What is the primary function of the early teleost gill? Evidence for Na\(^+\)/NH\(_4\)^+ exchange in developing rainbow trout (Oncorhynchus mykiss)

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Post-hatch fishes lack a functional gill and use cutaneous surfaces for exchange with the surrounding environment. The ionoregulatory hypothesis posits that ionoregulation is the first physiological process to be limited by cutaneous exchange, necessitating its shift to the gills. We hypothesized that the ontogeny of branchial ammonia excretion (\(J_{\text{amm}}\)) is coupled to Na\(^+\) uptake (\(J_{\text{Na}^+}\)) in accordance with the current model for Na\(^+\)/NH\(_4\)^+ exchange in freshwater. Using divided chambers, branchial and cutaneous \(J_{\text{amm}}\), \(J_{\text{Na}^+}\) and oxygen consumption (MO\(_2\)) by larval rainbow trout were assessed. Following hatch, the skin accounted for 97% and 86% of total \(J_{\text{amm}}\) and \(J_{\text{Na}^+}\), respectively. \(J_{\text{amm}}\) and \(J_{\text{Na}^+}\) shifted to the gills simultaneously at 15 days post-hatch (dph) and were highly correlated (\(R^2 = 0.951\)) at the gills, but not the skin, over development. Contrastingly, MO\(_2\) shifted significantly later at 27 dph, in agreement with the ionoregulatory hypothesis. Moreover, the mRNA expression and/or enzymatic activity of Rhesus proteins, Na\(^+\)/H\(^+\)-exchanger, H\(^+\)-ATPase, Na\(^+\)/K\(^+\)-ATPase and carbonic anhydrase, all key components of the Na\(^+\)/NH\(_4\)^+ exchange system, increased in the gills over larval development. We propose that the ontogeny of branchial \(J_{\text{Na}^+}\) occurs as Na\(^+\)/NH\(_4\)^+ exchange and provide evidence for a novel element to the ionoregulatory hypothesis, the excretion of potentially lethal metabolic ammonia.

1. Introduction

The gill in most teleost fishes is the major site for physiological exchanges with the surrounding environment, participating in gas exchange, ion acquisition, acid-base regulation and nitrogenous waste excretion (reviewed in [1]). In larval fishes, however, the gill is undeveloped following hatch and contributes little to physiological exchange (e.g. [2,3]). Initially, the skin is the dominant site, and as the gills develop, these exchanges eventually shift to become primarily branchial [2,3]. The selective pressures underlying gill ontogeny in both a developmental and evolutionary context have been debated over several decades.

In larval fishes, metabolic rate increases more rapidly over development than skin surface area, leading to a problematic limitation in cutaneous transport, necessitating the development of the gills (reviewed in [4]). August Krogh [5] first suggested that the earliest function of the developing gill is O\(_2\) uptake and this hypothesis, termed the oxygen hypothesis, was the accepted view of gill development for half a century. More recently, however, this view has been challenged. In many fish species, the primary site of ionoregulatory exchange, precedes the formation of gill respiratory structures (i.e. filaments or lamellae) [6–8]. These observations led to the ionoregulatory hypothesis, which posits that ionoregulation is the earliest gill function (reviewed in [4,9]). Most recently, Fu et al. [3], using divided chambers, directly demonstrated that in rainbow trout larvae reared in soft or hard water, Na\(^+\) uptake shifts from the skin to the gills prior to O\(_2\) uptake, strongly supporting the ionoregulatory hypothesis.
The goal of this study was to determine the relationship between the ontogeny of Na\(^+\) uptake and the ontogeny of excretion of ammonia, the third respiratory gas [10], at this early life stage in an ammonephotic teleost fish. As recently suggested [9], the ontogeny of branchial ion uptake may occur as a function of homeostatic processes which are coupled to ion uptake such as acid-base balance and/or ammonia excretion, rather than being solely for the acquisition of mineral nutrients. We hypothesized that branchial ammonia excretion in larval trout would occur as a function of Na\(^+\)/NH\(_4\)\(^+\) exchange and that this coupling of Na\(^+\) uptake (\(J_{Na}\)) and ammonia excretion (\(I_{ammon} \)) might represent the earliest function of the developing gill. Moreover, \(I_{ammon} \) in larval fishes is particularly important given that the catabolism of yolk sac proteins, which fuels metabolism at this stage, generates a metabolic ammonia load [11] which can be potentially lethal at elevated levels [12].

