Ions first: Na\(^+\) uptake shifts from the skin to the gills before O\(_2\) uptake in developing rainbow trout, Oncorhynchus mykiss

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This is the first direct physiological evidence in support of the ionoregulatory hypothesis, challenging the long-held assumption that teleost gills develop initially for gas exchange. Resting unidirectional sodium (Na\(^+\)) uptake and oxygen (O\(_2\)) uptake across the skin and gills were measured simultaneously in larval rainbow trout, Oncorhynchus mykiss, during development. In soft and hard water, Na\(^+\) uptake shifted to the gills by 15 and 16 days post-hatch (dph) while O\(_2\) uptake took 50–80% longer and shifted by 23 and 28 dph, respectively. This suggests that gills are required for ion regulation prior to gas exchange in developing rainbow trout. The age of transition for Na\(^+\) uptake, gill Na\(^+\), K\(^+\)-ATPase (NKA) α-subunit protein expression and gill NKA enzyme activity were not significantly different between soft and hard water-reared groups, which suggests a lack of plasticity in gill ionoregulatory development.

In rainbow trout, the gills assume a dominant role in ion regulation before gas exchange, suggesting that ion regulation may be the initial driving force for gill development. Further investigation is required to determine whether this pattern is consistent with other teleosts and more basal fishes during early development to gain insight into the role of ion regulation in vertebrate gill evolution.

Keywords: ionoregulation; gas exchange; development; rainbow trout; gill; metabolic rate

1. INTRODUCTION

The gills of adult fish are a multifunctional organ involved in gas exchange, ionoregulation, acid–base regulation and ammonia excretion (reviewed in Evans et al. 2005). Of these functions, gas exchange, in particular O\(_2\) uptake, is generally considered most critical. Blocking O\(_2\) uptake at the gill of adult fish rapidly leads to death by asphyxiation. Oxygen uptake is also generally considered the primary function driving gill development (Krogh 1941).

Gills form relatively late in larval development and until they appear, O\(_2\) uptake is restricted to the skin (Rombough 1988). Since skin surface area (SA) expands at a rate proportional to \(M^{2/3}\) (\(M\) = mass), while the demand for O\(_2\) expands at a rate proportional to \(M^{1.0}\), skin SA eventually becomes limiting (Rombough 2007).

According to Krogh (1941), the solution to this problem is to develop gills which because of their morphology are not subject to the same geometric constraints as the skin. To date, the view that gills develop for gas exchange (often termed the oxygen hypothesis) is the accepted explanation for why fish develop gills.

However, recent evidence suggests that ion regulation may be more important than O\(_2\) uptake as a driving force during the initial stages of gill development (reviewed in Rombough 2007). Like O\(_2\), ion uptake in freshwater is initially entirely cutaneous and is subject to similar, if not more stringent geometric constraints (Rombough 2007). The major site of ion uptake in developing fish, as in adult fish, is the mitochondrion-rich cells (MRCs; reviewed in Hwang 2009; Hwang & Lee 2007). MRCs are initially restricted to cutaneous surfaces but begin to appear on the gills soon after they start to develop (Li et al. 1995; Rombough 1999). In all freshwater and marine fish species examined to date, MRCs appear on the gills in advance of gill lamellae (reviewed in Rombough 2007). Since gill lamellae are considered the definitive site of O\(_2\) uptake, this suggests that the gills initially may be more important for ion exchange than for gas exchange. Functional ablation experiments with zebrafish (Danio rerio) appear to confirm this: the ionoregulatory functions normally provided by the developing gills become critical for survival at about 7 days post-fertilization (dpf) in zebrafish larvae while respiratory services do not become critical until about 14 dpf (Rombough 2002). Based on the balance of evidence, there would appear to be a strong case for ion exchange being the more important factor driving gill development. This view has been termed the ionoregulatory hypothesis (Rombough 2007).

