et al. (42). Branchial and extra-branchial J_{urea} ($\mu\text{mol}\cdot\text{g}\cdot\text{h}$) were determined using the following equations:

$$\text{Branchial } J_{\text{urea}} = \text{anterior } J_{\text{urea}} - \left[\text{anterior SA} \times \left(\text{average posterior } J_{\text{urea}} / \text{posterior SA} \right) \right] \quad (2)$$

$$\text{Extra-branchial } J_{\text{urea}} = \text{total } J_{\text{urea}} - \text{branchial } J_{\text{urea}} \quad (3)$$

where anterior J_{urea} is J_{urea} measured in the anterior chamber, anterior SA is the cutaneous surface area localized to the anterior chamber (cm^2), average posterior J_{urea} is the mean of J_{urea} measured in the posterior chamber, and posterior SA is the cutaneous surface area localized to the posterior chamber (cm^2). A caveat to this correction, used here to make comparisons to previous studies (7, 41, 42), is that any posterior J_{urea} , which is not cutaneous (e.g., renal, gastrointestinal), is considered to be cutaneous when subtracted from anterior J_{urea} . Therefore, this correction may underestimate the branchial contribution to anterior J_{urea} by overestimating the cutaneous contribution.

Whole body urea concentration measurement. Frozen whole body samples of larvae from *series 1* and *2* were ground to a fine powder under liquid nitrogen in a liquid nitrogen-cooled mortar and pestle (1–4 fish per replicate depending on fish mass). Samples were then deproteinized by adding ~100 mg of powdered tissue to 500 μl of

ice-cold 8% perchloric acid, vortexing the mixture, and incubating for 5 min on ice. Samples were centrifuged for 30 s at 5,000 g, and the resulting supernatant was used to determine urea concentration via the protocol described above for water samples. Urea turnover time (h) was determined using the following equation:

$$\text{Turnover time} = \text{mean whole body urea concentration} / \text{total } J_{\text{urea}} \quad (4)$$

where mean whole body urea concentration ($\mu\text{mol/g}$) is the mean whole body urea concentration within a given time and total J_{urea} ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) is the sum of branchial and extra-branchial J_{urea} within the respective time. For both whole body urea concentration and J_{urea} , data are presented in urea-N units, accounting for 2 moles of nitrogen per mole of urea.

UT gene expression. Frozen gill, yolk sac skin, and body skin samples from *series 1* and 2 were homogenized individually on ice in 600 μl of ice-cold commercial lysis buffer (PureLink RNA mini kit, Ambion, Austin, TX) using a motorized rotor and stator homogenizer, and RNA was extracted from homogenates of individual tissue samples using the PureLink RNA mini kit (Ambion), following the manufacturer's protocol. DNase treatment was performed using an on-column treatment (PureLink DNase set; Ambion), also according to the manufacturer's protocol. RNA concentration and purity were determined spectrophotometrically using a Nanodrop system (ND-1000; Nanodrop Technologies, Wilmington, DE), and RNA quality was assessed by running samples through a 1% agarose gel using Redsafe (FroggaBio) staining. cDNA was synthesized from 200 ng total RNA using an oligo(dT₁₇) primer and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using published primers for UT-A and reference genes (EF1 α and β -actin) (9). Rainbow trout UT-A (accession no. EF688013.1) shares 79%, 78%, and 76% sequence identity with UT-A genes from the toadfish (*Opsanus beta*, AF165893.2), Japanese eel (*Anguilla japonica*, AB049726.1), and zebrafish (*Danio rerio*, BC115203.1), respectively (alignment not shown). Reactions were performed using a total volume of 10 μl [4 μl diluted cDNA template, 5 μl 2 \times SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA), 0.4 μl each of 100 $\mu\text{mol/l}$ forward and reverse primers of the genes of interest, and 0.2 μl nuclease-free water] in a CFX Connect real-time PCR detection system (Bio-Rad) and were initiated by heating to 98°C for 2 min to activate the polymerase followed by 40 cycles of 2 s at 98°C and 5 s at 60°C. No-template and non-reverse transcribed controls were run for each gene. Melt curve analysis revealed a single product for each primer pair, and amplification efficiency was between 90 and 110% for each gene. UT gene expression was expressed relative to the geometric mean of EF1 α and β -actin expression using Genorm software, which is integrated into the CFX software. The mean coefficient of variation and M value for the reference gene pair were 0.1660 and 0.4751, respectively, below the thresholds of 0.25 and 0.5 considered to be stable reference pair expression in a homogenous tissue sample. In *series 1*, relative UT mRNA expression was normalized to expression in the gill at 3 dph and in *series 2*, expression was normalized to that in the gill or body skin, respectively, at 12 dph of control larvae.