In adult rainbow trout, \(I_{ammon} \) is believed to be coupled to \(J_{Na}\) via an Na\(^+\)/NH\(_4\)\(^+\)-exchange complex in which Rhesus (Rh) glycoproteins play a critical role (reviewed in [13]). However, it is not clear if this would be the case in larval trout where \(J_{Na}\) shifts from the skin to the gills over development [3]. We used the same divided chamber approach as employed by Fu et al. [3] to examine these processes. We predicted that, similar to \(J_{Na}\) and oxygen consumption (MO\(_2\)) [2, 3], \(I_{ammon} \) would initially occur via cutaneous routes, but would shift to the gills in synchrony with \(J_{Na}\) and not with MO\(_2\). Such a result would indicate that the ontogeny of branchial Na\(^+\) uptake occurs as part of Na\(^+\)/NH\(_4\)\(^+\)-coupled exchange, adding a critical new element to the ionoregulatory hypothesis. Alternatively, if \(I_{ammon} \) at this stage is not coupled to \(J_{Na}\), we might expect its shift to be closer to that of another respiratory gas, oxygen. An additional related hypothesis was that \(I_{ammon} \) and \(J_{Na}\) in larval trout would occur via the components of the Na\(^+\)/NH\(_4\)\(^+\)-exchange complex. We predicted that the increase in branchial \(I_{ammon} \) and \(J_{Na}\) over development would occur in conjunction with an increase in gene expression and/or enzymatic activity of the key components of the complex (Rh proteins, N\(_a\)/H\(^+\)-exchanger (NHE), Na\(^+\)/K\(^+\)-ATPase, H\(^+\)-ATPase and carbonic anhydrase (CA); reviewed in [13]) in the gills, while a decreased mRNA expression and/or activity of these proteins was expected in skin tissues.

2. Material and methods

(a) Fish husbandry

Rainbow trout (Oncorhynchus mykiss) embryos were purchased in the eyed-stage stage from Rainbow Springs Hatchery (Thamesford, Ontario, Canada) and held at 12°C in hatching trays with flow-through Hamilton dechlorinated tapwater (moderately hard: [Na\(^+\)] = 0.6 mequiv l\(^{-1}\), [Cl\(^-\)] = 0.8 mequiv l\(^{-1}\), [Ca\(^{2+}\)] = 0.8 equiv l\(^{-1}\), [Mg\(^{2+}\)] = 0.3 mequiv l\(^{-1}\), [K\(^+\)] = 0.05 mequiv l\(^{-1}\); titration alkalinity 2.1 mequiv l\(^{-1}\); pH approx. 8.0; hardness approx. 140 mg l\(^{-1}\) as CaCO\(_3\) equivalents). All larvae used in flux experiments and measurements of whole-body ammonia content were from the same batch of embryos which hatched at the same time. Hatching took place approximately one week after purchase and this marked the beginning of the experimental period. Complete yolk sac absorption (CYA) occurred approximately 30 days post-hatch (dph) at which point exogenous feeding began, with daily meals of approximately 5% fish body mass. All experiments performed after CYA were conducted on 48 h fasted fish; experimental temperature was 12°C.

