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REVIEW

Ammonia and urea handling by early life stages of fishes

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ABSTRACT

Nitrogen metabolism in fishes has been a focus of comparative physiologists for nearly a century. In this Review, we focus specifically on early life stages of fishes, which have received considerable attention in more recent work. Nitrogen metabolism and excretion in early life differs fundamentally from that of juvenile and adult fishes because of (1) the presence of a chorion capsule in embryos that imposes a limitation on effective ammonia excretion, (2) an amino acid-based metabolism that generates a substantial ammonia load, and (3) the lack of a functional gill, which is the primary site of nitrogen excretion in juvenile and adult fishes. Recent findings have shed considerable light on the mechanisms by which these constraints are overcome in early life. Perhaps most importantly, the discovery of Rhesus (Rh) glycoproteins as ammonia transporters and their expression in ion-transporting cells on the skin of larval fishes has transformed our understanding of ammonia excretion by fishes in general. The emergence of larval zebrafish as a model species, together with genetic knockdown techniques, has similarly advanced our understanding of ammonia and urea metabolism and excretion by larval fishes. It has also now been demonstrated that ammonia excretion is one of the primary functions of the developing gill in rainbow trout larvae, leading to new hypotheses regarding the physiological demands driving gill development in larval fishes. Here, we highlight and discuss the dramatic changes in nitrogen handling that occur over early life development in fishes.

KEY WORDS: Fish development, Gill ontogeny, Ion regulation, Ornithine urea cycle, OUC, Rhesus proteins, Urea transporter

Introduction

Ammonia is generated as a waste product of protein and amino acid catabolism. In this Review, 'ammonia' refers to total ammonia, whereas 'NH_3' and 'NH_4^+' refer to non-ionic and ionic ammonia, respectively. The pK of this equilibrium ($NH_3+H_2O \leftrightarrow NH_4^+ + OH^-$) ranges from 9.3 to 9.8 at 28–10°C (tropical and temperate fishes) and thus the majority of ammonia exists as NH_4^+ at physiological pH and plasma ionic strength (Cameron and Heisler, 1983). Ammonia excretion and/or detoxification is vitally important, as ammonia is toxic when present at elevated levels within blood and tissues. While some animals must rely on ammonia detoxification via the ATP-consuming synthesis of less-toxic compounds such as glutamine, urea or uric acid, most fishes simply excrete ammonia into the surrounding environment. The study of ammonia excretion by fishes has its roots in some of the pioneers of comparative physiology. Homer Smith (1929) first demonstrated that the gills were the dominant site of ammonia excretion in adult fishes and, a decade later, August Krogh (1939) presented evidence suggesting that NH_4^+ excretion is coupled to Na^+ uptake, a physiological process that has since been debated extensively. These discoveries founded nearly nine decades of research into the production and excretion of nitrogenous waste in fishes. While most studies have employed adult fishes, many others have addressed nitrogen metabolism specifically in early life stages – i.e. embryonic and larval fishes.

Fish early life stages face three physiological problems with respect to ammonia excretion. Firstly, in embryos, the presence of the acellular chorion (see Glossary) imposes a limitation on effective ammonia elimination. Secondly, yolk proteins and amino acids are the main source of fuel in embryos and larvae, generating a constant surfeit of ammonia. Thirdly, immediately following hatching, larvae generally lack a functional gill, which is the primary site of ammonia excretion in adult fishes. In this Review, we will explore the features of early life stage fishes that help to overcome these critical problems.

A number of recent discoveries have advanced our understanding of nitrogen handling in fishes, and these will also be reviewed here. For instance, in 2007, the discovery of a role for Rhesus (Rh) glycoproteins as ammonia transport channels in fishes drastically changed how fish physiologists viewed ammonia excretion (Hung et al., 2007; Nakada et al., 2007a; Nawata et al., 2007) and led to updated transport models (Weihrauch et al., 2009; Wright and Wood, 2009). Similarly, the use of genetic knockdown approaches has allowed researchers to more precisely understand the role of Rh glycoproteins in ammonia excretion (e.g. Braun et al., 2009a; Kumai and Perry, 2011; Shih et al., 2012), and the roles of carbamoyl phosphate synthetase (LeMoine and Walsh, 2013) and urea transporters (Braun et al., 2009a) in the production and excretion of urea by larval zebrafish. We will conclude with a discussion of ammonia excretion as the primary function of the developing gill and contrast this view to the oxygen and ionoregulatory hypotheses that posit that oxygen uptake and ionoregulation, respectively, are the dominant gill functions in developing fishes.

Nitrogen metabolism

Ammonia and urea are the two major forms of nitrogen that are produced and excreted to the surrounding environment in most fishes (Wood, 1993). In this section we will focus specifically on the production of ammonia and urea by embryonic and larval fishes.

Ammonia

The majority of ammonia produced by typical fish species results from amino acid catabolism via specific deaminases or by transdeamination through the combined actions of aminotransferases and glutamate dehydrogenase (GDH). Transdeamination is generally considered to be the major route of ammonia production (Wright and Fyhn, 2001). Early life stages of fish rely on the catabolism of yolk amino acids and proteins for energy production during the period of yolk sac absorption. Although the developmental timing varies, nearly every fish species studied to date experiences a period where amino acid

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List of abbreviations

CA	carbonic anhydrase
CpG	cytosine-phosphate-guanine
CPS	carbamoyl phosphate synthetase
CRISPR	clustered regularly interspaced palindromic repeats
CYA	complete yolk sac absorption
dpf	days post-fertilization
dph	days post-hatching
GDH	glutamate dehydrogenase
GS	glutamine synthetase
HEA	high environmental ammonia
HR cells	H^+-ATPase-rich cells
J_{amm}	ammonia excretion
$J_{Na,in}$	Na^+ uptake
J_{urea}	urea excretion
miRNA	microRNA
$\dot{M}_{O_2}$	O_2 consumption
NHE	Na^+/H^+-exchanger
OUC	ornithine–urea cycle
PVF	perivitelline fluid
Rh	rhesus
SIET	scanning ion-selective electrode technique
UT	urea transporter

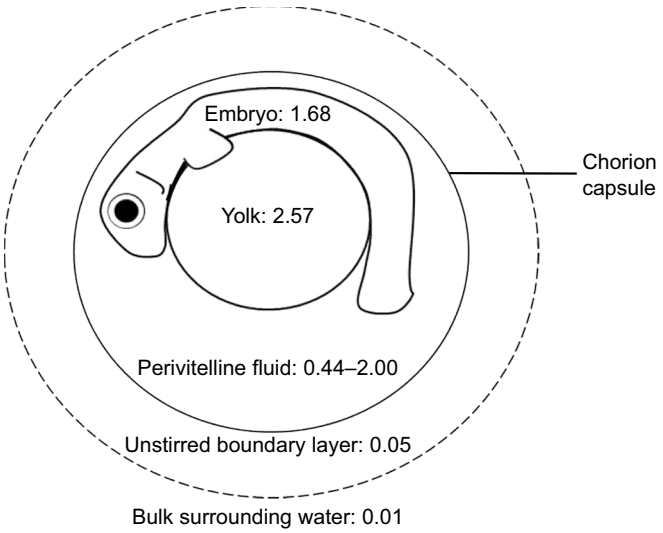

Fig. 1. Distribution of ammonia within the rainbow trout embryo and its microenvironment. Ammonia concentration (mmol l^{-1}) within the embryo body, yolk, surrounding perivitelline fluid, unstirred boundary layer surrounding the chorion and bulk water. Figure and values adapted from Dhiyebi et al. (2013) and Wright and Fyhn (2001).

catabolism is the major source of energy production (Finn et al., 1991, 1995; Fyhn and Serigstad, 1987; Rønnestad et al., 1992a,b, 1999). In some instances, metabolic rate is supported entirely by the catabolism of free amino acids within the yolk (Finn et al., 1995). Thus, embryonic and larval fishes produce a substantial ammonia load as a by-product of an amino acid-based metabolism. This is reflected by developmental increases in whole-body ammonia content (Braun et al., 2009a; Chadwick and Wright, 1999; Essex-Fraser et al., 2005; Finn et al., 1991; Fyhn and Serigstad, 1987; Kharbuli et al., 2006; Rice and Stokes, 1974; Rønnestad et al., 1992a,b; Wright et al., 1995a; Zimmer et al., 2014b) and ammonia excretion rate (Braun et al., 2009a; Chadwick and Wright, 1999; Essex-Fraser et al., 2005; Finn et al., 1991; Kharbuli et al., 2006; Rønnestad et al., 1992a; Wright et al., 1995a; Zimmer et al., 2014b).

Internal ammonia levels increase over embryonic development as a result of the presence of a chorion capsule (Fig. 1). The chorion capsule has pore channels that are permeable to small solutes such as NH_3/NH_4^+ (Groot and Alderdice, 1985; Peterson and Martin-Robichaud, 1987), but it constrains ammonia excretion by limiting convective diffusion away from the embryo. There is an unstirred boundary layer surrounding the embryo that has elevated levels of ammonia relative to the bulk water (Fig. 1) (Dhiyebi et al., 2013). Furthermore, there is an ammonia gradient between the perivitelline fluid (PVF; see Glossary; Fig. 1) surrounding the embryo and the unstirred boundary layer surrounding the chorion, demonstrating the diffusion barrier across the chorion (Dhiyebi et al., 2013; Rahaman-Noronha et al., 1996). Within the embryo, ammonia is generally found at higher concentrations in the yolk than in the body (Fig. 1; Bucking et al., 2013; Essex-Fraser et al., 2005). This sequestration may be a function of an acidic yolk (Rahaman-Noronha et al., 1996) relative to the rest of the body, which would act to trap NH_3 as NH_4^+. The yolk sac membrane may also be permeable to NH_4^+, and it has a negative potential relative to the surrounding PVF (Rahaman-Noronha et al., 1996), perhaps indicating that NH_4^+ is also sequestered in the yolk based on membrane potential, as occurs in the muscle of adult trout (Wang et al., 1996; Wright and Wood, 1988). The dominant sites of ammonia production in embryos and larvae and the mechanisms underlying ammonia distribution within the body and yolk are currently not well understood and should be addressed in future research.

Glossary

Ammonotelic
Excreting the majority of nitrogen as ammonia.
Branchial
Pertaining to the gill.
Chorion
Acellular capsule surrounding the fish embryo.
Gnathostomes
Jawed fishes.
Ionocyte
Epithelial cell type involved in ion transport.
Metabolon
Structural or functional complex of enzymes or proteins.
Perivitelline fluid
Fluid surrounding the embryo contained within the chorion.
Ureotelic
Excreting the majority of nitrogen as urea.

Urea

While storage of ammonia in the yolk may represent one mechanism whereby ammonia levels within the body are minimized, the primary mechanism is undoubtedly the production of urea. In typical ammonotelic (see Glossary) juvenile and adult fishes, urea is produced primarily by hydrolysis of dietary arginine (arginolysis), and also by the breakdown of uric acid (uricolysis). In early life stages, urea is additionally synthesized from glutamine and HCO_3^- via the ornithine–urea cycle (OUC; Fig. 2), which is active in early life but later repressed. In most fishes, carbamoyl phosphate synthetase III (CPSIII) is responsible for assimilating one amino-nitrogen into the cycle, preferentially accepting glutamine as a nitrogen donor (Anderson, 1976, 1980, 1981; Anderson and Casey, 1984). As such, the glutamine-forming enzyme glutamine synthetase (GS), which converts glutamate and ammonia into glutamine (Fig. 2), is generally considered as an accessory enzyme of the OUC in fishes. However, in at least one ureotelic (see Glossary) teleost, CPSIII also accepts ammonia as a nitrogen donor