Statistical analyses. All data are presented as means \pm SE (n = number of fish or number of pooled replicates), and statistical significance was accepted at the $P < 0.05$ level. In general, comparisons between two data sets were performed using an unpaired two-tailed t -test, comparisons among three or more means were performed using a one-way ANOVA with a Holm-Sidak post hoc test, and two-factor comparisons among multiple means were performed using a two-way ANOVA with a Holm-Sidak post hoc test using SigmaStat software (SigmaStat v. 3.5, Systat Software). Specific details of statistical approaches for each data set are also included in corresponding figure captions.

RESULTS

Ontogeny of urea handling by posthatch rainbow trout. Whole body urea concentration in developing trout larvae ranged from 2.0 to 2.5 $\mu\text{mol N/g}$ over the first 9 days following hatch (Fig. 1A). By 12 dph, these values were approximately halved, reaching a minimum of $1.02 \pm 0.24 \mu\text{mol N/g}$ by 18 dph, and thereafter, whole body urea concentration remained relatively constant (Fig. 1A). Urea turnover time (Eq. 4) demonstrated an overall $\sim 80\%$ decrease from 12 to 55 dph, the only exception being a transient, nonsignificant rise seen at 18 dph, which was absent by 21 dph (Fig. 1B).

Total J_{urea} (sum of branchial and extra-branchial J_{urea}) increased nearly four-fold over the study period from $0.047 \pm 0.009 \mu\text{mol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ at 12 dph to $0.182 \pm 0.018 \mu\text{mol N}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ by 55 dph (Fig. 2A), contributing to the decreases in urea turnover time (Fig. 1B) in the face of approximately stable whole body urea concentration over this period (Fig. 1A). In the divided chamber system used in this study, it was not possible to reliably detect J_{urea} prior to 12 dph, as many fish excreted no detectable amounts of urea prior to this time. From 12 to 44 dph, extra-branchial routes accounted for the majority of total J_{urea} , ranging from 60 to 95% of total (Fig. 2B). By 55 dph, the gills represented the dominant site for J_{urea} , with 67% of the total occurring branchially (Fig. 2B). Thus, the gills became the dominant site for J_{urea} between 40 and 55 dph.

mRNA transcripts for UT-A were detected in gill, yolk sac skin, and body skin tissues at 3 dph, and UT gene expression increased significantly in all tissues over development (Fig. 3). By 40 dph, UT gene expression increased significantly by 30- and 10-fold in the gills and body skin, respectively, relative to 3 dph (Fig. 3). In the yolk sac skin, there was a significant, five-fold increase in UT gene expression by 21 dph, relative to 3 dph (Fig. 3).

Responses to exogenous urea loading. Following 12 h of exposure to high urea (25 mmol/l), whole body urea concentration increased nearly 7-fold and 40-fold in 12 and 55 dph trout, respectively (Fig. 4). At 12 dph, total J_{urea} (measured after transfer to urea-free water) was elevated by nearly four-

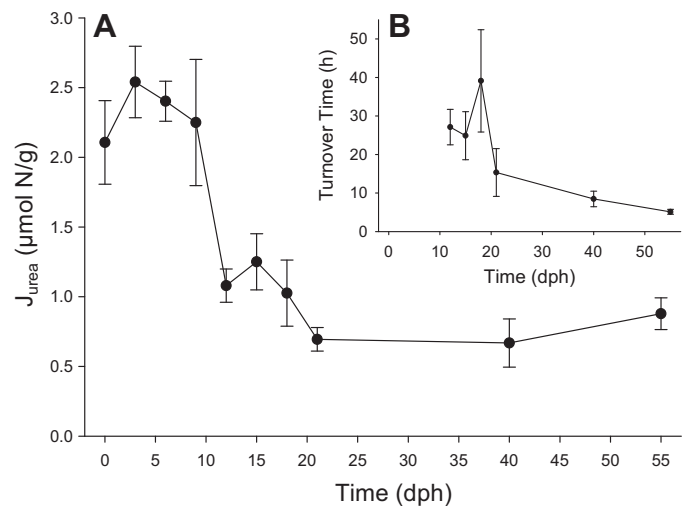


Fig. 1. A: whole body urea concentration (expressed as urea N). B: urea turnover time in rainbow trout larvae over 0–55 days posthatch (dph); $n = 5$ or 6 pooled replicates of 1–4 fish for whole body urea concentration; $n = 5$ –9 individual fish for turnover time.