(b) Experimental design for flux measurements

At 0, 3, 6, 9, 12, 15, 18, 21 dph, and following CYA, experiments were conducted to estimate branchial and cutaneous contributions to ammonia excretion (\(I_{ammon} \)) and oxygen consumption (MO\(_2\)) using a divided chamber protocol. Prior to experimentation, fish were anaesthetized in 50–100 ppm clove oil, similar to that used in a previous divided chamber study [5]. After anaesthesia to stage 3 [14], each fish was loaded into a small hole in a latex dental dam, such that its head, operculae and pectoral fins were separated spatially from the rest of its body. The fish and dam were then mounted between two 5-ml half-chambers containing dechlorinated tapwater with 10–15 ppm clove oil. Fresh aerated, clove oil-spiked tapwater was then circulated through both chambers using a peristaltic pump at a rate of 0.2 ml min\(^{-1}\) for a 1 h acclimation period.

An additional control series of experiments was conducted on non-anaesthetized, unrestrained larvae at 3 dph, which revealed that the anaesthetized and divided chamber set-up had no significant effect on \(I_{ammon} \), \(J_{Na}\) or MO\(_2\) (data not shown). All experimental procedures, measurements and analyses used for control experiments were identical to those used in the divided chamber experiments described below.

(c) \(I_{ammon} \) and \(J_{Na}\)

Following the 1 h acclimation period, water recirculation was stopped and 0.5 μCi \(^{22}\)Na (Amersham Pharmacia Biotech Inc.) was added to either the anterior or posterior chamber; it was not possible to measure anterior and posterior Na\(^+\) fluxes on the same fish. Following 5 min of mixing by aeration, initial 1.25 ml samples were taken from both chambers to measure water total ammonia (\(I_{ammon} \)). Radioactivity (as counts per minute; cpm) and total [Na\(^+\)]*. Following a 1.5 h flux period, identical final samples were taken, and the remaining water volume was measured in both chambers. Larvae were removed from the divided chambers and were rinsed three times with 5 mM NaCl and once with double-distilled water to remove any surface-bound isotope. Larvae were then euthanized using 0.2 g l\(^{-1}\) MS-222, weighed, and counted for \(^{22}\)Na radioactivity. Samples for water \(I_{ammon} \) were stored at −20°C until later analysis while samples for \(^{22}\)Na radioactivity were counted immediately and stored at 4°C until later [Na\(^+\)]* analysis. For each flux, a maximum of 10% isotope leak to the unloaded chamber was accepted as a successful dam and all fish recovered completely from anaesthetic treatment within 5 min, prior to euthanasia.

All flux values are presented as flux per gram of whole fish including the yolk sac. Total ammonia concentrations (\(T_{ammon} \)) of water samples were assayed using the protocol described by Verdouw et al. [15]. Anterior and posterior ammonia flux rates (\(I_{ammon} \) \(\mu\)mol g\(^{-1}\) h\(^{-1}\)) were determined using the following equation:

\[
I_{ammon} = \frac{(T_{ammon} f - T_{ammon i}) \times V}{15} ,
\]

where \(T_{ammon} f \) and \(T_{ammon i} \) are the final and initial concentrations of ammonia (\(\mu\)mol l\(^{-1}\)) in the water samples within the anterior or posterior chambers, \(V \) is volume (l) of the given chamber, \(f \) is flux duration (h) and \(M \) is mass (g) of the fish, yolk sac included. Total \(I_{ammon} \) was determined by adding anterior and posterior flux rates from the same fish. Radioactivity of \(^{22}\)Na in counts per minute (cpm) of water samples and whole larvae was measured via gamma counting (Perkin Elmer Wizard 1480 3rd Auto Gamma Counter), and [Na\(^+\)]* of water samples was measured using atomic absorption spectrophotometry (SpectAA 220FS Atomic Absorption Spectrophotometer). Anterior and posterior sodium influx rates (\(J_{Na} \) \(\mu\)mol g\(^{-1}\) h\(^{-1}\)) were calculated as

\[
J_{Na} = \frac{R_{fish}}{SA_{average} \times f \times M} ,
\]

where \(R_{fish} \) is radioactivity of \(^{22}\)Na in anterior chamber, \(f \) is time period during which radioactivity was measured, \(M \) is mass (g) of fish, \(SA \) is total surface area of anterior chamber and \(f \) is flux duration (h).