To date, no direct physiological evidence exists to differentiate between the O\(_2\) and ionoregulatory hypotheses. For the most part, the evidence is either theoretical (e.g. skin SA:M ratios) or circumstantial (e.g. relative timing of the appearance of branchial...
MRCs and lamellae) and, as such, is open to interpretation. To our knowledge, no one has directly measured ion and gas uptake across the skin and gills at the same stage of development in the same species under the same conditions. The primary goal of our study was to determine how the partitioning of Na\(^+\) and O\(_2\) uptake between the skin and gills changes during early gill development. Comparison of the partitioning patterns would then allow us to directly test whether the ionoregulatory or respiratory hypothesis applies for that species under our experimental conditions. We picked rainbow trout, *Onchorhyncus mykiss*, as our animal model because (i) the methodologies for measuring skin and gill O\(_2\) uptake (Rombough 1998) and whole body Na\(^+\) uptake (Brauner & Wood 2002a,b) have already been developed and proven reliable and (ii) the respiratory and ionoregulatory physiology of juvenile and adult rainbow trout is well understood. This allowed us to put the results of the current study into perspective. Our study had two main objectives:

— To determine whether ion or gas uptake is first to make the transition from the skin to the gills in larval rainbow trout. This was assessed by comparing the relative contributions of the skin and gills to total O\(_2\) and total Na\(^+\) uptake at various stages throughout the course of larval development.

— To determine whether the rate at which ionoregulatory activity shifts from the skin to the gills is affected by water hardness. Shen & Leatherland (1978) reported that the ionic strength of the rearing medium had no impact on the number or appearance of gill MRCs in developing rainbow trout. This suggests that water hardness is unlikely to affect the timing of the shift of ionoregulatory activity to the gills, yet the possibility remains since gill ion uptake rates were not measured directly in that study. Hence, a subsidiary goal of this study was to determine whether water hardness indeed has no significant effect on the ionoregulatory activity of the developing gill. This was evaluated by comparing Na\(^+\) uptake rates, gill Na\(^+\), K\(^+\)-ATPase (NKA) concentrations and NKA activities in soft and hard water.

### 2. MATERIAL AND METHODS

(a) Animals and rearing

Rainbow trout, *O. mykiss*, were obtained as eyed embryos from Rainbow Springs Trout Hatchery in Thamesford, Ontario, Canada, and transported to the University of British Columbia, Vancouver, Canada. The soft water-acclimated group was reared in March 2008 in soft dechlorinated tap water from the City of Greater Vancouver (in mM: Na\(^+\), 0.06; Cl\(^-\), 0.05; Ca\(^2+\), 0.03; Mg\(^2+\), 0.007; K\(^+\), 0.004; alkalinity, 3.3 mg as CaCO\(_3\); hardness 3.55 mg as CaCO\(_3\); pH 6.2–6.7; Metro Vancouver 2007), and the hard water-acclimated group was reared in April 2008 in artificial hard water made by adding salts to distilled deionized water (nominal concentrations in mM: Na\(^+\), 2.3; Cl\(^-\), 0.1; Ca\(^2+\), 0.9; Mg\(^2+\), 1.0; K\(^+\), 0.1; HCO\(_3\)^\(^-\), 2.3; alkalinity, 110–120 mg as CaCO\(_3\); hardness 160–180 mg as CaCO\(_3\); pH 7.6–8.0). Both groups were reared in the anterior and posterior chambers as mg O\(_2\) l\(^{-1}\) in an egg incubation tray supplied by a 40 l recirculating system.

(b) Flux chamber design and use

Flux chambers similar to those of Wells & Pinder (1996) were designed to partition larvae into two compartments, enabling *in vivo* measurement of O\(_2\) and Na\(^+\) uptake by the anterior compartment (consisting of the head and opercula epithelia and gills) separately from the posterior compartment (consisting of the remaining body and yolk sac epithelia). Each flux chamber consisted of two identical rectangular chambers made of acrylic plastic, each approximately 5 ml in volume and had a micro-stir bar, an air inlet and an O\(_2\) probe/radioisotope injection port.

Larvae were randomly selected for either oxygen (O\(_2\)) or sodium (Na\(^+\)) uptake measurements at 0, 6, 9, 12 and 18 days post-hatch (dph), with the exception that no measurements were performed on 12 dph in soft water larvae due to technical difficulties.

Uptake measurements required positioning an individual larva into a hole in a stretched rubber membrane of dental dam latex. Prior to positioning, larvae were calmed with 100 ppm clove oil (Rougier Pharma, Quebec, Canada) for approximately 5 min to attain stage 5 anaesthesia (total loss of equilibrium and swimming motion with weak opercular motion and total loss of reactivity; Keene et al. 1998). Water in flux chambers contained 10 ppm clove oil to reduce struggling without impaireing opercular motion. During a 1 h recovery period prior to the start of measurements, flux chambers were aerated and submerged in a 10°C water bath (actual temperature 9.6 ± 0.1°C).