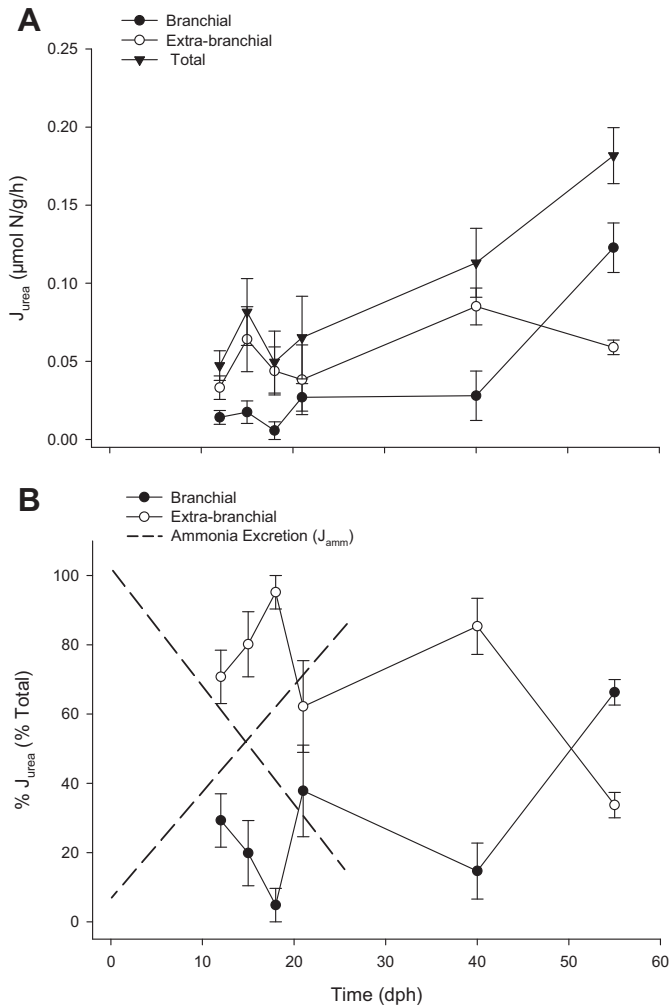


Fig. 2. Absolute and relative urea excretion rates (J_{urea} , expressed as urea N) by rainbow trout larvae over 12–55 days posthatch (dph) measured in divided chambers. *A*: absolute branchial (●), extra-branchial (○), and total (▼; sum of branchial and extra-branchial) J_{urea} . *B*: relative (% of total) branchial (●) and extra-branchial (○) J_{urea} . Intersect of dashed vertical lines in *B* represents the skin-to-gill shift for ammonia excretion (J_{amm} ; 15 dph) reported by Zimmer et al. (42); $n = 5-9$.

fold in response to urea loading (Fig. 5A). Interestingly, while extra-branchial routes accounted for the majority (70%) of J_{urea} at this stage, branchial J_{urea} increased 5.4-fold in response to high urea, while extra-branchial J_{urea} increased by only 2.8-fold (Fig. 5A). However, even under urea-loaded conditions, extra-branchial J_{urea} still accounted for the majority of total J_{urea} in 12 dph trout, though to a lesser degree (55%) than under control conditions. Similar to the results seen in whole body urea concentration, high urea exposure at 55 dph led to a much more dramatic change in J_{urea} than that observed at 12 dph. In 55 dph trout, total J_{urea} increased by 21-fold in response to exogenous urea loading, with 22- and 19-fold increases in branchial and extra-branchial J_{urea} , respectively (Fig. 5B). Although both sites appeared to respond equally to urea loading at 55 dph, the gills accounted for the majority of total J_{urea} under both control (67%) and urea-loaded (70%) conditions.