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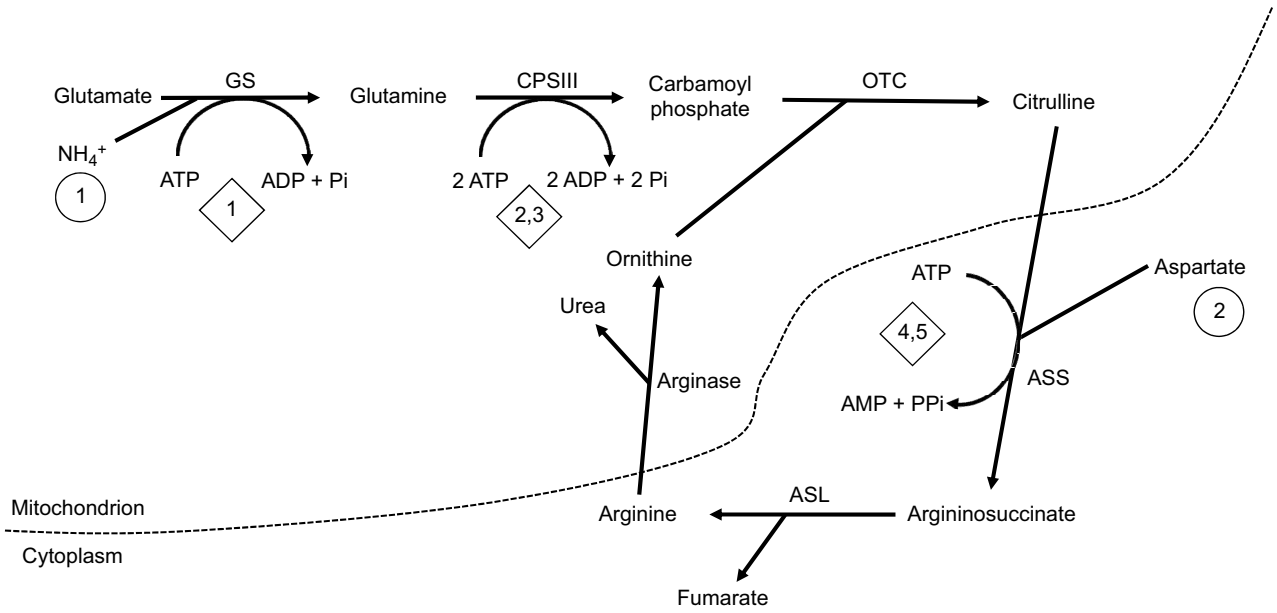

Fig. 2. The ornithine–urea cycle in early life stages of fishes. The ornithine–urea cycle (OUC) of fishes consists of five major enzymes: carbamoyl phosphate synthetase III (CPSIII), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase. GS, glutamine synthetase, is a key accessory enzyme. In the figure, numbers within circles represent the nitrogen-donating steps of the cycle and numbers within diamonds represent the ATP-consuming steps of the cycle. Note that ASS utilizes only 1 ATP but releases free AMP and pyrophosphate (PPi). AMP requires the input of ATP to replenish the ADP pool and thus this reaction is considered to consume 2 moles of ATP overall, assuming that this AMP remains within the AMP–ATP–ADP pool. A total of 2 moles of nitrogen are donated to form urea and the cycle consumes 5 moles of ATP for every mole of urea formed. Adapted from Ballantyne (1997).

(Lindley et al., 1999). In mammals, CPSI accepts ammonia as a nitrogen donor, and in both fish and mammals, CPSII accepts both glutamine and NH_4^+ as donors and plays a role in pyrimidine synthesis. In all animals possessing the OUC, the second amino-nitrogen is donated by aspartate, when argininosuccinate synthetase converts citrulline to argininosuccinate. The production of urea is energetically expensive, consuming 5 moles of ATP for every mole of urea produced (Fig. 2; see Ballantyne, 1997; Wright and Fyhn, 2001, for further review).

An active OUC is present in fish early life stages. Dépêche et al. (1979) first demonstrated that urea content in developing embryos of oviparous rainbow trout (*Oncorhynchus mykiss*) and viviparous guppies (*Poecilia reticulata*) increased over development in conjunction with the biological incorporation of radiolabelled HCO_3^- into urea extracts. While previous studies had concluded that the OUC was not functional in rainbow trout embryos and larvae (based on the absence of detectable CPS activity; Rice and Stokes, 1974), Wright et al. (1995a) later demonstrated that CPSIII activity was detected when glutamine was included in the reaction buffer. The mRNA expression and/or activity of CPSIII and other OUC enzymes has now been demonstrated in embryos and larvae of ammonotelic species [rainbow trout (Korte et al., 1997; Steele et al., 2001; Wright et al., 1995a), zebrafish (*Danio rerio*; LeMoine and Walsh, 2013), Atlantic cod (*Gadus morhua*; Chadwick and Wright, 1999), Atlantic halibut (*Hippoglossus*; Terjesen et al., 2000)] and ureotelic species [Magadi tilapia (*Alcolapia grahami*; Randall et al., 1989; Lindley et al., 1999); African catfish (*Clarias batrachus*; Kharbuli et al., 2006; Terjesen et al., 2001) and gulf toadfish (*Opsanus beta*; Barimo et al., 2004)]. In ammonotelic fishes, CPSIII activity and OUC functionality are eventually repressed during later development. Therefore, the levels of urea accumulated during embryogenesis generally decrease as larval development proceeds (Dépêche et al., 1979; Essex-Fraser et al., 2005; Kharbuli et al., 2006; Rice and Stokes, 1974; Wright et al., 1995a; Zimmer and Wood, 2016), probably owing to both an increase in excretion and a decrease in OUC activity. The increase in urea excretion may also be attributed to the catabolism of arginine, as whole-body levels of arginine tend to decrease over development (Finn et al., 1991; Rice and Stokes, 1974; Rønnestad et al., 1992a,b), in parallel with an increase in arginase activity (Chadwick and Wright, 1999; Rice and Stokes, 1974; Terjesen et al., 2002; Wright et al., 1995a) and urea transporter mRNA expression (Hung et al., 2008), which is discussed further below. The mechanism by which CPSIII and overall OUC activities are silenced over development in ammonotelic fish is currently not known but, at least in zebrafish, it does not appear to involve the methylation of cytosine-phosphate-guanine (CpG) islands in the promoter region of the CPSIII gene (LeMoine and Walsh, 2013). The role of regulatory small RNAs or microRNAs (miRNAs), which may be differentially expressed over zebrafish development (Pasquinelli et al., 2000) and which can target mRNAs for degradation (Giraldez et al., 2006; see Mishima, 2012, for a review), may be an interesting avenue for future research on CPSIII gene silencing.

Ammonia detoxification

Griffith (1991) proposed that the production of urea via the OUC in fish early life stages probably evolved in early gnathostomes (see Glossary) to prevent ammonia toxicity during prolonged, amino acid-fuelled embryogenesis. Several studies have used high environmental ammonia (HEA) or high pH (pH 9–9.5) exposures, which inhibit ammonia excretion in adult fishes (Wilkie and Wood, 1991, 1994; Wilson et al., 1994; Wright and Wood, 1985), to assess the capacity of fishes in early life stages to detoxify ammonia to urea. In rainbow trout embryos, exposure to HEA led to an inhibition or reversal of ammonia excretion (J_{amm}), but no change in urea excretion (J_{urea}) (Sashaw et al., 2010; Wright and Land, 1998). The urea content of the

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embryos, however, did increase (Sashaw et al., 2010), perhaps suggesting that the presence of the chorion also limits urea diffusion at this stage. Indeed, in free-swimming larvae lacking a chorion, J_{urea} increased in response to HEA or high pH exposure, in conjunction with a decrease in J_{amm} (Wright and Land, 1998); the same has been observed in larval zebrafish (Braun et al., 2009b). The capacity to convert ammonia to urea was detected as early as 1 day post-fertilization (dpf) in zebrafish that were treated with injections of ammonia into the yolk (Bucking et al., 2013), suggesting the presence of an active OUC capable of detoxifying exogenous ammonia. However, in trout larvae exposed to chronic HEA, the activity of CPSIII could only account for 50% of increased urea production (Steele et al., 2001). This is similar to adult fishes chronically exposed to high pH, where J_{amm} decreases and J_{urea} transiently increases (Wilkie et al., 1996; Wright, 1993), despite the absence of a functional OUC. It is unclear how or why the excretion of non-OUC-derived urea increases in response to ammonia loading in these fishes. Interestingly, Steele et al. (2001) also showed that ammonia accumulation during HEA exposure in trout embryos was limited to the yolk, similar to other findings (Bucking et al., 2013; Essex-Fraser et al., 2005; Rahaman-Noronha et al., 1996), highlighting this potential mechanism for limiting ammonia toxicity. However, the role of the OUC in detoxifying ammonia cannot be discounted. In zebrafish larvae injected with ammonia, morpholino antisense oligonucleotide knockdown of CPSIII led to a decrease in urea production and a consequently elevated whole-body ammonia content relative to fish treated with sham morpholino (LeMoine and Walsh, 2013). Overall, it is clear that early life stages of fishes possess the capacity to limit ammonia toxicity, and in some instances are extremely tolerant to ammonia, surviving exposure to 27 mmol l^{-1} NH_4Cl for up to 6 days (Atlantic halibut; Terjesen et al., 1998).

Ammonia excretion

The major nitrogenous waste excreted can vary between ammonia and urea in developing fishes (Braun et al., 2009a; Chadwick and Wright, 1999; Essex-Fraser et al., 2005; Wright et al., 1995a); however, ammonia predominates following hatching (Braun et al., 2009a; Chadwick and Wright, 1999; Essex-Fraser et al., 2005; Terjesen et al., 2002; Wright et al., 1995a) (Fig. 3). Indeed, ammonia accounts for 70–90% of total nitrogen excretion in juvenile and adult stages of most ammonotelic fishes (Wood, 1993).

Sites of excretion

It is only in the past decade that researchers have focused on understanding cutaneous J_{amm} by fish early life stages. In juvenile and adult fishes, the dominant site of J_{amm} is the gill (branchial ammonia excretion; see Glossary), while gastrointestinal, renal and cutaneous routes generally contribute less than 10–20% of total J_{amm} (Wood, 1993). Immediately following hatching, however, most larval fishes possess only a rudimentary gill with minimal respiratory and ionoregulatory capacity owing to its small surface area and low density of ionocytes (see Glossary) (Gonzalez et al., 1996; Rombough, 1999, 2007; Varsamos et al., 2002; Wells and Pinder, 1996a). Therefore, larval fishes must rely on extra-branchial routes for J_{amm} and other processes that are typically performed by the gill of developed fishes. The skin of larvae effectively serves this purpose. Early in development, the skin comprises the majority of functional surface area (Rombough, 1999; Rombough and Moroz, 1990; Wells and Pinder, 1996a) and has higher ionocyte density than the gill (Katoh et al., 2000; Rombough, 1999). In fact, the skin of larval fishes accounts for the majority of both gas exchange and ion regulation following hatching (Fu et al., 2010; Wells and Pinder, 1996b; Zimmer et al., 2014b), and the majority of J_{amm} also occurs via the skin in rainbow trout post-hatching (Zimmer and Wood, 2015; Zimmer et al., 2014b) (Fig. 4A). To date, no study to our knowledge has assessed the contribution of gastrointestinal and/or renal routes to overall J_{amm} in early life, despite the fact that the kidney is functional early in development (Varsamos et al., 2005). Our understanding of these extra-branchial contributions may be increased through the use of *in vitro* approaches using isolated guts or nephrons of embryonic and larval fishes. One example of such an approach is the scanning ion-selective electrode technique (SIET) that has been used to measure epithelial NH_4^+ fluxes in Malpighian tubules of fruit flies (Browne and O'Donnell, 2013).

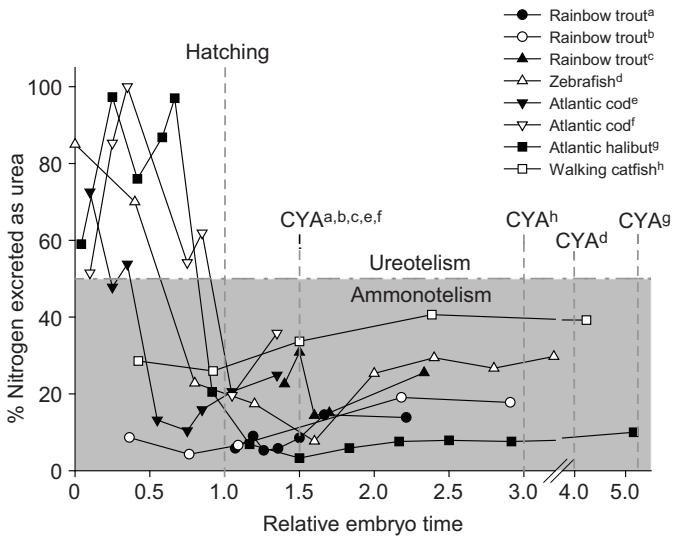

Fig. 3. Ureotelism in early life stages of fish. The graph shows the percentage of nitrogen excreted as urea in early life stages of several ammonotelic species and one facultatively ureotelic species (walking catfish). Relative embryo time was calculated by dividing age in days post-fertilization (dpf) by hatching time in dpf, such that a relative embryo time of 1 represents the hatching time for each species. The first dashed vertical line represents hatching time and the subsequent dashed lines represent the time at complete yolk sac absorption (CYA) for each species (superscript letters). The shaded lower half of the plot represents ammonotelism, where less than 50% of nitrogen is excreted as urea; the upper half represents ureotelism. Data for each species: [a]*Oncorhynchus mykiss* (Wright et al., 1995a); [b]*Oncorhynchus mykiss* (Essex-Fraser et al., 2005); [c]*Oncorhynchus mykiss* (Zimmer et al., 2014b; Zimmer and Wood, 2016); [d]*Danio rerio* (Braun et al., 2009a); [e]*Gadus morhua*, Newfoundland population (Chadwick and Wright, 1999); [f]*Gadus morhua*, New Brunswick population (Chadwick and Wright, 1999); [g]*Hippoglossus hippoglossus* (Terjesen et al., 2002); [h]*Clarias batrachus* (Kharbuli et al., 2006).