\[
J_{Na} = \frac{R_{fish}}{SA_{average} \times f \times M} \cdot \frac{22}{12} 
\]

Where \(R_{fish} \) is radioactivity of \(^{22}\)Na in anterior chamber, \(f \) is time period during which radioactivity was measured, \(M \) is mass (g) of fish, \(SA \) is total surface area of anterior chamber and \(f \) is flux duration (h).

\[
J_{Na} = \frac{R_{fish}}{SA_{average} \times f \times M} \cdot \frac{22}{12} 
\]
where $R_{fish}$ is the gamma-radioactivity of the fish (cpm) at the end of the flux period and $SA_{average}$ is the mean of the initial and final specific activities (cpm/µmol) of the water from the $^{22}$Na-loaded chamber. Total $F_{i}$, (anterior + posterior) was calculated by sorting replicate fish by mass and pairing corresponding anterior and posterior replicates together.

(d) MO$_2$

O$_2$ consumption rates were measured on separate fish at the same time periods. Following the 1 h acclimation period described above, air lines were removed and anterior and posterior chambers were filled completely with clove oil-spiked tapwater. Initial 600-µl samples were taken from both chambers and replaced with an equal volume of water of known PO$_2$. The PO$_2$ of each initial sample was read immediately using a Clarke-type oxygen electrode (Cameron Instruments) connected to a Model 1900 Polarographic Amplifier (AM Systems) kept at 12 C. Following 0.5–1 h, a second 600 µl sample was taken from each chamber to assess final PO$_2$. This procedure was done simultaneously for both anterior and posterior chambers for each replicate fish. Larvae were then removed from the divided chambers, euthanized and weighed. As in the previous series, 0.5 µCi $^{22}$Na was added to one side for the assessment of dam integrity. Anterior and posterior oxygen consumption rates (MO$_2$: µmol g$^{-1}$ h$^{-1}$) were calculated as

$$PMO_2 = \frac{(PO_2 i - PO_2 f) \times A_{O_2} \times V}{I \times M},$$  

where PO$_2$i (mmHg) is the initial PO$_2$ of the chamber which was corrected for the replacement water of a known PO$_2$. The PO$_2$o (mmHg) is the final PO$_2$ of the chamber and $A_{O_2}$ ($\mu$mol l$^{-1}$ mmHg$^{-1}$) is the solubility constant for O$_2$ in water at 12 C [16].

(e) Skin surface area measurements and calculation of branchial and cutaneous flux rates

In a separate batch of larvae, lateral images of larvae at each developmental time point (n = 3–6) were taken using a digital camera attached to a Leica EZ4D dissecting microscope. Images were then traced using IMAGEJ software (Wayne Rasband, National Institutes of Health, USA) and the two-dimensional cutaneous surface area. Cutaneous flux rates (µmol g$^{-1}$ h$^{-1}$) were then determined as

$$= \frac{\text{cutaneous flux rate}}{\text{cutaneous surface area}} \times \text{cutaneous surface area},$$

where anterior flux rate (µmol g$^{-1}$ h$^{-1}$) is the flux measured in the anterior chamber, anterior SA (cm$^2$) is average anterior cutaneous surface area, average posterior flux rate (µmol g$^{-1}$ h$^{-1}$) is the average flux measured in the posterior chamber and posterior SA (cm$^2$) is the average posterior cutaneous surface area. Cutaneous flux rates (µmol g$^{-1}$ h$^{-1}$) were calculated as

$$= \frac{\text{cutaneous flux rate}}{\text{cutaneous surface area}} \times \text{cutaneous surface area},$$

where total flux rate (µmol g$^{-1}$ h$^{-1}$) is the sum of anterior and posterior flux rates. As in [3], these calculations assume that cutaneous flux per unit skin area is the same in the