In *series 2*, UT gene expression in the gills increased significantly by twofold from 12 to 55 dph, while UT gene

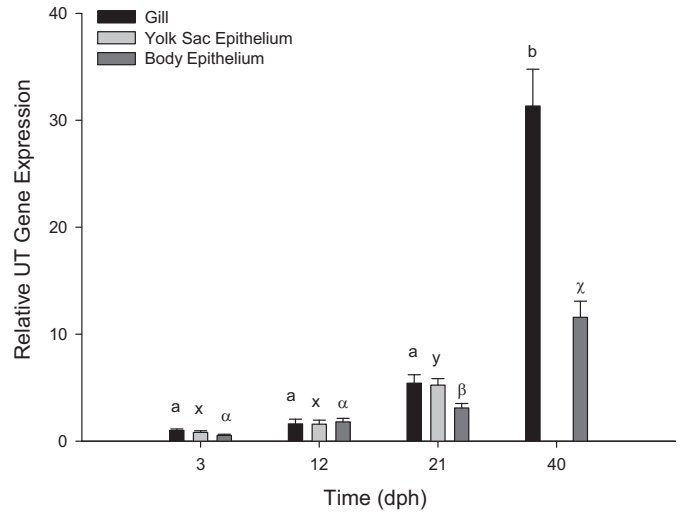


Fig. 3. Relative urea transporter (UT) gene expression in the gills (black bars), yolk sac epithelium (light gray bars), and body epithelium (dark gray bars) of rainbow trout larvae at 3, 12, 21, and 40 days posthatch (dph). Expression is relative to the geometric mean of EF1 α and β -actin expression and is normalized to the relative expression measured in the gill at 3 dph. Means not sharing the same letters between time points within a given tissue (abc for gill, xyz for yolk sac epithelium, and $\alpha\beta\gamma$ for body epithelium) are significantly different from one another, as determined by a one-way ANOVA with a Holm-Sidak post hoc test. ($P < 0.05$; $n = 4-6$)

expression in the skin did not increase significantly (Fig. 6). These differences were much less than those observed in *series 1*, where UT gene expression increased 20-fold and 6-fold in the gills and skin, respectively, from 12 to 40 dph (Fig. 3). Exposure to high urea in *series 2* led to a significant 50% increase in gill UT gene expression (Fig. 6A). UT mRNA expression in the body epithelium was not significantly altered by high urea exposure (Fig. 6B).

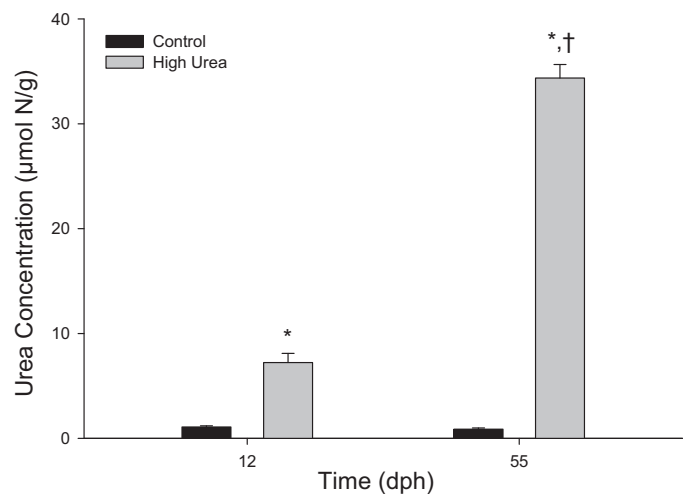


Fig. 4. Whole body urea concentration (expressed as urea N) of rainbow trout larvae at 12 and 55 days posthatch (dph) under control conditions (black bars) or following a 12 h exposure to 25 mmol/l urea (gray bars). Asterisks represent significant differences between control and high urea means at either time point, and daggers represent a significant difference between 12 and 55 dph larvae in either treatment group. Significant differences were determined using a two-way ANOVA with a Holm-Sidak post hoc test; a statistically significant interaction between time and treatment was detected. ($P < 0.05$; $n = 6$ individual larvae).