(c) Measurement of unidirectional Na\(^+\) uptake rate

After the recovery period, either the anterior or the posterior chamber (chosen randomly) was injected with 0.5 μCi of the radioisotope \(^{22}\)Na (PerkinElmer, MA, USA). Both chambers were gently aerated and stirred for the duration of the 1.5 h flux period. Water samples of 25 μl were removed in duplicate 10 min post-injection and again at 1.5 h for measurement of water radioactivity. At the end of the flux period, larvae were immediately removed from the dam and flux chambers, rinsed three times with 5 mM NaCl to displace surface-bound \(^{22}\)Na and then rinsed once with deionized water. Water samples and larvae were measured for radioactivity in counts per minute using a gamma counter (PerkinElmer, Turku, Finland). Na\(^+\) uptake, expressed in μmol Na\(^+\) h\(^{-1}\) larvæ\(^{-1}\), was calculated as in Brauner & Wood (2002b). Water concentration of Na\(^+\) was determined by flame atomic absorption spectrometry (Varian Australia Pty Ltd, Australia).

(d) Measurement of O\(_2\) uptake rate

Following the recovery period, air bubbles were removed from the flux chambers and the chambers were completely filled. Needle-type housing fibre-optic O\(_2\) microoptodes (Loligo Systems ApS, Denmark) were positioned in both chambers. A data acquisition system (Precision Sensing GmbH, Germany) recorded the decline of O\(_2\) in the water in the anterior and posterior chambers as mg O\(_2\) h\(^{-1}\) min\(^{-1}\) at 10°C for 30 min. After the completion of measurements, larvae were placed in aerated, dechlorinated water to recover for 1 h. Larvae were euthanized with 1000 ppm tricaine methane sulphonate (MS-222, Finquel, Argent Chemical Laboratories), and body wet mass (with and without yolk) was determined to the nearest 0.1 mg. Data were discarded for individuals that failed to recover within 1 h or had visible physical damage. Data are expressed as mg O\(_2\) h\(^{-1}\) larvæ\(^{-1}\).
(e) **Measurement of skin SA**

Animals were fixed in paraformaldeyde solution in phosphate-buffered saline (pH 7.4, as described in Presnell & Schreibman 1997) for 24 h at 4°C, then stored in 70 per cent ethanol at 4°C until analysis. To measure total skin SA, the body was divided into the head, pectoral fins, trunk and associated fins, tail and yolk sac regions and skinned. All skin components were immersed briefly in Wright--Giemsa stain (SureStain, Fisher Scientific, Pittsburgh, USA) and rinsed with deionized water prior to mounting on coverslips to improve visibility for imaging. Slides were photographed with a Leica MZ16A dissecting microscope with a JVC KY-F75U digital camera using the software AUTO-MONTAGE PRO 5.0.0005 (Synoptics Ltd). SAs were then measured using the area measurement function in trace mode using the software SIGMA SCAN PRO IMAGE ANALYSIS 5.0.0 (SPSS). The average percentages of total skin SA in the anterior and posterior chambers were calculated for each age group (n = 3–6 per group), with the exception of hard water larvae at 0 dph because they were too small to handle.

(f) **Calculation of gill and skin Na\(^+\) and O\(_2\) uptake rates**

Rates of O\(_2\) and Na\(^+\) uptake by the gills and skin (consisting of head, opercula, body and yolk sac epitelia) were calculated from the uptake rates measured in the anterior and posterior chambers as described in Wells & Pinder (1996). Gill and skin absolute value uptake rates were also expressed as percentages of total uptake rates, where total O\(_2\) uptake rate = anterior O\(_2\) uptake rate + posterior O\(_2\) uptake rate (obtained simultaneously on the same larvae) and total Na\(^+\) uptake rate = mean anterior Na\(^+\) uptake rate + mean posterior Na\(^+\) uptake rate (measurements were on different larvae, and consequently, in some cases, addition of the two deviates slightly from 100%).