The contributions of the intestine and kidney to J_{amm} are likely to be minimal in early life, however, given that renal routes contribute less to overall J_{amm} than cutaneous routes even in adult trout (Zimmer et al., 2014a), and gastrointestinal excretion is negligible (Kajimura et al., 2004). Overall, J_{amm} by fish early life stages is defined by an early cutaneous phase followed by a later shift to branchial J_{amm} (Fig. 4A).

Mechanisms of excretion

Branchial ion and ammonia transport models can vary between species (Dymowska et al., 2012). Therefore, this section will begin with a general overview of the mechanisms of J_{amm} by freshwater fishes, followed by a more detailed discussion of the specific mechanisms utilized by the larval stages of zebrafish, trout and freshwater-acclimated Japanese medaka (*Oryzias latipes*). Notably,

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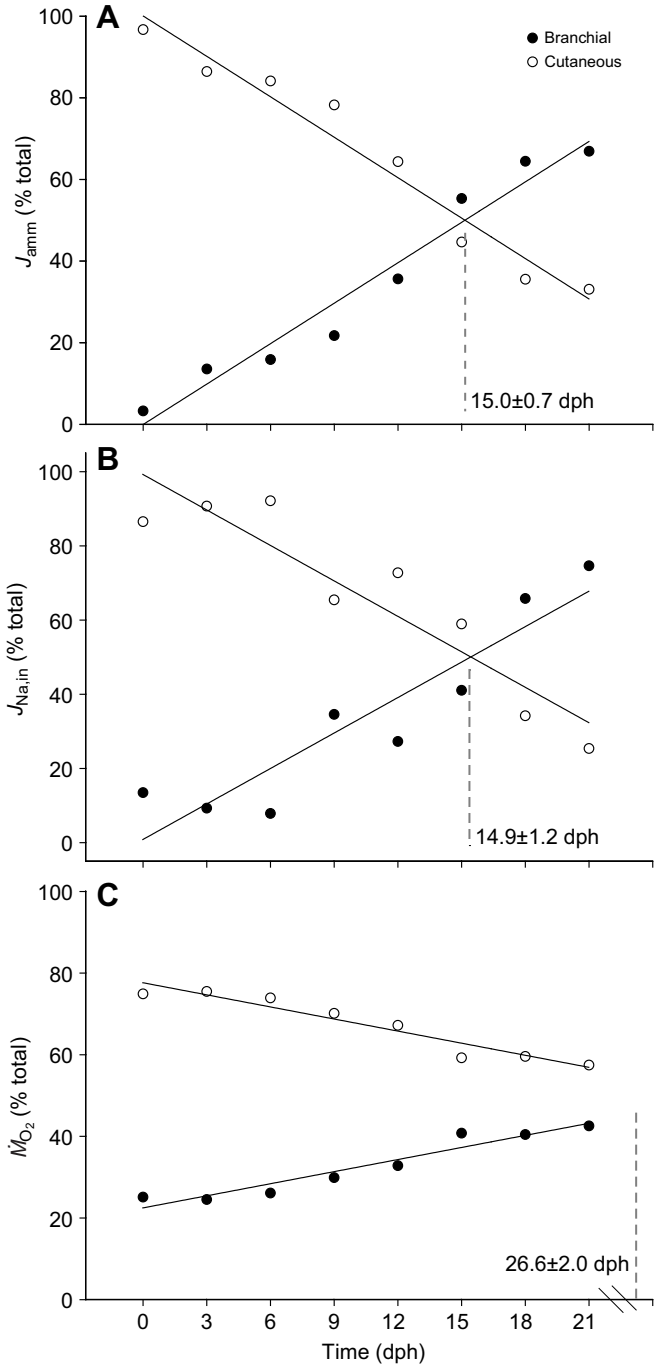

Fig. 4. Cutaneous and branchial contributions to ammonia excretion, Na^+ uptake and O_2 consumption in developing rainbow trout larvae. Relative branchial and cutaneous ammonia excretion (J_{amm}; A), Na^+ uptake ($J_{Na,in}$; B) and oxygen consumption ($\dot{M}_{O_2}$; C), expressed as a percentage of the total, by rainbow trout (*Oncorhynchus mykiss*) over the first 21 days post-hatching (dph). Dashed vertical lines represent the skin-to-gill transition for each process. Data and calculations from Zimmer et al. (2014b).

much less is known regarding the embryonic stage when fish are surrounded by the PVF within the chorion.

Arguably one of the most important advances in the nearly century-long study of J_{amm} in fishes was made only in the past decade with the discovery of Rh glycoprotein expression in the gills and skin (Hung et al., 2007; Nakada et al., 2007a,b; Nawata et al., 2007). Since this discovery, a role for Rh glycoproteins in facilitating bi-directional (Nawata et al., 2010a) epithelial ammonia transport has been demonstrated in many species (Braun et al., 2009a; Nakada et al., 2010; Nawata et al., 2007, 2010a,b; Sinha et al., 2013; Wood et al., 2013; Wu et al., 2010). Nakada et al. (2007b) showed that the apical Rh glycoprotein Rhcg1 (see Box 1) was expressed in the yolk sac epithelium of zebrafish, and that J_{amm} and the number of Rhcg1-expressing cells on the yolk sac increased in conjunction over development. The expression levels of Rh genes in zebrafish embryos and larvae also increased in parallel with J_{amm} over development (Braun et al., 2009a), and this same phenomenon has been observed in early life stages of trout (Hung et al., 2008; Zimmer et al., 2014b). Additionally, many studies have demonstrated strong evidence of a role for Rh glycoproteins in J_{amm} by larval zebrafish using morpholino gene knockdown (Braun et al., 2009a; Kumai and Perry, 2011; Shih et al., 2008, 2012, 2013).

Krogh (1939) first posited that NH_4^+ is excreted by direct exchange with Na^+ in freshwater fishes, thereby maintaining electroneutrality. While this idea was controversial for many years, the current consensus (which has emerged since the discovery of Rh glycoproteins in fishes) supports Krogh's idea, although the mechanism is more complicated than he probably envisaged. Branchial J_{amm}, mediated by ammonia-conductive Rh channel glycoproteins, is thought to be loosely coupled to Na^+ uptake via an indirect 'Na^+/NH_4^+-exchange complex' (Tsui et al., 2009; Wright and Wood, 2009). This mechanism, which is distinctly different from direct Na^+/NH_4^+ exchange (Krogh, 1939; Maetz and Garcia-Romeu, 1964), is a flexible, indirect coupling of Na^+ uptake and NH_4^+ excretion that appears to be facilitated by two primary mechanisms, Na^+/H^+-exchangers (NHEs) and H^+-ATPase (Dymowska et al., 2012; Weihrauch et al., 2009; Wright and Wood, 2009, 2012).

NHEs are believed to facilitate indirect Na^+/NH_4^+ exchange by forming a functional metabolon (see Glossary) with Rh glycoproteins (Fig. 5A; Ito et al., 2013). Notably, the thermodynamic feasibility of NHEs in freshwater has been challenged previously, as intracellular $[Na^+]$ (2–80 mmol l^{-1}) is much greater than $[Na^+]$ of most freshwater (<1 mmol l^{-1}) (Parks et al., 2008). However, closely associated apical Rh glycoproteins are believed to establish an intracellular acidic microenvironment by stripping off H^+ from cytosolic NH_4^+ and subsequently conducting NH_3, thereby providing a sufficiently large pH gradient to drive Na^+/H^+ exchange via NHE (Fig. 5A; Conroy et al., 2005; Khademi, 2004; Knepper and Agre, 2004; Nawata et al., 2010a; Wright and Wood, 2012). In the apical boundary layer, these protons from Na^+/H^+ exchange titrate NH_3 to NH_4^+, maintaining the partial pressure gradient of NH_3 (P_{NH_3}). Previous work has demonstrated that Rhcg1 knockdown reduced Na^+ uptake in larval zebrafish (see 'Zebrafish' section, below). Furthermore, Ito et al. (2014) showed that zebrafish NHE3b expressed in *Xenopus* oocytes facilitated Na^+ uptake from a medium containing only 0.5 mmol l^{-1} Na^+ when oocytes were acidified, supporting the notion that favourable pH gradients can drive Na^+/H^+ exchange against unfavourable $[Na^+]$ gradients. These researchers also presented evidence that NH_4^+ may take the place of H^+ on zebrafish NHE3b (Ito et al., 2014), though this idea has been debated previously (see Wright and Wood, 2009, for review).

H^+-ATPase also facilitates indirect Na^+/NH_4^+ exchange by simultaneously promoting J_{amm} by boundary layer acidification (thereby maintaining the P_{NH_3} gradient) and promoting electrogenic Na^+ uptake via putative Na^+ channels, which may be acid-sensing ion channels (Dymowska et al., 2014, 2015). It has been argued that this electrogenic Na^+ uptake model is more favourable than the NHE model on thermodynamic grounds (Avella and Bornancin, 1989; Parks et al., 2008). It is also important to note that boundary

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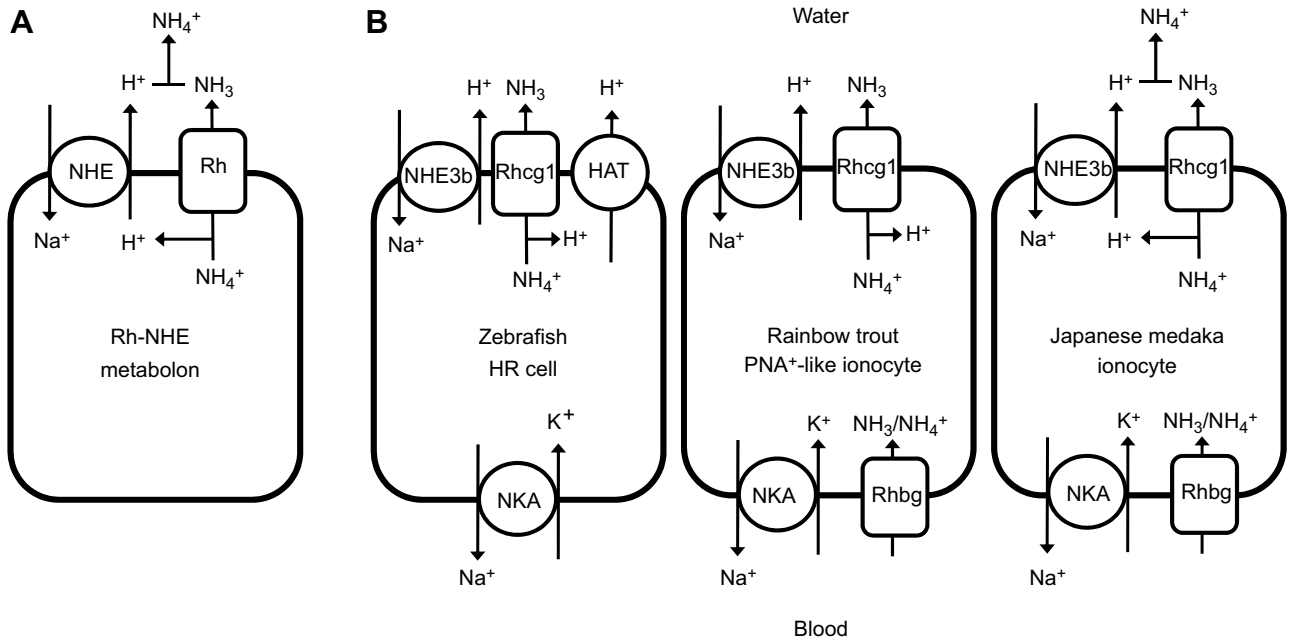

Fig. 5. Models of ammonia excretion and Na^+ uptake by cutaneous ionocytes of larval freshwater fishes. (A) Generalized diagram of Rhesus-Na^+/H^+-exchanger (Rh-NHE) metabolon function in freshwater fishes. (B) Models of apical ammonia excretion and Na^+ uptake by H^+-ATPase-rich (HR) cells of zebrafish, peanut lectin agglutinin-positive (PNA^+)-like ionocytes of rainbow trout and ionocytes of Japanese medaka. HAT, H^+-ATPase; NHE, Na^+/H^+-exchanger; NKA, Na^+/K^+-ATPase; Rhcg1, Rhesus glycoprotein Rhcg1. See 'Ammonia excretion' section for details.

layer acidification may also be achieved by the hydration of excreted CO_2 by apical carbonic anhydrase (CA). Both apical and cytosolic CA can also influence Na^+ uptake (Ito et al., 2013; Lin et al., 2008) and have been implicated in coordinating indirect Na^+/NH_4^+ exchange (see Hwang and Chou, 2013; Wright and Wood, 2009, for review).