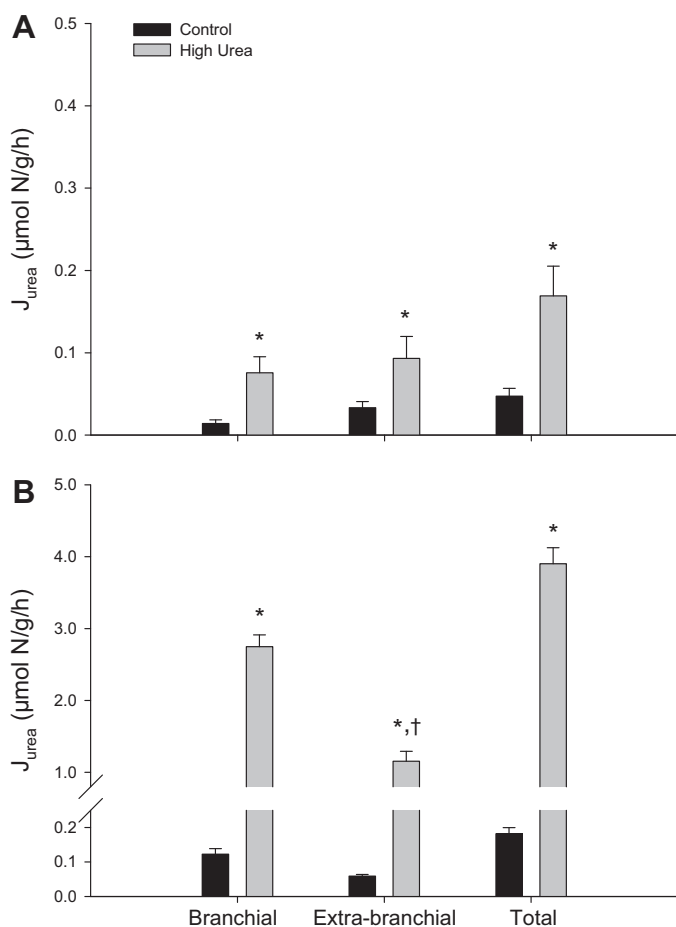


Fig. 5. Branchial, extra-branchial, and total J_{urea} (expressed as urea N) by rainbow trout larvae at 12 (A) and 55 days (B) posthatch (dph) measured in divided chambers under control conditions (black bars) or following a 12-h exposure to 25 mmol/l urea (gray bars). In both groups, excretion rates were measured in urea-free water. *Significant differences between control and high urea means of branchial, extra-branchial, and total J_{urea} . †Significant difference between branchial and extra-branchial means in a given treatment. Significant differences within each time point were analyzed separately using a two-way ANOVA with a Holm-Sidak post hoc test; a statistically significant interaction between time and treatment was detected at 55 dph, but not 12 dph ($P < 0.05$; $n = 6$).

DISCUSSION

Nitrogen handling by ELS rainbow trout. Following hatch, whole body urea concentration in rainbow trout declined over developmental time (Fig. 1A), while total J_{urea} increased from 12 to 55 dph (Fig. 2A); thus urea turned over much more rapidly, as development proceeded (Fig. 1B). These appear to be common developmental features of posthatch fish (1, 3, 6, 37, 38). In rainbow trout, whole body urea content accumulates in embryos and is generally high at hatching (4, 6, 24, 37), likely due to the production of urea via the OUC in the embryonic stage. The accumulation of urea in embryos perhaps indicates that prior to hatch, similar to the case of J_{amm} (5), the excretion of urea is also impeded by the presence of the chorion, despite the fact that the chorion is permeable to both urea and ammonia (20, 21). In studies using high environmental ammonia (HEA) exposure to experimentally increase urea production in ELS trout, both embryos (prehatch) and larvae (~45 dph) produced urea in response to HEA (27, 38). How-

ever, in embryos, whole body urea concentration increased, but J_{urea} was unchanged (27, 38), while in larvae, J_{urea} increased (38). This indicates that while both life stages have the capacity to produce urea in response to ammonia loading, it is only after hatch that fish are able to excrete this extra urea to the surrounding environment. Interestingly, even though whole body urea concentration in rainbow trout stabilized at ~12 dph (Fig. 1A), J_{urea} continued to increase until well after yolk sac absorption (Fig. 2A), in agreement with all previous studies on ELS rainbow trout (6, 37, 38). This more rapid urea turnover (Fig. 1B) occurred despite the fact that by CYA, over 80% of total N excretion occurs as ammonia (6, 37), and fish at this stage possess an effective mechanism for branchial J_{amm} (42). Moreover, ureagenic capacity also decreases over developmental time, which would presumably reduce the input urea load to the system, although the timing of the loss of ureagenic capacity via the OUC in developing rainbow trout is not clear at present.