(g) **Measurement of NKA protein concentration**

Five to seven larvae from each age group in soft and hard water groups were euthanized with 1000 ppm MS-222, immediately frozen intact in liquid nitrogen and stored at -80°C until analysis. Samples of the gills (on larvae 6 dpf and older), body epithelium and yolk sac epithelium were homogenized by sonication (Kontes Micro Ultrasonic Cell Disrupter, Mandel Scientific Co., Guelph, Canada) in 300 µl SEI buffer on ice and then centrifuged at 5000 g for 5 min. Activity was measured in the supernatant according to McCormick (1993) at 25°C using a plate reader (Spectra-MAX 190, Molecular Devices, Sunnyvale, USA) and is expressed in µmol ADP mg\(^{-1}\) protein h\(^{-1}\). Protein content of the homogenate supernatant was measured using Bradford reagent (Sigma-Aldrich) and bovine serum albumin as a standard.

(i) **Statistical analyses**

O\(_2\) and Na\(^+\) uptake rates, NKA α-subunit protein expression and NKA enzyme activity were plotted against age. In general, the F-test showed that linear regressions were a better fit to the data than second-order polynomial regressions (PRISM 5.02, GraphPad Software, Inc., CA, USA). The F-test was used to test for slopes significantly different from zero and significant differences between pairs of slopes (PRISM 5.02, GraphPad Software, Inc.).

Gill and skin O\(_2\) and Na\(^+\) uptake rates as percentages of total uptake rates were plotted against age. The age at which each pair of gill and skin lines intersected (corresponding to 50% gill uptake and 50% skin uptake) was determined. The mean and standard error for points of intersection were calculated by fitting data to the equation \(Y = Y_\text{cross} + (X - X_\text{cross})\) slope, where \(X_\text{cross}\) and \(Y_\text{cross}\) were defined as shared parameters to find a best-fit value that applies to both the gill and skin lines. Pairs of crossing points were tested for significant differences using Student’s t-test (PRISM 5.02, GraphPad Software, Inc.). Statistical significance was assumed at \(p < 0.05\). All values are reported as mean ± s.e.m.

3. **RESULTS AND DISCUSSION**

During early development, the gills take on a primary role in Na\(^+\) uptake in just over half the time relative to that observed for O\(_2\) uptake in rainbow trout (15–16 versus 23–28 dpf) highlighting the importance of the developing fish gill to ionoregulation. Furthermore, the timing of the transition to the gills for Na\(^+\) uptake, gill NKA α-subunit protein expression and gill NKA activity levels were similar between soft and hard water groups, suggesting that gill ionoregulatory development is
unaffected by water hardness. These findings support the ionoregulatory hypothesis and have important implications for our current understanding of developmental physiology and the function of larval gills.

(a) Concerns with measurement under calmed, resting conditions in normoxia
Measurements of resting O₂ uptake rate in normoxia usually underestimate metabolic rate in the natural environment (Wieser 1985), but this discrepancy is probably minimal in developing fish larvae in general because of their relatively low factorial aerobic scope (ratio of maximum to standard metabolic rate) due to the high O₂ requirements of development (Killen et al. 2007). The factorial aerobic scope of rainbow trout larvae is only 2.7, considerably lower than 5.2 for fry (Wieser 1985) and 6 to 20 for adults (Wilson et al. 1994; Shingles et al. 2001). Consequently, resting measurements in larval rainbow trout may more closely represent levels in their natural habitats relative to juvenile and adult stages.

Larvae must be calmed to prevent struggling and injury while in the rubber dam. Preliminary studies revealed that calming with 10 ppm clove oil only reduced resting Na⁺ uptake by 12 per cent (not statistically significant, data not shown) and O₂ uptake by 24 per cent (p = 0.003), while MS 222 had much greater effects on Na⁺ uptake precluding its use. Given that the effects of clove oil were relatively small, it is unlikely that its use significantly influenced the timing of the skin-to-gill transition for Na⁺ and O₂ uptake, but we have assumed that the effect of clove oil was similar on gills and skin and that they were consistent throughout development.

(b) Na⁺ uptake rate partitioning between the gills and the skin
Total Na⁺ uptake rate increased with development (figure 1). Total Na⁺ uptake rate was lower overall and increased at a slower rate in soft water than in hard water, most probably because Na⁺ uptake rate is dependent on water Na⁺ content (figure 1 and table S2 in the electronic supplementary material; Kirschner 1970). Mass-specific total Na⁺ uptake rates are comparable to those reported in Brauner & Wood (2002a) at equivalent developmental stages.