(f) Estimation of cutaneous-to-branchial shifting point

In order to estimate the cutaneous-to-branchial shifting point for a given process (where 50% occurs via branchial or cutaneous routes), per cent of total branchial and cutaneous values were plotted as x-values against time (dph; y-values). $x = 0$ was then set to 50%, such that the y-intercept (in dph) occurred at x = 50% and would correspond to the shifting point for any given process. y-intercept values and corresponding standard errors of estimate were obtained using SIGMAPLOT v. 10.0 (Systat Software, Inc.).
(g) Whole-body tissue $J_{\text{amm}}$ and turnover time
At 0, 3, 6, 9, 12, 15, 18, 21 dph, and following CYA, a random set of larvae were removed from hatching trays, euthanized in neutralized MS-222; whole gill baskets, yolk sac epithelium and body epithelium samples were taken for gene expression or enzymatic analyses. Tissue samples for gene expression were placed individually directly into 600 μl of ice-cold commercial lysis buffer (PureLink RNA mini kit, Ambion) and homogenized using a small plastic pestle and lysed by passing the homogenate through a 23-gauge needle three times. RNA was extracted from tissue samples using the PureLink RNA mini kit according to the manufacturer’s protocol. DNAase 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 (Nanodrop ND-1000, Nanodrop Technologies) and RNA quality was assessed by running samples through a 1% agarose gel stained with RedSafe (FroggBio). cDNA was synthesized from 200 ng total RNA using an oligo(dT)17 primer and superscript II reverse transcriptase (Invitrogen). mRNA expression of reference and target genes was determined by quantitative polymerase chain reaction (qPCR). Total reactions (10 μl) consisted of 4 μl of diluted cDNA template, 5 μl of 2X SsoFast EvaGreen Supermix (Bio-Rad), 0.4 μl each of 100 μmol l$^{-1}$ forward and reverse primers of the genes of interest (electronic supplementary material, table S2), and 0.2 μl nuclease-free water, and were performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad). The reaction mix was first heated to 98°C for 2 min to activate polymerase, followed by 40 amplification cycles of 2 s at 98°C and 5 s at the annealing temperature specific to the given primer pair (electronic supplementary material, table S2) which had been optimized beforehand. No template controls were conducted with every run and non-reverse transcribed controls were performed for every primer pair. Melt curve analyses confirmed the presence of a single PCR product for every gene of interest and the efficiency of amplification for every primer pair in each tissue type was between 95% and 110%. Relative expression of target genes was determined via the ΔΔCq method using both EF1-α and β-actin as reference genes. Expression was normalized to that of the gill at 3 dph using the CFX MANAGER v. 3.0 software.

For enzymatic analyses, each tissue was placed directly into 250 μl of ice-cold EGTA-Na deoxycholate homogenization buffer, flash frozen and stored at −80°C for later analysis.

(h) Branchial and cutaneous enzyme activity and gene expression
At 3, 12, 21 dph, and following CYA, a random set of larvae were removed from hatching trays, euthanized using an overdose of neutralized MS-222; whole gill baskets, yolk sac epithelium and body epithelium samples were taken for gene expression or enzymatic analyses. Tissue samples for gene expression were placed individually directly into 600 μl of ice-cold commercial lysis buffer (PureLink RNA mini kit, Ambion) and homogenized using a small plastic pestle and lysed by passing the homogenate through a 23-gauge needle three times. RNA was extracted from tissue samples using the PureLink RNA mini kit according to the manufacturer’s protocol. DNAase 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 (Nanodrop ND-1000, Nanodrop Technologies) and RNA quality was assessed by running samples through a 1% agarose gel stained with RedSafe (FroggBio). cDNA was synthesized from 200 ng total RNA using an oligo(dT)17 primer and superscript II reverse transcriptase (Invitrogen). mRNA expression of reference and target genes was determined by quantitative polymerase chain reaction (qPCR). Total reactions (10 μl) consisted of 4 μl of diluted cDNA template, 5 μl of 2X SsoFast EvaGreen Supermix (Bio-Rad), 0.4 μl each of 100 μmol l$^{-1}$ forward and reverse primers of the genes of interest (electronic supplementary material, table S2), and 0.2 μl nuclease-free water, and were performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad). The reaction mix was first heated to 98°C for 2 min to activate polymerase, followed by 40 amplification cycles of 2 s at 98°C and 5 s at the annealing temperature specific to the given primer pair (electronic supplementary material, table S2) which had been optimized beforehand. No template controls were conducted with every run and non-reverse transcribed controls were performed for every primer pair. Melt curve analyses confirmed the presence of a single PCR product for every gene of interest and the efficiency of amplification for every primer pair in each tissue type was between 95% and 110%. Relative expression of target genes was determined via the ΔΔCq method using both EF1-α and β-actin as reference genes. Expression was normalized to that of the gill at 3 dph using the CFX MANAGER v. 3.0 software.