Zebrafish

The mechanisms of J_{amm} and Na^+ uptake ($J_{Na,in}$) by larval zebrafish are the best described for any fish species (Hwang, 2009; Hwang and Chou, 2013; Kwong et al., 2014). Lin et al. (2006) first identified H^+-ATPase-rich cells (HR cells; Fig. 5B) in the yolk sac membrane of larval zebrafish. These cells, which are also present in the gills, secrete acid (Lin et al., 2006), express NHE3b (Ito et al., 2013; Yan et al., 2007) and Rhcg1 (Nakada et al., 2007b), and are major sites of Na^+ accumulation, which is blocked by inhibition of H^+-ATPase and NHE with bafilomycin and EIPA, respectively (Esaki et al., 2007). They are also believed to be an important site of J_{amm} (Shih et al., 2012, 2013). Although HR cells in the yolk sac skin possess the hallmark components of Na^+/NH_4^+ exchange, there does not appear to be any coupled exchange under normal laboratory conditions [neutral pH (7–8); 0.5–0.8 mmol l^{-1} Na^+] (Kumai and Perry, 2011; Shih et al., 2012). However, when zebrafish larvae were reared under low pH (pH 4) (Kumai and Perry, 2011) or low [Na^+] (0.005 mmol l^{-1}) (Shih et al., 2012), substantial Na^+/NH_4^+ exchange was observed. In 4 dpf larvae reared at neutral pH, knockdown of Rhcg1 led to a significant inhibition of whole-body J_{amm}, but had no effect on $J_{Na,in}$. However, in low pH, Rhcg1 knockdown substantially reduced both J_{amm} and $J_{Na,in}$ (Kumai and Perry, 2011). Similarly, Rhcg1 or NHE3b knockdown led to a concomitant decrease in NH_4^+ efflux and Na^+ influx across the yolk sac skin of zebrafish reared in low Na^+, but coupling of these processes was generally absent under normal conditions (Shih et al., 2012). When larvae were reared in HEA [5 mmol l^{-1} NH_4^+ as $(NH_4)_2SO_4$], cutaneous NH_4^+ efflux increased, but Na^+ influx was unchanged. However, significant increases in both NH_4^+ flux and Na^+ flux, measured in the absence of external ammonia, were observed when larvae were simultaneously reared in low Na^+ and HEA (Shih et al., 2012). Therefore, the Rh-NHE metabolon (Fig. 5A) probably plays an important role in coordinating Na^+/NH_4^+ exchange by HR cells (Fig. 5B) only under conditions where the system is challenged, such as low pH or low Na^+, despite these findings being in contradiction to thermodynamic assumptions of Na^+/H^+ exchange in freshwater (Parks et al., 2008). An important caveat of these studies is that these acidic and dilute conditions probably do not reflect the natural environment of this species (Engeszer et al., 2007), and thus indirect Na^+/NH_4^+ exchange in larval zebrafish may not occur in natural conditions. Nevertheless, the zebrafish is a useful model species for those fishes that do inhabit acidic and dilute environments (Kwong et al., 2014).

Keratinocytes in the yolk sac membrane are also important sites of NH_4^+ efflux. In zebrafish larvae, keratinocytes express basolateral and apical Rhbg (Shih et al., 2013), whereas in other species, Rhbg is generally restricted to the basolateral membrane (Nakada et al., 2007a). In these cells, there is no coupling of NH_4^+ efflux to H^+ efflux or Na^+ influx, and J_{amm} is probably passive, in contrast to H^+-ATPase- and/or NHE3b-facilitated J_{amm} by HR cells. The

Box 1. Rhcg gene nomenclature in fish

In 2011, the nomenclature of the rhcg genes in zebrafish was changed in order to conform to zebrafish gene nomenclature guidelines. *drRhcg1*, *drRhcg2* and *drRhcg3* were renamed *rhcgb*, *rhcgl1* and *rhcga*, respectively (see Table 1). In trout, these names have yet to be adopted, although this may change during the annotation process of the sequenced trout genome (Berthelot et al., 2014). In this Review, we have opted to maintain the commonly used nomenclature of cited studies in order to avoid confusion.

Table 1. Rhcg nomenclature in zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*)

Species	Common gene name	Gene symbol*	Accession no. in original reference	Reference
D. rerio	Rhcg1	*rhcgb*	AB286865	(Nakada et al., 2007b)
D. rerio	Rhcg2	*rhcgl1*	AB286866	(Nakada et al., 2007b)
D. rerio	Rhcg3	*rhcga*	AB286867	(Nakada et al., 2007b)
O. mykiss	Rhcg1	*rhcg1-b*	EF051115/DQ431244	(Nawata et al., 2007)
O. mykiss	Rhcg2	*rhcg*	AY619986	(Huang and Peng, 2005)

*As listed on ZFIN and GenBank on 9 February 2017.

concentration against which ammonia efflux can be maintained is 2 mmol l^{-1} greater in HR cells than in keratinocytes (Shih et al., 2013). However, when NH_4^+ efflux was measured using SIET at the yolk sac surface, Rhbg knockdown caused a much greater reduction in flux than Rhcg1 knockdown, suggesting that keratinocytes may be the dominant site of J_{amm} under normal conditions (Shih et al., 2013), whereas HR cells play a greater role under challenging conditions. In a whole-body study, Braun et al. (2009a) conversely found that knockdown of Rhcg1 and Rhbg had similar inhibitory effects on whole-body J_{amm} of larval zebrafish, which perhaps was related to the inclusion of branchial contributions to J_{amm} in the whole-body approach that were not included using the yolk sac-specific SIET. Another important caveat with respect to this work in larval zebrafish is that recent studies have demonstrated that effects of morpholino knockdown can differ from those of gene knockout, partly as a result of off-target effects or compensatory responses (Kok et al., 2015; Rossi et al., 2015), complicating the interpretation of these findings. The use of genetic knockout approaches such as the clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 technique is imperative in future work.

Rainbow trout

In rainbow trout larvae reared in neutral waters with sufficient Na^+ (>0.5 mmol l^{-1}), J_{amm} and $J_{Na,in}$ increased over development (Fu et al., 2010; Zimmer and Wood, 2015; Zimmer et al., 2014b), and these processes were tightly coupled to one another at the gill, but not the skin (Zimmer et al., 2014b). Cutaneous mechanisms of J_{amm} and $J_{Na,in}$ by larval trout are thus similar to those of zebrafish, where indirect Na^+/NH_4^+ exchange is absent under comparable conditions. In a follow-up study (Zimmer et al., 2017), trout were exposed to HEA (500 µmol l^{-1} NH_4HCO_3) for 12 h either soon after hatching [6–8 days post-hatching (dph); referred to here as 'post-hatch fish'], where the skin is the major site of J_{amm} and $J_{Na,in}$, or following CYA (30 dph; 'CYA fish'), where the gill is the dominant site of flux. Following HEA exposure, J_{amm} by the yolk sac skin of post-hatch trout and the gill of CYA trout increased after transfer to ammonia-free control water. In the CYA stage, this was accompanied by an increase in branchial $J_{Na,in}$, in accordance with the indirect Na^+/NH_4^+ exchange complex model. In post-hatch fish, however, cutaneous $J_{Na,in}$ was unchanged (Zimmer et al., 2017). Expression of NHE2 (but not NHE3b) and H^+-ATPase mRNA increased significantly in the CYA gill in response to HEA, but was unchanged in the post-hatch yolk sac skin, consistent with a lack of $J_{Na,in}$ stimulation. Furthermore, while ionocytes expressing Rhcg1, NHE2 and NHE3b were identified in CYA gill sections, yolk sac skin ionocytes were immunoreactive for Rhcg1 and NHE3b only [peanut lectin agglutinin-positive (PNA^+)-like ionocytes; Fig. 5B]. In gill sections, Rhcg2 appeared to be mainly present in pavement cells, but Rhcg2 immunoreactivity was not detectable in the yolk sac epithelium, even after HEA exposure (Zimmer et al., 2017). It is currently not known whether trout yolk sac PNA^+-like ionocytes express apical H^+-ATPase (Fig. 5B).

Clearly, ionoregulatory mechanisms can change over development as the primary site of J_{amm} and $J_{Na,in}$ changes from the skin to the gills (Zimmer et al., 2017). NHE2 appears to play an important role in coordinating Na^+/NH_4^+ exchange by the gill, and its functional absence in the yolk sac skin may account for the lack of Na^+/NH_4^+ coupling. The upregulation of NHE2 mRNA expression in the gills in response to HEA has been demonstrated repeatedly in juvenile and adult trout (Sinha et al., 2013; Tsui et al., 2009; Wood and Nawata, 2011; Zimmer et al., 2010). In future work, it will be of interest to test whether challenging larval trout with low pH and/or low Na^+ will activate functional Na^+/NH_4^+ exchange, as has been described in zebrafish.

Japanese medaka

In Japanese medaka larvae, the linkage between NH_4^+ excretion and Na^+ uptake is different from that of larval zebrafish and rainbow trout. Cutaneous NH_4^+ efflux at the yolk sac membrane has been demonstrated using SIET, and acclimation to either low Na^+ (nominal 0 mmol l^{-1} Na^+) or HEA [5 mmol l^{-1} NH_4^+ as $(NH_4)_2SO_4$] led to simultaneous increases in both NH_4^+ efflux and Na^+ influx (Wu et al., 2010). This is different from larval zebrafish or trout, where acclimation to low Na^+ or HEA led to independent increases in cutaneous Na^+ influx and NH_4^+ efflux, respectively (Shih et al., 2012; Zimmer et al., 2017). Moreover, in medaka acclimated to normal conditions, acute exposure to low pH (pH 6) led to a simultaneous upregulation of Na^+ influx and NH_4^+ efflux at the surface of individual ionocytes (Wu et al., 2010). Wu et al. (2010) concluded that a Rh-NHE mechanism for coordinating Na^+/NH_4^+ exchange was present in yolk sac skin ionocytes of medaka (Fig. 5B) acclimated to normal conditions or to low Na^+ or HEA. NH_4^+-excreting keratinocytes are also expressed in the yolk sac of larval medaka and, similar to zebrafish keratinocytes, these cells have lower intrinsic rates of NH_4^+ efflux than ionocytes and do not contribute to Na^+/NH_4^+ exchange (Wu et al., 2010). Notably, the acid-secreting ionocytes of medaka are not believed to express apical H^+-ATPase (Fig. 5B), unlike the HR cells of zebrafish (Wu et al., 2010). Overall, the mechanism of J_{amm} by the medaka yolk sac differs from that of zebrafish or trout in that Na^+/NH_4^+ exchange is present under normal conditions.

Urea excretion

Urea accounts for only 10–20% of total nitrogen excretion in adult and juvenile ammonotelic fishes (Wood, 1993), but this can vary in early life (Fig. 3). In rainbow trout, J_{urea} generally increases over larval development, despite the eventual loss of OUC activity (Essex-Fraser et al., 2005; Wright et al., 1995b; Zimmer and Wood, 2016), probably as a result of the switch from endogenous to exogenous feeding, coupled to increases in arginase activity, discussed above, and urea transporter expression, discussed below.

Sites of excretion

The site of J_{urea} changes over development. In adult fishes, the primary site of J_{urea} is the gill, whether fishes are ammonotelic

(McDonald and Wood, 2004a; Smith, 1929; Wright et al., 1995b) or ureotelic (e.g. Magadi tilapia, gulf toadfish; Wood et al., 1994; Wood et al., 1995). In post-hatch larval rainbow trout, the skin initially accounts for the majority of J_{urea} (Zimmer and Wood, 2016). In adult fishes, only one study (Smith, 1929) has measured cutaneous J_{urea}, but found it to be negligible in goldfish and common carp. However, J_{urea} through the skin might be substantial in some marine teleosts (Morii et al., 1979; Sayer and Davenport, 1987). Unfortunately, it is not possible to use the divided chamber approach adopted in Zimmer and Wood (2016) to address the overall contributions of renal J_{urea} versus cutaneous J_{urea} in larval trout, as urinary catheterization of fish this small is not feasible. It is possible that urinary excretion plays a significant role in J_{urea}, given that the kidneys are functional in larval fish (Varsamos et al., 2005) and that renal J_{urea} accounts for approximately 25% of total excretion in adult trout (McDonald and Wood, 2004a). Perhaps an *in vitro* approach could be used to assess J_{urea} by isolated nephrons and thus better understand renal contributions to J_{urea} in fish early life stages.