Gill Na⁺ uptake increased linearly with age (figure 1). This is consistent with the increases in gill NKA α-subunit expression and NKA activity (figure 2) and the number of MRCs per filament which increased approximately three-fold from 0 to 18 dph (Rombough 1999). Na⁺ uptake rate increased at a slower rate at the skin than the gills indicating that the ionoregulatory capacity of the gills increased more rapidly than the skin (figure 1).

Ion uptake efficiency at the skin probably declines with age due to thickening of the skin, increasing distance of MRCs from blood vessels, decreasing MRC density after hatch and reabsorption of the yolk sac epithelia (Rombough 1999, 2004).

(c) Demands for increased total Na⁺ uptake rate with age
The observed increase in total Na⁺ uptake rate with development in both soft and hard water (figure 1) may be attributed to two major demands for Na⁺. Firstly, uptake rate must be elevated to supply ions to growing tissues. This is evident in the near doubling in whole body Na⁺ content in rainbow trout during the first 14 dph (Brauner & Wood 2002a). Secondly, expansion of respiratory SA exacerbates diffusive ion loss at the lamellae, requiring greater ion uptake rates to balance increased efflux (Evans et al. 1999).

Based on changes in whole body Na⁺ content reported in Brauner & Wood (2002a), we estimate that approximately 90 per cent (hard water) and 75 per cent (soft water) of Na⁺ uptake balances diffusive Na⁺ loss, while only 10 and 25 per cent of Na⁺ uptake, respectively, is incorporated into the body. Assuming that there were no differences in Na⁺ incorporated into larvae reared in soft and hard water of the present study (which were not measured) Na⁺ efflux must have been lower in soft water. The specific mechanisms that may reduce ion loss in a low ion content environment are not known, but may consist of tightening of paracellular junctions or reduction in gill perfusion. It is not known why diffusive efflux in developing trout is so high and represents...
such a large proportion of unidirectional influx, but it may be associated with acid–base regulation and the need to modulate net Na\(^+\)/H\(^+\) exchange; however, this remains to be investigated.

Given that the majority of Na\(^+\) uptake appears to balance efflux, Na\(^+\) uptake is expected to scale proportionately with total gill SA, assuming that unidirectional Na\(^+\) uptake is not environmentally limited (i.e. uptake rates are near \(V_{\text{max}}\)) and total gill SA is an indication of potential Na\(^+\) efflux. Indeed, gill Na\(^+\) uptake rate in larvae reared in hard water (where Na\(^+\) uptake rates are near maximal in adult rainbow trout; Kirschner et al. 1973) scaled to unity with gill SA (estimated from Rombough 1999; see figure S1 in the electronic supplementary material).

(d) \(O_2\) uptake rate and partitioning between the gills and skin
Total \(O_2\) uptake rate increased with development at the same rate in soft and hard water (figure 3). Total \(O_2\) uptake rates in mass-matched larvae were similar to those reported by Rombough (1998). The trends of increasing total, gill and skin \(O_2\) uptake rate per larva with tissue mass were consistent with those reported for larval chinook and Atlantic salmon in earlier partitioning studies (data not shown; Rombough & Ure 1991; Wells & Pinder 1996).

(e) Contribution of the gills to Na\(^+\) and \(O_2\) uptake rate
Studies on the function of larval gills have traditionally focused on their role as a gas exchange organ, while their importance in ionoregulation has been seen as secondary. Direct comparison of the contribution of the gills to total Na\(^+\) and \(O_2\) uptake suggests that the gills develop more rapidly for ionoregulation than gas exchange in rainbow trout. At hatch, the gills account for 21 per cent (soft water) and 25 per cent (hard water) of total \(O_2\) uptake (figure 4). These values are in agreement with published values of 27 per cent in rainbow trout (Rombough 1998), 26 per cent in chinook salmon (Rombough & Ure 1991) and 28 per cent in Atlantic salmon (Wells & Pinder 1996). By contrast, the gills at hatch accounted for only 9 per cent (soft water) and 14 per cent (hard water) of Na\(^+\) uptake rate (figure 4). By 18 dph, the gills account for 57 per cent (soft water) and 61 per cent (hard water) of total Na\(^+\) uptake while at this stage they only accounted for 37 per cent (hard water) to 43 per cent (soft water) of total \(O_2\) uptake (figure 4). Thus, the gills increase their involvement in Na\(^+\) uptake faster than for \(O_2\) uptake.