For enzymatic analyses, each tissue was placed directly into 250 μl of ice-cold EGTA-Na deoxycholate homogenization buffer, flash frozen and stored at −80°C for later analysis.
Figure 4. Relative gene expression of (a) Rhcg1, (b) Rhcg2, (c) Rhbg, (d) Na\(^+/\)H\(^+\)-exchange-2 (NHE-2), (e) Na\(^+/\)K\(^+\)-ATPase, (f) H\(^+\)-ATPase and (g) CA at 3, 12 and 21 days post-hatch (dph) and after CYA in the gills (black bars), yolk sac epithelium (grey bars) and body epithelium (dark grey bars) of developing rainbow trout larvae. Gene expression is relative to the expression of both EF1-α and β-actin and is normalized to the expression seen in the gill at 3 dph. Means within a given tissue across time points which do not share the same letter are significantly different from one another. Asterisks represent yolk sac epithelium means which differ significantly from gill means within a given time point and double asterisks represent body epithelium means which differ significantly from both gill and yolk sac epithelium means within a given time point. (n = 4–6) CYA, complete yolk sac absorption.
Pool of samples (up to five) was necessary to detect enzyme activity in small tissues. Pooled samples were later homogenized in the EGTA-Na deoxycholate buffer in which they were stored. Na+/K+-ATPase, H+-ATPase and CA activities in homogenates were assayed using methods described previously [18,19]. Protein concentration of homogenates was measured with the Bradford reagent (Sigma) using a bovine serum albumen (Sigma) standard curve.

(i) Statistical analyses
All data are represented as means ± s.e.m. (n = sample size) and statistical significance was accepted at the p < 0.05 level. All statistical and regression analyses were performed using SIGMAPLOT v. 10.0 with SIGMASTAT v. 3.5 integration (Systat Software, Inc.). In general, analyses comparing two means were performed using a two-tailed t-test, whereas analyses comparing three or more means were performed using a one-way analysis of variance (ANOVA) with a Holm–Sidak post hoc test. In the case of a failed normality test, data were square root or log transformed. Specific tests and normalization procedures used are described further in corresponding figure captions.

3. Results and discussion
The ontogeny of \( J_{\text{ass}} \) and \( J_{\text{in}}^{\text{NaH}} \) in larval rainbow trout appears to occur via an Na\(^+\)/NH\(_4\)\(^+\)-exchange complex as seen in the gills of adult trout [13]. Both \( J_{\text{ass}} \) and \( J_{\text{in}}^{\text{NaH}} \) shifted from the skin to the gills at the same time (15 dph) and were highly correlated over development at the gill \( (r^2 = 0.951) \). Moreover, this shift occurred significantly earlier than that of MO\(_2\) (27 dph), confirming previous work [3] and providing strong support for the ionoregulatory hypothesis. The mRNA expression and/or enzyme activity of several of the key components of the Na\(^+\)/NH\(_4\)\(^+\)-exchange system also increased in the gills, including Rh and NHE, in accordance with the configuration observed in larval zebrafish [20]. The evolution of such exchange systems and metabolons may have occurred as an effective single solution to limitations in a number of cutaneous exchange processes (ions, acid equivalents, nitrogen wastes) over larval development.