Mechanisms of excretion

Like J_{amm}, J_{urea} depends on facilitated diffusion by a carrier protein. Urea transporters (UTs) are part of the solute carrier (SLC) family of proteins and have been well described in the mammalian literature (e.g. Shayakul et al., 2013). In fish, the first UT was cloned from the kidney of the ureotelic dogfish shark (*Squalus acanthias suckleyii*), and its expression in *Xenopus* oocytes increased the uptake of radiolabelled urea 10-fold (Smith and Wright, 1999). Importantly, this uptake was saturable and phloretin sensitive, which are hallmarks of facilitated urea transport by UTs. UTs have since been identified in the gills of the facultatively ureotelic gulf toadfish (Walsh et al., 2000), the completely ureotelic Lake Magadi tilapia (Walsh et al., 2001a) and the ammonotelic eel *Anguilla japonica* (Mistry et al., 2001), zebrafish (Braun et al., 2009b) and rainbow trout (Zimmer and Wood, 2016). Indeed, UTs appear to occur in a wide variety of ammonotelic teleosts (Walsh et al., 2001b). Considerable functional evidence also supports the presence of UT-facilitated urea transport in the gills of adult ammonotelic rainbow trout. This includes the saturability of J_{urea} at higher rates of urea infusion (McDonald and Wood, 1998), phloretin sensitivity (McDonald and Wood, 2004b) and a profile of urea versus urea analogue permeability (urea>acetamide>thiourea) (McDonald and Wood, 2003) that is identical to that seen in ureotelic teleosts where UTs are known to function (McDonald et al., 2000, 2002; Walsh et al., 2001a). J_{urea} by adult zebrafish is also phloretin sensitive, consistent with the presence of branchial UT (Braun et al., 2009b).

UT-facilitated urea transport has also been demonstrated in embryos and larvae of some ammonotelic species. In newly hatched trout, J_{urea} is inhibited by phloretin and by competition from urea analogues (Pilley and Wright, 2000). Uptake of radiolabelled urea by larvae was also determined to be saturable, with evidence for the presence of a bi-directional facilitated transporter (Pilley and Wright, 2000). In zebrafish embryos, J_{urea} was inhibited by phloretin exposure as early as 1 dpf (Braun et al., 2009b), when embryos are presumably completely reliant upon cutaneous exchange. Furthermore, a role for UT was demonstrated by morpholino knockdown, which resulted in a significant decrease in J_{urea} relative to sham-treated larvae at 4 dpf (Braun et al., 2009a). In trout, mRNA expression of UT in the whole body increased in conjunction with J_{urea} over embryonic and larval development (Hung et al., 2008) and UT transcripts were also detected in the gills, yolk sac skin and body skin of rainbow trout larvae as early as 3 dph; the level of these transcripts increased over development in all three tissues (Zimmer and Wood, 2016). When J_{urea} was experimentally increased by pre-exposure to 25 mmol l^{-1} urea for 12 h, UT mRNA expression in the gills of 55 dph trout increased significantly (Zimmer and Wood, 2016). Overall, these studies support the presence of a UT-facilitated mechanism in the skin (and gills) early in development in trout and zebrafish, when J_{urea} represents a relatively large proportion of total nitrogen excretion. Poorly understood, however, is the localization of UTs in the yolk sac membrane of larval fish (Braun et al., 2009a). Understanding UT localization may shed light on the differential timing of the ontogeny of branchial J_{amm} and J_{urea} that has been observed in rainbow trout (Fig. 6).

Ammonia excretion as the primary function of the developing gill

There has been considerable debate around the question of which physiological process can be considered the primary function of the developing gill. In this section, we review three views of the development of the freshwater fish gill – the oxygen, ionoregulatory

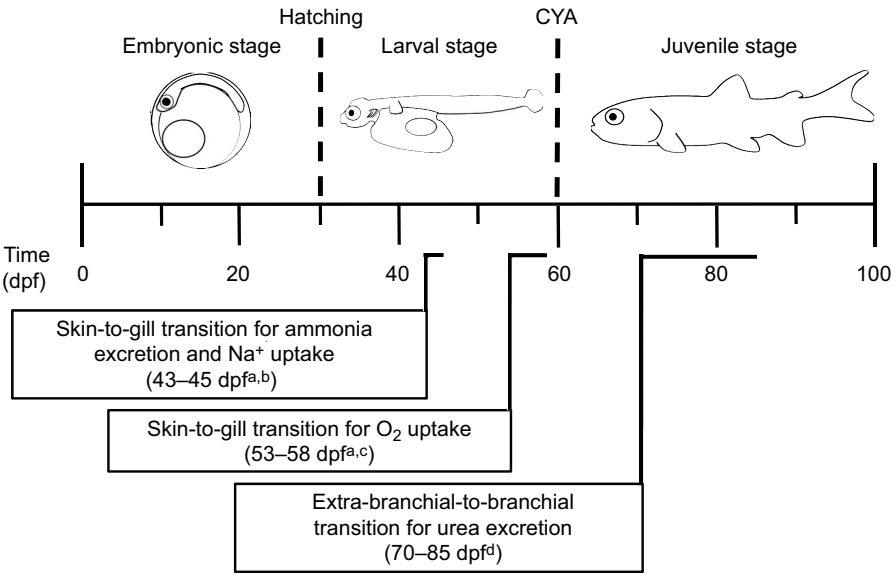

Fig. 6. Schematic drawing of the timing of ontogenetic transitions from cutaneous or extra-branchial exchange to branchial exchange in larval rainbow trout. Data for skin-to-gill (ammonia excretion, Na^+ uptake and O_2 uptake) and extra-branchial-to-branchial (urea excretion) transitions in developing larval rainbow trout were obtained from [a]Zimmer et al. (2014b); [b]Zimmer and Wood (2015); [c]Fu et al. (2010); [d]Zimmer and Wood (2016). Note that urea excretion is referred to specifically as extra-branchial and branchial because of potential renal contributions to overall excretion; see 'Sites of excretion' sections for details. The rearing temperature was 10–12°C.

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and ammonia hypotheses, which posit that the primary functions of the developing gill are to support oxygen uptake, or ionoregulation, or the removal of ammonia, respectively. The oxygen and ionoregulatory hypotheses have been reviewed in detail previously (Brauner and Rombough, 2012; Rombough, 2007), and thus will be discussed only briefly here. Another function of the developing gill is filter feeding (e.g. van der Meeren, 1991), though we are aware of no study to date that has addressed its ontogeny in relation to other physiological functions of the gill.

Oxygen hypothesis

August Krogh (1939) first posited that the gill develops in larval fishes to support increased demand for oxygen uptake over development, coupled to a decrease in effective skin surface area for gas diffusion. Theoretically, this argument is logical. Over larval development, O_2 consumption increases rapidly (Fu et al., 2010; Gilmour et al., 2009; Wells and Pinder, 1996b; Zimmer et al., 2014b), while the skin becomes thicker and scales form, both of which would impede gas diffusion. Moreover, metabolic rate scales with body size at near unity in early life (mass exponent ~0.9), while cutaneous surface area scales with a mass exponent of approximately 0.6 (Rombough and Moroz, 1997). Theoretically, this would eventually lead to a body size at which the cutaneous surface available for O_2 uptake would be unable to match demands in metabolic rate.

Although it is theoretically attractive, there is little experimental evidence for the oxygen hypothesis. Based on a survey of several marine and freshwater larvae and on gas exchange efficiency of adult fish skin, a body size of 100 mg was estimated as the size at which cutaneous surfaces become limiting for gas exchange (Rombough and Moroz, 1997). However, in this survey, most fish began to develop gills long before the 100 mg stage, suggesting that gills initially develop to serve a purpose other than O_2 acquisition. Using O_2 microelectrodes, Rombough (1992) further demonstrated that the P_{O_2} in the efferent branchial arteries of larval trout was slightly lower than in the venous circulation, suggesting that the gill is actually an O_2 sink at this stage.

Ionoregulatory hypothesis

The ionoregulatory hypothesis emerged as a competing view to the oxygen hypothesis, positing that the primary function of the developing gill is ion regulation. Initial evidence supporting this hypothesis was demonstrated in 3 dph tilapia (*Oreochromis mossambicus*) where numerous ionocytes were present on the gill prior to the development of respiratory lamellae (Li et al., 1995). Similar observations on the timing of gill ionocyte appearance have since been reported in larvae of other species (Katoh et al., 2000; Rombough, 1999; Varsamos et al., 2002). Moreover, the same arguments with respect to cutaneous surface area, diffusion distance and overall demand used to support the oxygen hypothesis can similarly be applied to the ionoregulatory hypothesis. In fact, Rombough (2007) argued that increased skin thickness may be more limiting for ionoregulation, as ionocytes require direct contact with the external environment and underlying blood vessels, whereas gas exchange can rely on simple diffusion across larger barriers. In support of the ionoregulatory hypothesis, a gill ablation approach in larval zebrafish demonstrated that cutaneous ion uptake becomes limiting to survival before cutaneous O_2 uptake (7 versus 14 dpf, respectively) (Rombough, 2002). The first study to directly test the ionoregulatory hypothesis used a divided chamber approach to separate cutaneous and branchial O_2 consumption ($\dot{M}_{O_2}$) and $J_{Na,in}$ in developing rainbow trout (Fu et al., 2010). The transition point at which the gill first accounted for 50% of the total whole-body flux for $J_{Na,in}$ occurred at 15–16 dph (45–46 dpf) in development, significantly earlier than that of $\dot{M}_{O_2}$ (23–28 dph; 53–58 dpf). Qualitatively similar data in rainbow trout were reported by Zimmer et al. (2014b). Although these findings have not yet been repeated in other fish species, the ionoregulatory hypothesis is now generally favoured over the oxygen hypothesis (Brauner and Rombough, 2012; Rombough, 2007).

Ammonia hypothesis

We developed the ammonia hypothesis as an extension of the ionoregulatory hypothesis, positing that the primary function of the developing gill is to clear metabolic ammonia via loosely coupled Na^+/NH_4^+ exchange (Zimmer et al., 2014b). The ammonia hypothesis specifically predicts that the ontogeny of Na^+/NH_4^+ exchange is a function of increased demand for ammonia clearance, rather than Na^+ acquisition. As discussed earlier, metabolic rate in larval fishes is largely fuelled by the catabolism of yolk amino acid stores, generating a large metabolic ammonia load. Simultaneously, the capacity to detoxify ammonia as urea is lost over larval development, which may necessitate the development of an effective excretion mechanism in order to avoid ammonia toxicity. Given that ammonia is excreted as both NH_3 and NH_4^+, the developmental increases in gill surface area and ionocyte density that occur over larval development would act to effectively promote branchial J_{amm} in larval fish.

We extended the approach of Fu et al. (2010) to demonstrate that the skin-to-gill 50% transition point for J_{amm} coincided perfectly with that of $J_{Na,in}$ (15 dph; 45 dpf), and long before that of $\dot{M}_{O_2}$ (27 dph; 57 dpf) (Figs 4 and 6) (Zimmer et al., 2014b). Notably, immediately following hatching, the gills accounted for a greater proportion of total $\dot{M}_{O_2}$ (25%) than J_{amm} (3%) or $J_{Na,in}$ (13%), similar to previous findings (Fu et al., 2010). However, it is clear from the rest of the developmental trajectory that the gills become the dominant ionoregulatory/nitrogen excretory organ prior to becoming the dominant O_2 exchange organ, in support of the ionoregulatory and ammonia hypotheses. Moreover, branchial J_{amm} was highly correlated with $J_{Na,in}$ over larval development, suggesting that the ionoregulatory hypothesis should be extended to include Na^+/NH_4^+ exchange as the physiological process which first shifts from the skin to the gills. In addition, ammonia turnover time (the time needed to completely clear the body of ammonia) was initially low following hatching, but increased to a peak at 12–15 dph, followed by a decrease over the rest of the larval developmental period (Fig. 7) (Zimmer et al., 2014b). These data suggested that ammonia clearance becomes less effective over larval development, potentially due to limitations in cutaneous exchange, and that effective clearance is restored after the skin-to-gill transition (thick black dashed line in Fig. 7). The mRNA expression of Rhcg1 and Rhcg2 also increased in the gill over development, consistent with the ontogeny of the gill as a site for J_{amm}. The mRNA expression levels and activities of proteins involved in $J_{Na,in}$ – such as NHE-2, Na^+/K^+-ATPase and H^+-ATPase – also increased as gills developed, in agreement with the gill ontogeny of Na^+/NH_4^+ exchange (Zimmer et al., 2014b). In contrast, urea turnover time was as low as that of ammonia prior to the transition to branchial excretion (thick grey dashed outline in Fig. 7), perhaps because of a renal contribution to J_{urea} in larvae.