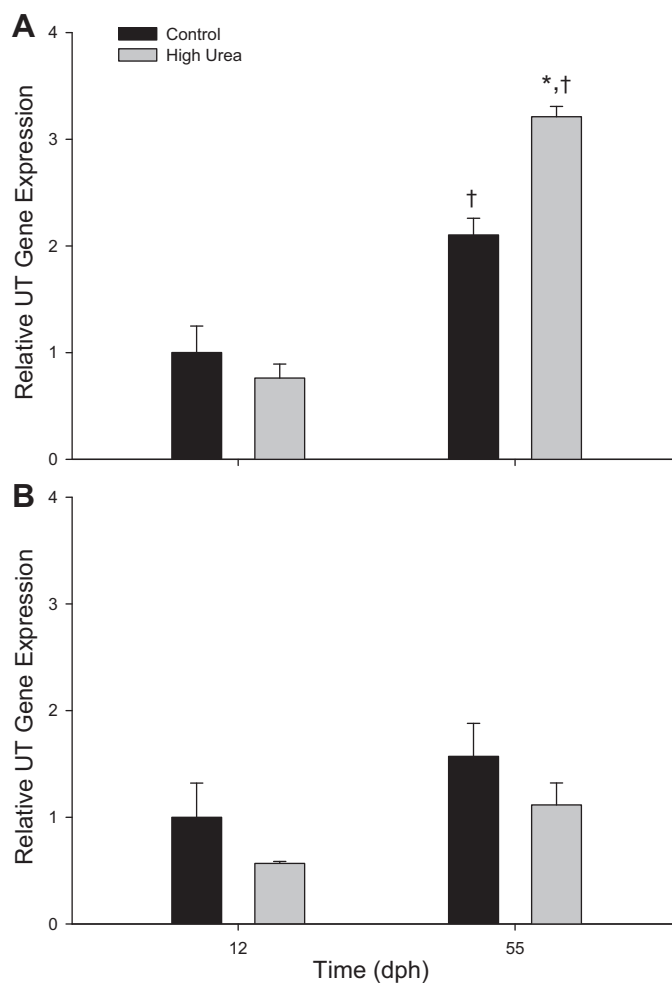


Fig. 6. Relative urea transporter (UT) gene expression in gills (A) and body epithelium (B) of rainbow trout larvae at 12 and 55 dph under control conditions (black bars) or following a 12-h exposure to 25 mmol/l urea (gray bars). Expression is relative to the geometric mean of EF1 α and β -actin expression and is normalized to the relative expression measured in control gills or body epithelium at 12 dph. *Significant differences between control and high urea means at either time point. †Significant difference between 12 and 55 dph larvae in either treatment group. Significant differences were determined using a two-way ANOVA with a Holm-Sidak post hoc test. ($P < 0.05$; $n = 3$ or 4).

Sites and mechanisms of J_{urea} in posthatch rainbow trout. Following hatch, extra-branchial excretion accounted for 70–90% of total J_{urea} (Fig. 2B), which is in agreement with all other physiological exchanges examined to date (7, 35, 41, 42). Note that unlike the aforementioned studies, in the present study we refer to extra-branchial excretion, as opposed to solely cutaneous excretion. This is because a contribution to posterior J_{urea} by the kidney cannot be discounted. Unlike Na^+ uptake, oxygen uptake, or J_{amm} , the latter of which only ~2–5% of total is attributed to renal excretion in adult trout (39), the kidney could represent an important site for total J_{urea} in developing fish. In adult trout, urinary J_{urea} accounts for ~25% of total (14), and there is evidence that the kidney functions for both osmoregulation and ionoregulation soon after hatch in some fish species (31). Therefore, we cannot discount the possible renal contribution to J_{urea} and opt to use the term extra-branchial excretion. To date, no study has determined the contribution of urinary output to J_{urea} or any other physiological function in ELS fish because obtaining urine samples from fish of this size would be technically difficult or perhaps impossible.