(a) The ontogeny of branchial Na\(^+\)/NH\(_4\)\(^+\) exchange
Immediately following hatch, the skin represented the dominant site for both \( J_{\text{ass}} \) and \( J_{\text{in}}^{\text{NaH}} \), accounting for 96.7 ± 2.0 and 86.5 ± 6.5% of total, respectively (figures 1 and 2). Over development, however, both absolute \( J_{\text{ass}} \) and absolute \( J_{\text{in}}^{\text{NaH}} \) by the gills increased while those by the skin remained relatively constant (figure 1). The cutaneous-to-branchial shift, at which 50% of a given process occurs via the gills, for \( J_{\text{ass}} \) occurred at 15.0 ± 0.7 dph, remarkably at exactly the same time as \( J_{\text{in}}^{\text{NaH}} \) (14.9 ± 1.2 dph), suggesting a coordinated ontogeny of both processes, potentially as Na\(^+\)/NH\(_4\)\(^+\) exchange. Indeed, both processes were observed to be highly correlated at the gills over development in rainbow trout \( (r^2 = 0.951; \text{figure 3}) \). While interestingly, such a relationship was not observed at the skin (data not shown). In adult fishes, this coupled exchange is coordinated by a complex consisting of a number of different components (reviewed in [13]). Perhaps the most integral part of the Na\(^+\)/NH\(_4\)\(^+\)-exchange complex is the Rh-NHE metabolon; recent evidence demonstrates that this metabolon is key to both \( J_{\text{ass}} \) and \( J_{\text{in}}^{\text{NaH}} \) in larval fishes [20–23]. In larval rainbow trout, gene expression of Rhcg2 in the gill, relative to 3 dph, increased significantly by 21 dph, whereas expression of Rhcg1 and NHE-2 increased significantly by CYA (figure 4e,f,g); Rhbg mRNA expression was unchanged in the gills over development (figure 4c). The branchial gene expression of three additional components, Na\(^+\)/K\(^-\)-ATPase, H\(^-\)-ATPase and CA, increased significantly over larval development only in the case of Na\(^+\)/K\(^-\)-ATPase (figure 4c). However, the enzymatic activity of these components increased 6.2-, 4.8- and 4.2-fold from 3–21 dph (figure 5) for Na\(^+\)/K\(^-\)-ATPase, H\(^-\)-ATPase and CA, respectively, potentially indicating the induction of at least part of the Na\(^+\)/NH\(_4\)\(^+\)-exchange system. Some discrepancies between gene expression and enzymatic activity may not be surprising.
given that H\(^+\)-ATPase, for example, has many regulatory inputs at the post-transcriptional level (reviewed in [24]).

These observations suggest that the ontogeny of branchial Na\(^+\)/NH\(_4\)\(^+\) exchange is coordinated by the same exchange complex as seen in the gill of adult freshwater fishes [13] and zebrafish larvae [20] and that this arrangement is present at the onset of branchial \(J_{\text{amm}}\) and \(J_{\text{Na}^+\text{in}}\). In the present larval trout, such a configuration does not appear to exist at the skin. Contrary to our initial hypothesis, cutaneous \(J_{\text{amm}}\) and \(J_{\text{Na}^+\text{in}}\) (figure 1) and the gene expression and enzyme activity of several of the key components of Na\(^+\)/NH\(_4\)\(^+\)-exchange in cutaneous epithelia (yolk sac and body; figures 4 and 5) remained relatively constant over larval development. This may suggest alternate mechanisms of transport, consistent with differentiation of distinct gill and skin epithelial surfaces. Moreover, the decrease in relative importance of cutaneous routes over development appears to be a function of increasing branchial exchange, rather than decreasing cutaneous exchange.