In an experiment to tease apart the relative importance of the two components of branchial Na^+/NH_4^+ exchange, rainbow trout larvae were reared in 60 mmol l^{-1} NaCl in order to decrease ionoregulatory demand without affecting the demand for J_{amm}

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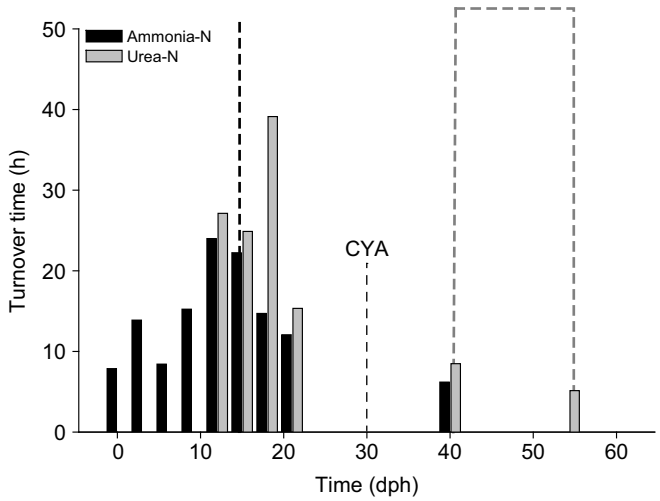

Fig. 7. Ammonia and urea turnover times in developing rainbow trout larvae. The thin dashed line represents the time at CYA. The thick black dashed line represents the skin-to-gill transition for ammonia excretion; the dashed grey bar outline represents the extra-branchial-to-branchial transition for urea excretion. Data and calculations from Zimmer et al. (2014b) and Zimmer and Wood (2016).

(Zimmer and Wood, 2015). We predicted that if the ammonia hypothesis was correct, rearing larvae in chronically high NaCl would have no effect on the timing of the skin-to-gill transition for J_{amm}. If the hypothesis was incorrect, and Na^+ uptake demand drives the ontogeny of the system, there would be a delay in the skin-to-gill transition when larvae were reared in high NaCl, as a result of a lower Na^+ uptake demand. We found that the skin-to-gill transition for J_{amm} occurred at 13 dph and was not affected by chronic high-NaCl rearing. Moreover, whole-body Na^+ levels were the same in the two groups up to the skin-to-gill transition but, by 18 dph, the high-NaCl group had significantly higher Na^+ levels than the control group (Zimmer and Wood, 2015). These data indicate the maintenance of the ontogenetic trajectory of Na^+-coupled J_{amm} at the expense of Na^+ balance, suggesting that J_{amm} drives the ontogeny of the branchial Na^+/NH_4^+ exchange complex.

In summary, J_{amm} and $J_{Na,in}$ transition to the gill at the same time (Figs 4 and 6), in agreement with both the ionoregulatory and ammonia hypotheses. However, the ammonia hypothesis argues that this is a function of the demand for J_{amm}, rather than Na^+ uptake. Importantly, the ammonia hypothesis is not a contradiction of the ionoregulatory hypothesis, but rather an extension of it.

Future directions

Our understanding of nitrogen handling in early life stages of fishes has increased substantially in the past decade, but many aspects have yet to be addressed. For instance, we have a very limited understanding of the timing of the loss of ureagenic capacity in early life. While many studies have demonstrated declines in enzyme activity or mRNA expression of key OUC enzymes over development (e.g. Korte et al., 1997; Wright et al., 1995a), no study has pinpointed the timing of complete loss. It is also not known whether OUC activity is plastic in development, perhaps being retained under conditions such as HEA or high pH, where J_{amm} is challenging. Indeed, the extremely alkaline environment (pH 9.6–10) of the Lake Magadi tilapia has resulted in the retention of ureotelism into adulthood in the only 100% ureotelic fish known to date (Randall et al., 1989). The ontogeny of nitrogen handling in early life stages of Magadi tilapia and other ureotelic fish (such as elasmobranchs) may also represent an interesting avenue for future research. Using genetic knockout approaches such as CRISPR/Cas9 to create mutant lines of zebrafish, or other model species, that lack one or more genes of the OUC may also improve our understanding of ammonia detoxification via urea production in early life. Indeed, the use of CRISPR/Cas9 and other genetic knockout approaches are an important next step in comparative and developmental physiology.

Furthermore, little is known regarding the ontogeny of J_{amm} in seawater teleosts, where J_{amm} coupling to ionoregulatory processes (i.e. active ion excretion, acid–base balance) is weak or absent. Determining the skin-to-gill shift of these processes in seawater fishes may help to further our understanding of the physiological pressures driving gill development in early life. It is also now clear that the mechanisms of ion and ammonia regulation can vary dramatically depending on the major site of exchange over development (skin versus gills) (Zimmer et al., 2017), which may necessitate the development of skin- and gill-specific transport models in future work. In this respect, future work should also be aimed at a better understanding of the role of NHEs in both J_{amm} and $J_{Na,in}$ in freshwater fishes. Finally, it is also important to keep in mind that we understand nitrogen handling in early life stages for only a handful of the >30,000 species of teleost fishes, and the detailed ontogenetic timing of branchial function in only one species (*Oncorhynchus mykiss*; Fig. 6). Pursuing a broader understanding of these processes in phylogenetically and ecologically distinct species will shed light on the mechanisms and evolution of nitrogen production and excretion in early life stages of fishes.

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Competing interests

The authors declare no competing or financial interests.

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References

Anderson, P. M. (1976). A glutamine- and N-acetyl-L-glutamate-dependent carbamoyl phosphate synthetase activity in the teleost *Micropterus salmoides*. *Comp. Biochem. Physiol.* **54**, 261-263.

Anderson, P. M. (1980). Glutamine- and N-acetylglutamate-dependent carbamoyl phosphate synthetase in elasmobranchs. *Science* **208**, 291-293.

Anderson, P. M. (1981). Purification and properties of the glutamine- and N-acetyl-L-glutamate dependent carbamoyl phosphate synthetase from liver of *Squalus acanthias*. *J. Biol. Chem.* **256**, 12228-12238.

Anderson, P. M. and Casey, C. A. (1984). Glutamine-dependent synthesis of citrulline by isolated hepatic mitochondria from *Squalus acanthias*. *J. Biol. Chem.* **259**, 456-462.

Avella, M. and Bornancin, M. (1989). A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **142**, 155-175.

Ballantyne, J. S. (1997). Jaws: the inside story. The metabolism of eleasmobranch fishes. *Comp. Biochem. Physiol. B* **118**, 703-742.

Barimo, J. F., Steele, S. L., Wright, P. A. and Walsh, P. J. (2004). Dogmas and controversies in the handling of nitrogenous wastes: ureotely and ammonia tolerance in early life stages of the gulf toadfish, *Opsanus beta*. *J. Exp. Biol.* **207**, 2011-2020.

Berthelot, C., Brunet, F., Chalopin, D., Juanchich, A., Bernard, M., Noël, B., Bento, P., Da Silva, C., Labadie, K., Alberti, A. et al. (2014). The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. *Nat. Commun.* **5**, 3657.

Braun, M. H., Steele, S. L., Ekker, M. and Perry, S. F. (2009a). Nitrogen excretion in developing zebrafish (*Danio rerio*): a role for Rh proteins and urea transporters. *Am. J. Physiol. Renal Physiol.* **296**, F994-F1005.

Braun, M. H., Steele, S. L. and Perry, S. F. (2009b). The responses of zebrafish (*Danio rerio*) to high external ammonia and urea transporter inhibition: nitrogen excretion and expression of rhesus glycoproteins and urea transporter proteins. *J. Exp. Biol.* **212**, 3846-3856.

Brauner, C. J. and Rombough, P. J. (2012). Ontogeny and paleophysiology of the gill: new insights from larval and air-breathing fish. *Respir. Physiol. Neurobiol.* **184**, 293-300.

Browne, A. and O'Donnell, M. J. (2013). Ammonium secretion by Malpighian tubules of *Drosophila melanogaster*: application of a novel ammonium-selective microelectrode. *J. Exp. Biol.* **216**, 3818-3827.

Bucking, C., Lemoine, C. M. R. and Walsh, P. J. (2013). Waste nitrogen metabolism and excretion in zebrafish embryos: effects of light, ammonia, and nicotinamide. *J. Exp. Zool.* **319**, 391-403.

Cameron, J. N. and Heisler, N. (1983). Studies of ammonia in the rainbow trout: physico-chemical parameters, acid-base behaviour and respiratory clearance. *J. Exp. Biol.* **105**, 107-125.

Chadwick, T. D. and Wright, P. A. (1999). Nitrogen excretion and expression of urea cycle enzymes in the Atlantic cod (*Gadus morhua* L.): a comparison of early life stages with adults. *J. Exp. Biol.* **202**, 2653-2662.

Conroy, M. J., Bullough, P. A., Merrick, M. and Avent, N. D. (2005). Modelling the human rhesus proteins: implications for structure and function. *Br. J. Haematol.* **131**, 543-551.

Dépêche, J., Gilles, R., Daufresne, S. and Chiapello, H. (1979). Urea content and urea production via the ornithine-urea cycle pathway during the ontogenic development of two teleost fishes. *Comp. Biochem. Physiol.* **63A**, 51-56.

Dhiyebi, H. A., O'Donnell, M. J. and Wright, P. A. (2013). Water chemistry in the microenvironment of rainbow trout *Oncorhynchus mykiss* embryos is affected by development, the egg capsule and crowding. *J. Fish Biol.* **82**, 444-457.

Dymowska, A. K., Hwang, P.-P. and Goss, G. G. (2012). Structure and function of ionocytes in the freshwater fish gill. *Respir. Physiol. Neurobiol.* **184**, 282-292.

Dymowska, A. K., Schultz, A. G., Blair, S. D., Chamot, D. and Goss, G. G. (2014). Acid-sensing ion channels are involved in epithelial Na^+ uptake in the rainbow trout *Oncorhynchus mykiss*. *Am. J. Physiol. Cell Physiol.* **307**, C255-C265.

Dymowska, A. K., Boyle, D., Schultz, A. G. and Goss, G. G. (2015). The role of acid-sensing ion channels in epithelial Na^+ uptake in adult zebrafish (*Danio rerio*). *J. Exp. Biol.* **218**, 1244-1251.

Engeszer, R. E., Patterson, L. B., Rao, A. A. and Parichy, D. M. (2007). Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish* **4**, 21-40.

Esaki, M., Hoshijima, K., Kobayashi, S., Fukuda, H., Kawakami, K. and Hirose, S. (2007). Visualization in zebrafish larvae of Na^+ uptake in mitochondria-rich cells whose differentiation is dependent on foxi3a. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292**, R470-R480.

Essex-Fraser, P. A., Steele, S. L., Bernier, N. J., Murray, B. W., Stevens, E. D. and Wright, P. A. (2005). Expression of four glutamine synthetase genes in the early stages of development of rainbow trout (*Oncorhynchus mykiss*) in relationship to nitrogen excretion. *J. Biol. Chem.* **280**, 20268-20273.

Finn, R. N., Fyhn, H. J. and Evjen, M. S. (1991). Respiration and nitrogen metabolism of Atlantic halibut eggs (*Hippoglossus hippoglossus*). *Mar. Biol.* **108**, 11-19.

Finn, R. N., Rønnestad, I. and Fyhn, H. J. (1995). Respiration, nitrogen and energy metabolism of developing yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus* L.). *Comp. Biochem. Physiol.* **111A**, 647-671.

Fu, C., Wilson, J. M., Rombough, P. J. and Brauner, C. J. (2010). Ions first: Na^+ uptake shifts from the skin to the gills before O_2 uptake in developing rainbow trout, *Oncorhynchus mykiss*. *Proc. R. Soc. B Biol. Sci.* **277**, 1553-1560.

Fyhn, H. J. and Serigstad, B. (1987). Free amino acids as energy substrate in developing eggs and larvae of the cod *Gadus morhua*. *Mar. Biol.* **96**, 334-341.

Gilmour, K. M., Thomas, K., Esbaugh, A. J. and Perry, S. F. (2009). Carbonic anhydrase expression and CO_2 excretion during early development in zebrafish *Danio rerio*. *J. Exp. Biol.* **212**, 3837-3845.

Giraldez, A. J., Mishima, Y., Rihel, J., Grocock, R. J., Van Dongen, S., Inoue, K., Enright, A. J. and Schier, A. F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312**, 75-79.