(f) Timing of skin-to-gill transitions for Na\(^+\) and \(O_2\) uptake
The time at which Na\(^+\) and \(O_2\) uptake transitions from skin to gills was defined as the age at which the gills account for 50 per cent of total uptake rate. This was estimated as the intersection between the gills’ and skin’s per cent contribution to total uptake (figure 4). As predicted by the ionoregulatory hypothesis, the skin-to-gill transition occurred significantly earlier for Na\(^+\) uptake than for \(O_2\) uptake in both soft and hard water environments (\(p < 0.003\)). The Na\(^+\) transition occurred at 15 ± 2...
dph in soft water and at 16 ± 2 dph in hard water (not statistically different; figure 4). Intersection points for O₂ uptake were extrapolated to occur at 23 ± 2 dph in soft water and 28 ± 3 dph in hard water (not statistically different; figure 4). This is consistent with predictions based on morphological data: MRCs appeared on the gills as early as 15 days prior to the appearance of lamellae (Gonzalez et al. 1996), and the gills accounted for 50 per cent of all MRCs by 18 dph while it took until day 35 dph for the gills to account for 50 per cent of total respiratory SA (Rombough 1999). In gill function ablation experiments on larval zebrafish, ionoregulation by the gills were essential to survival by 7 dpf but O₂ uptake by the gills were only essential to survival by 14 dpf (Rombough 2002). It appears that the gills play a major role in ionoregulation before doing so for gas exchange in both rainbow trout and zebrafish.

**The lack of plasticity of gill ionoregulatory development: timing of skin-to-gill transition for Na⁺ uptake, NKA α-subunit protein expression and NKA activity**

The timing of skin-to-gill transition for Na⁺ uptake was not affected by water hardness. Hardness also had little effect on NKA α-subunit expression and NKA activity. This is in agreement with an early study on rainbow trout embryos and larvae which reported that gill MRCs did not differ in number or appearance in larvae reared at different salinities (deionized water, 11 ppm and 13 ppm sea water; Shen & Leatherland 1978).

Taken together, it appears that variations in the ion content of water have little impact on the ionoregulatory development of the rainbow trout gill, at least within the relatively broad range in ion content employed in this study.

**Conclusion and future directions**

Until the ionoregulatory hypothesis was proposed (Li et al. 1995), it was assumed that the gills of all fish developed initially for the purpose of gas exchange. Ionoregulation was assumed to be a secondary function acquired at a later stage. Our findings suggest the reverse is true in at least some species. In rainbow trout, the gills become the primary site of Na⁺ uptake 8–12 days sooner than for O₂ uptake. In environments that differ markedly in ion content (hard versus soft water), there were no differences in the timing of skin-to-gill transition for Na⁺ uptake during development in rainbow trout. Furthermore, no differences in gill NKA protein and activity during development were observed between soft and hard water rearing environments, suggesting that variations in water ion content may have little impact on the rate of ionoregulatory development of the gills.

The findings of our study may have important implications on our current understanding of gill evolution.
It is commonly assumed that gills evolved from the branchial baskets of protochordates to complement O$_2$ uptake across the skin as O$_2$ demands increased due to the evolution of more active, predatory lifestyles and increased body size (reviewed in Coolidge et al. 2007). Such pressures are similar to those encountered by larvae during gill development (Rombough 2004). It has been suggested that exchange at the skin became limiting for ionoregulation before gas exchange during the course of gill evolution, as is believed to be the case for the gills during development (Rombough 2004). This hypothesis is supported by studies on the gill function of primitive fishes. Studies on gill function in osmoconforming marine hagfishes, extant members of the earliest fish lineage, suggest that the first function of the vertebrate gills may be acid–base regulation (Mallat et al. 1987; reviewed in Evans et al. 2005). It has been proposed that the mechanism of acid–base relevant NaCl exchange at the gills were an exaptation for ionoregulatory functions that became important when fish invaded freshwater habitats (Evans 1984; reviewed in Wright 2007). In subsequently evolving species including lampreys (reviewed in Wright 2007), the gills are also believed to play an important, if not a primary, role in acid–base regulation and/or ionoregulation. In summary, we emphasize the need to investigate the functions of gills in phylogenetically varied species. The role of larval fish gills in ionoregulation is a good starting point for future research. We can then make a well-informed hypothesis on the relative strength of the forces that drive ontogenic gill development and, on a larger scale, the selective pressures that may have influenced the evolution of the vertebrate gill.

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