We had initially hypothesized that the transition to branchial J_{urea} would occur in conjunction with that of J_{amm} at 13–15 dph (41, 42), as both fluxes function to clear the body of nitrogenous waste. However, contrary to our hypothesis, the gill did not account for the majority of total J_{urea} until 55 dph when 66% of total excretion occurred branchially, while at 40 dph, the gill accounted for only 15% of total J_{urea} (Fig. 2B). Given that the transition to branchial exchange for Na^+ uptake and oxygen uptake occur at 13–15 and 26–28 dph, respectively (7, 41, 42), J_{urea} has the most developmentally delayed branchial ontogeny of any physiological process measured to date.

In fish, urea transport across epithelia is facilitated by the urea transporter (UT). In developing rainbow trout, changes in whole body UT-A gene expression match the temporal increase in J_{urea} over development time (9), and the transport kinetics of urea display all of the hallmarks of UT-facilitated transport (19). In 3 dph larvae, UT-A mRNA was expressed in the gill, yolk sac skin, and body skin (Fig. 3), supporting the presence of UT-facilitated urea transport across these epithelia at this stage. In larval zebrafish, UT-A, the mRNA expression, which is present in both the gills and skin, is critical to J_{urea} as morpholino knockdown of UT nearly abolishes J_{urea} (1). In the present study on trout, UT-A gene expression increased by ~20- and 30-fold, respectively, in the gill and body skin of posthatch trout from 3 to 40 dph (Fig. 3). These changes occurred in conjunction with increases in branchial and extra-branchial J_{urea} (Fig. 2A), although the relative developmental increases in J_{urea} were much less than those of UT-A gene expression. While UT-A gene expression in the gills and skin increased 20-fold and 6-fold, respectively, from 12 to 40 dph in *series 1* (Fig. 3), UT-A gene expression increased only twofold in the gills from 12–55 dph in *series 2* and did not increase significantly in the skin (Fig. 6). Furthermore, when measured in the whole body, UT-A gene expression increased only approximately five-fold from posthatch to 30 dph (9). Thus, it is possible that the large increase in UT-A gene expression observed in both the skin and gills at 40 dph in the present study is a transient developmental phenomenon. In previous work on posthatch (3 dph) trout, ^{14}C -urea was shown to be accumulated in the body when present in the surrounding

environment, and its uptake was both saturable at concentrations below 10 mmol/l and inhibited by phloretin and the urea analogs acetamide and thiourea (19), suggesting the presence of UT-facilitated transport. This suggests that the skin is a likely site for urea transport via UT at this stage given that 3 dph larvae lack a functional gill (7, 42), and the skin of these larvae accounts for more than 90% of total surface area (26). In future studies, it will be very informative to examine the developmental patterns of UT-A expression at the protein level in gills and skin of posthatch trout.

Responses to urea loading. To our knowledge, this is the first study to examine the effects of exogenous urea loading by high urea exposure on whole body urea concentration, J_{urea} , and UT gene expression in ELS rainbow trout. Exposure to 25 mmol/l urea for 12 h caused 7- and 38-fold increases in whole body urea concentration in fish at 12 and 55 dph, respectively (Fig. 4). The dramatic increase in urea accumulation observed between the two developmental time points may be related to increased UT gene expression over posthatch development (9) (Figs. 3 and 6). Indeed, in 3 dph larvae, the uptake of urea at an external concentration of 20 mmol/l is facilitated by UT (19). However, increased urea accumulation could also simply be attributed to increasing surface area for uptake over posthatch development (26). Following high urea exposure, J_{urea} in urea-free water measured in divided chambers was elevated relative to control fish at both 12 and 55 dph. As would be predicted, the overall increase in total J_{urea} was greater at 55 dph (20-fold higher than respective control) than at 12 dph (4-fold higher than respective control) (Fig. 5). Overall, the results from the high urea exposures support our initial findings in terms of the relative role of the gills in J_{urea} over development. At 12 dph, branchial and extra-branchial routes of excretion contributed equally to total J_{urea} following high urea exposure (Fig. 5A), while at 55 dph, the gills played a greater role in the clearance of the exogenous urea load (Fig. 5B). Overall, these data further demonstrate an increase in urea transport capacity over time in posthatch fish. Most notably, the high urea treatment also led to an increase in UT gene expression in the gills of 55 dph fish only (Fig. 6A), while body skin UT gene expression was unchanged at either life stage (Fig. 6B). This marks, to our knowledge, the first evidence that direct urea loading can alter UT gene expression in rainbow trout. More work is now needed to determine the effect of urea loading on posttranscriptional regulation of UT.