González, M. E., Blánquez, M. J. and Rojo, C. (1996). Early gill development in the rainbow trout, *Oncorhynchus mykiss*. *J. Morphol.* **229**, 201-217.

Griffith, R. W. (1991). Guppies, toadfish, lungfish, coelocanths and frogs: a scenario for the evolution of urea retention in fishes. *Environ. Biol. Fishes* **32**, 199-218.

Groot, E. P. and Alderdice, D. F. (1985). Fine structure of the external egg membrane of five species of Pacific salmon and steelhead trout. *Can. J. Zool.* **63**, 552-566.

Huang, C.-H. and Peng, J. (2005). Evolutionary conservation and diversification of Rh family genes and proteins. *Proc. Natl. Acad. Sci.* **102**, 15512-15517.

Hung, C. Y. C., Tsui, K. N. T., Wilson, J. M., Nawata, C. M., Wood, C. M. and Wright, P. A. (2007). Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. *J. Exp. Biol.* **210**, 2419-2429.

Hung, C. C., Nawata, C. M., Wood, C. M. and Wright, P. A. (2008). Rhesus glycoprotein and urea transporter genes are expressed in early stages of development of rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Zool.* **309**, 262-268.

Hwang, P.-P. (2009). Ion uptake and acid secretion in zebrafish (*Danio rerio*). *J. Exp. Biol.* **212**, 1745-1752.

Hwang, P.-P. and Chou, M.-Y. (2013). Zebrafish as an animal model to study ion homeostasis. *Pflüg. Arch. - Eur. J. Physiol.* **465**, 1233-1247.

Ito, Y., Kobayashi, S., Nakamura, N., Miyagi, H., Esaki, M., Hoshijima, K. and Hirose, S. (2013). Close association of carbonic anhydrase (CA2a and CA15a), Na^+/H^+ exchanger (Nhe3b), and ammonia transporter Rhcg1 in zebrafish ionocytes responsible for Na^+ uptake. *Front. Physiol.* **4**, 59.

Ito, Y., Kato, A., Hirata, T., Hirose, S. and Romero, M. F. (2014). Na^+/H^+ and Na^+/NH_4^+ exchange activities of zebrafish NHE3b expressed in *Xenopus* oocytes. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **306**, R315-R327.

Kajimura, M., Croke, S. J., Glover, C. N. and Wood, C. M. (2004). Dogmas and controversies in the handling of nitrogenous wastes: the effect of feeding and fasting on the excretion of ammonia, urea and other nitrogenous waste products in rainbow trout. *J. Exp. Biol.* **207**, 1993-2002.

Katoh, F., Shimizu, A., Uchida, K. and Kaneko, T. (2000). Shift of chloride cell distribution during early life stages in seawater-adapted killifish, *Fundulus heteroclitus*. *Zoolog. Sci.* **17**, 11-18.

Khademi, S. (2004). Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 A. *Science* **305**, 1587-1594.

Kharbuli, Z. Y., Datta, S., Biswas, K., Sarma, D. and Saha, N. (2006). Expression of ornithine–urea cycle enzymes in early life stages of air-breathing walking catfish *Clarias batrachus* and induction of ureogenesis under hyper-ammonia stress. *Comp. Biochem. Physiol.* **143**, 44-53.

Knepper, M. A. and Agre, P. (2004). Structural biology: the atomic architecture of a gas channel. *Science* **305**, 1573-1574.

Kok, F. O., Shin, M., Ni, C.-W., Gupta, A., Grosse, A. S., van Impel, A., Kirchmaier, B. C., Peterson-Maduro, J., Kourkoulis, G., Male, I. et al. (2015). Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Dev. Cell* **32**, 97-108.

Korte, J. J., Salo, W. L., Cabrera, V. M., Wright, P. A., Felskie, A. K. and Anderson, P. M. (1997). Expression of carbamoyl-phosphate synthetase III mRNA during the early stages of development and in muscle of adult rainbow trout (*Oncorhynchus mykiss*). *J. Biol. Chem.* **272**, 6270-6277.

Krogh, A. (1939). *Osmotic Regulation in Aquatic Animals*. Cambridge: Cambridge University Press.

Kumai, Y. and Perry, S. F. (2011). Ammonia excretion via Rhcg1 facilitates Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic water. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **301**, R1517-R1528.

Kwong, R. W. M., Kumai, Y. and Perry, S. F. (2014). The physiology of fish at low pH: the zebrafish as a model system. *J. Exp. Biol.* **217**, 651-662.

LeMoine, C. M. R. and Walsh, P. J. (2013). Ontogeny of ornithine-urea cycle gene expression in zebrafish (*Danio rerio*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **304**, R991-R1000.

Li, J., Eygensteyn, J., Lock, R. A. C., Verbost, P. M., van der Heijden, A. J. H., Wendellar Bonga, S. E. and Flik, G. (1995). Branchial chloride cells in larvae and juveniles of freshwater tilapia *Oreochromis mossambicus*. *J. Exp. Biol.* **198**, 2177-2184.

Lin, L.-Y., Horng, J.-L., Kunkel, J. G. and Hwang, P.-P. (2006). Proton pump-rich cell secretes acid in skin of zebrafish larvae. *Am. J. Physiol. Cell Physiol.* **290**, C371-C378.

Lin, T.-Y., Liao, B.-K., Horng, J.-L., Yan, J.-J., Hsiao, C.-D. and Hwang, P.-P. (2008). Carbonic anhydrase 2-like a and 15a are involved in acid-base regulation and Na^+ uptake in zebrafish H^+-ATPase-rich cells. *Am. J. Physiol. Cell Physiol.* **294**, C1250-C1260.

Lindley, T. E., Scheiderer, C. L., Walsh, P. J., Wood, C. M., Bergman, H. L., Bergman, A. L., Laurent, P., Wilson, P. and Anderson, P. M. (1999). Muscle as the primary site of urea cycle enzyme activity in an alkaline lake-adapted tilapia, *Oreochromis alcalicus grahami*. *J. Biol. Chem.* **274**, 29858-29861.

Maetz, J. and Garcia-Romeu, F. (1964). The mechanism of sodium and chloride uptake by the gills of a fresh-water fish, *Carassius auratus*. II. Evidence for NH_4^+/Na^+ and HCO_3^-/Cl^- exchanges. *J. Gen. Physiol.* **47**, 1209-1227.

McDonald, M. D. and Wood, C. M. (1998). Reabsorption of urea by the kidney of the freshwater rainbow trout. *Fish Physiol. Biochem.* **18**, 375-386.

McDonald, M. D. and Wood, C. M. (2003). Differential handling of urea and its analogues suggests carrier-mediated urea excretion in freshwater rainbow trout. *Physiol. Biochem. Zool.* **76**, 791-802.

McDonald, M. D. and Wood, C. M. (2004a). The effect of chronic cortisol elevation on urea metabolism and excretion in the rainbow trout (*Oncorhynchus mykiss*). *J. Comp. Physiol. B* **174**, 71-81.

McDonald, M. D. and Wood, C. M. (2004b). Evidence for facilitated diffusion of urea across the gill basolateral membrane of the rainbow trout (*Oncorhynchus mykiss*). *Biochim. Biophys. Acta BBA - Biomembr.* **1663**, 89-96.

McDonald, M. D., Wood, C. M., Wang, Y. and Walsh, P. J. (2000). Differential branchial and renal handling of urea, acetamide and thiourea in the gulf toadfish *Opsanus beta*: evidence for two transporters. *J. Exp. Biol.* **203**, 1027-1037.

McDonald, M. D., Walsh, P. J. and Wood, C. M. (2002). Branchial and renal excretion of urea and urea analogues in the plainfin midshipman, *Porichthys notatus*. *J. Comp. Physiol. B* **172**, 699-712.