(b) Evidence supporting a novel element of the ionoregulatory hypothesis

The coordinated cutaneous-to-branchial shifts for \(J_{\text{amm}}\) and \(J_{\text{Na}^+\text{in}}\) at 15 dph occurred significantly earlier in development than the shift for MO\(_2\) at 26.6 \(\pm\) 2.0 dph (by extrapolation, figure 2). These results are in agreement with and are nearly identical to those of Fu et al. [3], which provided support for the ionoregulatory hypothesis using a direct physiological approach. The independent replication of these results [3] and the wealth of indirect histological evidence that exists (reviewed in [4,9]) provide concrete support for the ionoregulatory hypothesis. Moreover, these findings provide evidence for an additional critical element of the ionoregulatory hypothesis, demonstrating that the ontogeny of branchial Na\(^+\) uptake occurs as Na\(^+\)/NH\(_4\)\(^+\) exchange.

(c) What is the earliest gill function?

An effective mechanism for excretion of ammonia, the third respiratory gas [10], may be critically important in developing fishes. During larval development, metabolism is fuelled by the catabolism of amino acids obtained from yolk proteins, leading to the release of potentially toxic ammonia. Embryonic fishes, surrounded by the chorion, accumulate a substantial load of metabolic ammonia prior to hatch [25,26]. Whole-body \(T_{\text{amm}}\), build-up, which continued even after hatch (figure 6), could potentially be attenuated by gill development. Prior to the cutaneous-to-branchial shift for \(J_{\text{amm}}\) whole-body \(T_{\text{amm}}\) accumulated at a rate of 0.18 \(\mu\text{mol g}^{-1}\text{d}^{-1}\) over the first 15 dph while from 15 to 21 dph, \(T_{\text{amm}}\) accumulation slowed to a rate of 0.06 \(\mu\text{mol g}^{-1}\text{d}^{-1}\) (figure 6).

Similarly, ammonia turnover time increased over development until reaching a peak at 12–15 dph (figure 6), again coinciding with the cutaneous-to-branchial shift for \(J_{\text{amm}}\) (figure 2). Thereafter, ammonia turnover time decreased steadily, suggesting that the ontogeny of branchial \(J_{\text{amm}}\) might allow for a more effective clearance of metabolic ammonia. This may be a particularly critical event in early development given the eventual loss of ureogenic capacity which is used in embryonic stages to limit the accumulation of metabolic ammonia [25,27].

The acquisition of mineral ions required by larval growth, however, is also critical to larval development (reviewed in [4]). The simultaneous ontogeny of branchial \(J_{\text{amm}}\) and \(J_{\text{Na}^+\text{in}}\) may function to coordinate the elimination of nitrogenous waste, the acquisition of Na\(^+\) and the removal of metabolic acid. At present, it is unclear which of these processes would be the first to be truly limited by cutaneous exchange, necessitating its branchial shift. Gill ablation studies have demonstrated clearly that branchial Na\(^+\) uptake is vital to larval survival earlier in development than branchial O\(_2\) uptake [28] and similar studies may be useful in determining
at which point in development branchial ammonia excretion and acid-base exchange become critical.

(d) Future perspectives
This study, in addition to further affirming and adding a novel element to the ionoregulatory hypothesis, provides evidence that the Na+/NH\textsubscript{4}+ exchange complex develops at the onset of branchial ontogeny. An interesting avenue for future research is the examination of the selective pressures which led to the evolution of such coupled exchanges. Interestingly, in hagfish, extant relatives of the most ancestral jawless fishes, all of the components of the Na+/NH\textsubscript{4}+ exchange complex are expressed in the gills [29,30] yet these fish display only Na+/H\textsuperscript{+} exchange and not Na+/NH\textsubscript{4}+ exchange [31], despite ammonia being the dominant form of nitrogenous waste excreted and the gills accounting for the majority of its excretion [29,32]. The question of why and when different branchial exchange metabolons evolved in fishes is an interesting one and may lead to a better overall understanding of the arrangement of branchial ionoregulatory, acid-base and nitrogen excretion mechanisms in modern fishes.

Ethics statement. All experimental procedures were approved by the animal care committee of McMaster University.

Data accessibility. All data used in figures are available through the Dryad repository (doi:10.5061/dryad.4b4rb).

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