Although UT gene expression appears to respond to exogenous urea loading, it is not clear whether endogenously produced urea can also elicit a similar response. When considering the mechanisms of ammonia transport in fish, exogenous ammonia loading by HEA increases the gene expression of ammonia-transporting Rhesus (Rh) proteins in the gills (18, 28, 36, 40), similar to the UT gene expression response to high urea (Fig. 6A). In addition, however, mRNA expression of Rh proteins in the gills is also increased in response to feeding (40), demonstrating that both exogenous and endogenous ammonia loads can alter Rh gene expression in trout. In rainbow trout embryos (4 and 48 h postfertilization), exposure to HEA led to an increase in endogenous urea production, but this did not alter UT gene expression (27). These observations may not be surprising given that UT gene expression was not altered by exogenous urea loading until 55 dph in the present study (Fig. 6A). In the facultatively ureotelic gulf toadfish, pulsatile J_{urea} is

associated with increases in gill UT gene expression, although only 12 h after a pulse event (25). No study to date has examined the effects of endogenous urea production on UT gene expression in an ammonotelic species.

Perspectives and Significance

The present study demonstrates a clear increase in urea excretion capacity and a more rapid urea turnover as development proceeds (Fig. 1B) in posthatch rainbow trout. What is unclear, however, is the ontogenetic pressure driving this increase. Griffith (8) first suggested that the production and accumulation of urea by embryonic fish is used as a strategy to avoid ammonia toxicity in fish species with a protracted embryonic stage. Moreover, Griffith (8) also proposed that the excretion of accumulated urea would be achieved once branchial and renal systems were fully developed following hatch. In reality, however, the ontogeny of J_{urea} in ELS fish may not be so simple. The initially high level of embryonically accumulated urea appears to be cleared within the first 12 dph (Fig. 1A) when J_{urea} is typically very low (6, 37). Moreover, contrary to Griffith's hypothesis (8), extra-branchial routes of excretion, not the gills, likely accounted for the majority of the initial clearance of urea because the gills did not become the dominant site for J_{urea} until 55 dph (Fig. 2B). Furthermore, J_{urea} may not increase appreciably until after CYA (6, 37) (Fig. 2A), well after the elimination of embryonically accumulated urea and also well after the onset of branchial ammonia excretion (13–15 dph) (41, 42). Thus, the later development of J_{urea} capacity in ELS fish is likely not driven by the need to clear urea produced in the detoxification of ammonia by the OUC.

In addition to the OUC, urea can also be produced by arginolysis and uricolysis. The former is particularly important in the developing fish because ornithine, the production of which is catalyzed by arginase, is essential as a substrate for the biosynthesis of polyamines, which are integral to growth (17). In addition, arginine is also a substrate for the synthesis of other important compounds such as nitric oxide and precursors of the phosphocreatine system (17). In developing trout, the activity of arginase increases exponentially following hatch (37), while the byproduct urea must be excreted. Interestingly, increases in J_{urea} are often coupled to increased growth rates in teleost fish (e.g., 2, 32). Thus, perhaps changes in J_{urea} in early life development are related to growth. Increased J_{urea} over development, in particular following CYA, might also be related to the switch from endogenous to exogenous feeding. It is possible that differences in arginine content between the yolk and commercial trout pellets and in overall feeding rates, both of which were not measured in the present study, could have contributed to an increased urea load following the onset of exogenous feeding. Recently, it has been suggested that the expression of UTs in nonureagenic tissues may be integral in limiting the internal accumulation of urea, preventing a "back up" of the arginase system via a mass action effect (13). This notion is supported by the observation that when 55 dph trout were loaded with exogenous urea, gill UT gene expression increased (Fig. 6A). Overall, we propose that the urea excretion mechanism developed in early life may be necessary to maintain low internal urea levels at this life stage. Faster urea turnover (Fig. 1B) may be critical for continued metabolism of endogenously produced arginine, and, following CYA, exog-

enous arginine as an important substrate for various critical biomolecules, including polyamines needed to support growth.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.M.Z. and C.M.W. conception and design of research; A.M.Z. performed experiments; A.M.Z. analyzed data; A.M.Z. and C.M.W. interpreted results of experiments; A.M.Z. prepared figures; A.M.Z. drafted manuscript; A.M.Z. and C.M.W. edited and revised manuscript; C.M.W. approved final version of manuscript.

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