Mishima, Y. (2012). Widespread roles of microRNAs during zebrafish development and beyond: miRNA functions in zebrafish. *Dev. Growth Differ.* **54**, 55-65.
Mistry, A. C., Honda, S., Hirata, T., Kato, A. and Hirose, S. (2001). Eel urea transporter is localized to chloride cells and is salinity dependent. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **281**, R1594-R1604.
Morii, H., Nishikata, K. and Tamura, O. (1979). Ammonia and urea excretion from mudskipper fishes *Periophthalamus cantonensis* and *Boleophthalamus pectinirostris* transferred from land to water. *Comp. Biochem. Physiol.* **63A**, 22-28.
Nakada, T., Westhoff, C. M., Kato, A. and Hirose, S. (2007a). Ammonia secretion from fish gill depends on a set of Rh glycoproteins. *FASEB J.* **21**, 1067-1074.
Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K. and Hirose, S. (2007b). Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **293**, R1743-R1753.
Nakada, T., Westhoff, C. M., Yamaguchi, Y., Hyodo, S., Li, X., Muro, T., Kato, A., Nakamura, N. and Hirose, S. (2010). Rhesus glycoprotein P2 (Rhp2) is a novel member of the Rh family of ammonia transporters highly expressed in shark kidney. *J. Biol. Chem.* **285**, 2653-2664.
Nawata, C. M., Hung, C. C. Y., Tsui, T. K. N., Wilson, J. M., Wright, P. A. and Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. *Physiol. Genomics* **31**, 463-474.
Nawata, C. M., Wood, C. M. and O'Donnell, M. J. (2010a). Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. *J. Exp. Biol.* **213**, 1049-1059.
Nawata, C. M., Hirose, S., Nakada, T., Wood, C. M. and Kato, A. (2010b). Rh glycoprotein expression is modulated in pufferfish (*Takifugu rubripes*) during high environmental ammonia exposure. *J. Exp. Biol.* **213**, 3150-3160.
Parks, S. K., Tresguerres, M. and Goss, G. G. (2008). Theoretical considerations underlying Na^+ uptake mechanisms in freshwater fishes. *Comp. Biochem. Physiol.* **148C**, 411-418.
Pasquinelli, A. E., Reinhart, B. J., Slack, F., Martindale, M. Q., Kuroda, M. I., Maller, B., Hayward, D. C., Ball, E. E., Degnan, B. and Müller, P. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* **408**, 86-89.
Peterson, R. H. and Martin-Robichaud, D. J. (1987). Permeability of the isolated Atlantic salmon (*Salmo salar*) chorion to ions as estimated by diffusion potentials. *Can. J. Fish. Aquat. Sci.* **44**, 1635-1639.
Pilley, C. M. and Wright, P. A. (2000). The mechanisms of urea transport by early life stages of rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **203**, 3199-3207.
Rahaman-Noronha, E., O'Donnell, M. J., Pilley, C. M. and Wright, P. A. (1996). Excretion and distribution of ammonia and the influence of boundary layer acidification in embryonic rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **199**, 2713-2723.
Randall, D. J., Wood, C. M., Perry, S. F., Bergman, H., Maloiy, G. M. O., Mommsen, T. P. and Wright, P. A. (1989). Urea excretion as a strategy for survival in a fish living in a very alkaline environment. *Nature* **337**, 165-166.
Rice, S. D. and Stokes, R. M. (1974). Metabolism of nitrogenous wastes in the eggs and alevins of rainbow trout, *Salmo gairdneri* Richardson. In *The Early Life History of Fish* (ed. J. H. S. Blaxter), pp. 325-337. New York: Springer-Verlag.
Rombough, P. J. (1992). Intravascular oxygen tensions in cutaneously respiring rainbow trout (*Oncorhynchus mykiss*) larvae. *Comp. Biochem. Physiol.* **101A**, 23-27.
Rombough, P. J. (1999). The gill of fish larvae. Is it primarily a respiratory or an ionoregulatory structure? *J. Fish Biol.* **55**, 186-204.
Rombough, P. (2002). Gills are needed for ionoregulation before they are needed for O_2 uptake in developing zebrafish, *Danio rerio*. *J. Exp. Biol.* **205**, 1787-1794.
Rombough, P. J. (2007). The functional ontogeny of the teleost gill: Which comes first, gas or ion exchange? *Comp. Biochem. Physiol.* **148A**, 732-742.
Rombough, P. J. and Moroz, B. M. (1990). The scaling and potential importance of cutaneous and branchial surfaces in respiratory gas exchange in young chinook salmon (*Oncorhynchus tshawytscha*). *J. Exp. Biol.* **154**, 1-12.
Rombough, P. J. and Moroz, B. M. (1997). The scaling and potential importance of cutaneous and branchial surfaces in respiratory gas exchange in larval and juvenile walleye *Stizostedion vitreum*. *J. Exp. Biol.* **200**, 2459-2468.
Rønnestad, I., Finn, R. N., Groot, E. P. and Fyhn, H. J. (1992a). Utilization of free amino acids related to energy metabolism of developing eggs and larvae of lemon sole *Microstomus kitt* reared in the laboratory. *Mar. Ecol. Prog. Ser.* **88**, 195-205.
Rønnestad, I., Fyhn, H. J. and Gravingen, K. (1992b). The importance of free amino acids to the energy metabolism of eggs and larvae of turbot (*Scophthalmus maximus*). *Mar. Biol.* **114**, 517-525.
Rønnestad, I., Thorsen, A. and Finn, R. N. (1999). Fish larval nutrition: a review of recent advances in the roles of amino acids. *Aquaculture* **177**, 201-216.
Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Hölper, S., Krüger, M. and Stainier, D. Y. R. (2015). Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature* **524**, 230-233.
Sashaw, J., Nawata, C. M., Thompson, S., Wood, C. M. and Wright, P. A. (2010). Rhesus glycoprotein and urea transporter genes in rainbow trout embryos are upregulated in response to alkaline water (pH 9.7) but not elevated water ammonia. *Aquat. Toxicol.* **96**, 308-313.
Sayer, M. D. J. and Davenport, J. (1987). The relative importance of the gills to ammonia and urea excretion in five seawater and one freshwater teleost species. *J. Fish Biol.* **31**, 561-570.
Shayakul, C., Clémencon, B. and Hediger, M. A. (2013). The urea transporter family (SLC14): physiological, pathological and structural aspects. *Mol. Aspects Med.* **34**, 313-322.
Shih, T.-H., Horng, J.-L., Hwang, P.-P. and Lin, L.-Y. (2008). Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. *Am. J. Physiol. Cell Physiol.* **295**, C1625-C1632.
Shih, T.-H., Horng, J.-L., Liu, S.-T., Hwang, P.-P. and Lin, L.-Y. (2012). Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **302**, R84-R93.
Shih, T.-H., Horng, J.-L., Lai, Y.-T. and Lin, L.-Y. (2013). Rhcg1 and Rhbg mediate ammonia excretion by ionocytes and keratinocytes in the skin of zebrafish larvae: H^+-ATPase-linked active ammonia excretion by ionocytes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **304**, R1130-R1138.
Sinha, A. K., Liew, H. J., Nawata, C. M., Blust, R., Wood, C. M. and De Boeck, G. (2013). Modulation of Rh glycoproteins, ammonia excretion and Na^+ fluxes in three freshwater teleosts when exposed chronically to high environmental ammonia. *J. Exp. Biol.* **216**, 2917-2930.
Smith, H. W. (1929). The excretion of ammonia and urea by the gills of fish. *J. Biol. Chem.* **81**, 727-742.
Smith, C. P. and Wright, P. A. (1999). Molecular characterization of an elasmobranch urea transporter. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **276**, R622-R626.
Steele, S. L., Chadwick, T. D. and Wright, P. A. (2001). Ammonia detoxification and localization of urea cycle enzyme activity in embryos of the rainbow trout (*Oncorhynchus mykiss*) in relation to early tolerance to high environmental ammonia levels. *J. Exp. Biol.* **204**, 2145-2154.
Terjesen, B. F., Mangor-Jensen, A. and Fyhn, H. J. (1998). Ammonia dynamics in relation to hatching in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Fish Physiol. Biochem.* **18**, 189-201.
Terjesen, B. F., Rønnestad, I., Norgberg, B. and Anderson, P. M. (2000). Detection and basic properties of carbamoyl phosphate synthetase III during teleost ontogeny: a case study in the Atlantic halibut (*Hippoglossus hippoglossus* L.). *Comp. Biochem. Physiol. B* **126**, 521-535.
Terjesen, B. F., Chadwick, T. D., Verreth, J. A. J., Ronnestad, I. and Wright, P. A. (2001). Pathways for urea production during early life of an air-breathing teleost, the African catfish *Clarias Gariepinus* Burchell. *J. Exp. Biol.* **204**, 2155-2165.
Terjesen, B. F., Finn, R. N., Norberg, B. and Rønnestad, I. (2002). Kinetics and fates of ammonia, urea, and uric acid during oocyte maturation and ontogeny of the Atlantic halibut (*Hippoglossus hippoglossus* L.). *Comp. Biochem. Physiol. A* **131**, 443-455.
Tsui, T. K. N., Hung, C. Y. C., Nawata, C. M., Wilson, J. M., Wright, P. A. and Wood, C. M. (2009). Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na^+/NH_4^+ exchange complex. *J. Exp. Biol.* **212**, 878-892.
van der Meeren, T. (1991). Algae as first food for cod larvae, *Gadus morhua* L.: filter feeding or ingestion by accident? *J. Fish Biol.* **39**, 225-237.
Varsamos, S., Diaz, J. P., Charmantier, G., Blasco, C., Connes, R. and Flik, G. (2002). Location and morphology of chloride cells during the post-embryonic development of the european sea bass, *Dicentrarchus labrax*. *Anat. Embryol. (Berl.)* **205**, 203-213.
Varsamos, S., Nebel, C. and Charmantier, G. (2005). Ontogeny of osmoregulation in postembryonic fish: a review. *Comp. Biochem. Physiol. A* **141**, 401-429.
Walsh, P. J., Heitz, M. J., Campbell, C. E., Cooper, G. J., Medina, M., Wang, Y. S., Goss, G. G., Vincek, V., Wood, C. M. and Smith, C. P. (2000). Molecular characterization of a urea transporter in the gill of the gulf toadfish (*Opsanus beta*). *J. Exp. Biol.* **203**, 2357-2364.
Walsh, P. J., Grosell, M., Goss, G. G., Bergman, H. L., Bergman, A. N., Wilson, P., Laurent, P., Alper, S. L., Smith, C. P., Kamunde, C. et al. (2001a). Physiological and molecular characterization of urea transport by the gills of the Lake Magadi tilapia (*Alcolapia grahami*). *J. Exp. Biol.* **204**, 509-520.
Walsh, P. J., Wang, Y. S., Campbell, C. E., De Boeck, G. and Wood, C. M. (2001b). Patterns of nitrogenous waste excretion and gill urea transporter mRNA expression in several species of marine fish. *Mar. Biol.* **139**, 839-844.
Wang, Y., Heigenhauser, G. J. and Wood, C. M. (1996). Ammonia movement and distribution after exercise across white muscle cell membranes in rainbow trout. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **271**, R738-R750.
Weihrauch, D., Wilkie, M. P. and Walsh, P. J. (2009). Ammonia and urea transporters in gills of fish and aquatic crustaceans. *J. Exp. Biol.* **212**, 2879-2879.
Wells, P. R. and Pinder, A. W. (1996a). The respiratory development of Atlantic salmon I. Morphometry of gills, yolk sac and body surface. *J. Exp. Biol.* **199**, 2725-2736.
Wells, P. R. and Pinder, A. W. (1996b). The respiratory development of Atlantic salmon II. Partitioning of oxygen uptake among gills, yolk sac and body surfaces. *J. Exp. Biol.* **199**, 2737-2744.

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Wilkie, M. P. and Wood, C. M. (1991). Nitrogenous waste excretion, acid-base regulation, and ionoregulation in rainbow trout (*Oncorhynchus mykiss*) exposed to extremely alkaline water. *Physiol. Zool.* **64**, 1069-1086.
Wilkie, M. P. and Wood, C. M. (1994). The effects of extremely alkaline water (pH 9.5) on rainbow trout gill function and morphology. *J. Fish Biol.* **45**, 87-98.
Wilkie, M. P., Simmons, H. E. and Wood, C. M. (1996). Physiological adaptations of rainbow trout to chronically elevated water pH (pH=9.5). *J. Exp. Zool.* **274**, 1-14.
Wilson, R. W., Wright, P. M., Munger, S. and Wood, C. M. (1994). Ammonia excretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for Na^+/NH_4^+ exchange. *J. Exp. Biol.* **191**, 37-58.
Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. Evans), pp. 379-425. Boca Raton, FL: CRC Press.
Wood, C. M. and Nawata, C. M. (2011). A nose-to-nose comparison of the physiological and molecular responses of rainbow trout to high environmental ammonia in seawater versus freshwater. *J. Exp. Biol.* **214**, 3557-3569.
Wood, C. M., Bergman, H. L., Laurent, P., Maina, J. N., Narahara, A. and Walsh, P. J. (1994). Urea production, acid-base regulation and their interactions in the Lake Magadi tilapia, a unique teleost adapted to a highly alkaline environment. *J. Exp. Biol.* **189**, 13-36.
Wood, C. M., Hopkins, T. E., Hogstrand, C. and Walsh, P. J. (1995). Pulsatile urea excretion in the ureagenic toadfish *Opsanus beta*: an analysis of rates and routes. *J. Exp. Biol.* **198**, 1729-1741.
Wood, C. M., Nawata, C. M., Wilson, J. M., Laurent, P., Chevalier, C., Bergman, H. L., Bianchini, A., Maina, J. N., Johannsson, O. E., Bianchini, L. F. et al. (2013). Rh proteins and NH_4^+-activated Na^+-ATPase in the Magadi tilapia (*Alcolapia grahami*), a 100% ureotelic teleost fish. *J. Exp. Biol.* **216**, 2998-3007.
Wright, P. A. (1993). Nitrogen excretion and enzyme pathways for ureagenesis in freshwater tilapia (*Oreochromis niloticus*). *Physiol. Zool.* **66**, 881-901.
Wright, P. A. and Fyhn, H. J. (2001). Ontogeny of nitrogen metabolism and excretion. In *Fish Physiology* (ed. P. Wright and P. Anderson), pp. 149-200. San Diego, CA: Academic Press.
Wright, P. A. and Land, M. D. (1998). Urea production and transport in teleost fishes. *Comp. Biochem. Physiol. A* **119**, 47-54.
Wright, P. A. and Wood, C. M. (1985). An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH change and sodium uptake blockade. *J. Exp. Biol.* **114**, 329-353.
Wright, P. A. and Wood, C. M. (1988). Muscle ammonia stores are not determined by pH gradients. *Fish Physiol. Biochem.* **5**, 159-162.
Wright, P. A. and Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J. Exp. Biol.* **212**, 2303-2312.
Wright, P. A. and Wood, C. M. (2012). Seven things fish know about ammonia and we don't. *Respir. Physiol. Neurobiol.* **184**, 231-240.
Wright, P. A., Felskie, A. K. and Anderson, P. M. (1995a). Induction of ornithine-urea cycle enzymes and nitrogen metabolism and excretion in rainbow trout (*Oncorhynchus mykiss*) during early life stages. *J. Exp. Biol.* **198**, 127-135.
Wright, P. A., Pärt, P. and Wood, C. M. (1995b). Ammonia and urea excretion in the tidepool sculpin (*Oligocottus maculosus*): sites of excretion, effects of reduced salinity and mechanisms of urea transport. *Fish Physiol. Biochem.* **14**, 111-123.
Wu, S.-C., Horng, J.-L., Liu, S.-T., Hwang, P.-P., Wen, Z.-H., Lin, C.-S. and Lin, L.-Y. (2010). Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae. *Am. J. Physiol. Cell Physiol.* **298**, C237-C250.
Yan, J.-J., Chou, M.-Y., Kaneko, T. and Hwang, P.-P. (2007). Gene expression of Na^+/H^+ exchanger in zebrafish H^+-ATPase-rich cells during acclimation to low-Na^+ and acidic environments. *Am. J. Physiol. Cell Physiol.* **293**, C1814-C1823.
Zimmer, A. M. and Wood, C. M. (2015). Ammonia first? The transition from cutaneous to branchial ammonia excretion in developing rainbow trout is not altered by exposure to chronically high NaCl. *J. Exp. Biol.* **218**, 1467-1470.
Zimmer, A. M. and Wood, C. M. (2016). 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.
Zimmer, A. M., Nawata, C. M. and Wood, C. M. (2010). Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and Na^+ uptake in freshwater rainbow trout. *J. Comp. Physiol. B* **180**, 1191-1204.
Zimmer, A. M., Brauner, C. J. and Wood, C. M. (2014a). Ammonia transport across the skin of adult rainbow trout (*Oncorhynchus mykiss*) exposed to high environmental ammonia (HEA). *J. Comp. Physiol. B* **184**, 77-90.
Zimmer, A. M., Wright, P. A. and Wood, C. M. (2014b). What is the primary function of the early teleost gill? Evidence for Na^+/NH_4^+ exchange in developing rainbow trout (*Oncorhynchus mykiss*). *Proc. R. Soc. B Biol. Sci.* **281**, 20141422.
Zimmer, A. M., Wilson, J. M., Wright, P. A., Hiroi, J. and Wood, C. M. (2017). Different mechanisms of Na^+ uptake and ammonia excretion by the gill and yolk sac epithelium of early life stage rainbow trout. *J. Exp. Biol.* **220**, 775-786.