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Commentary

Transition in organ function during the evolution of air-breathing; insights from *Arapaima gigas*, an obligate air-breathing teleost from the Amazon

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Summary

The transition from aquatic to aerial respiration is associated with dramatic physiological changes in relation to gas exchange, ion regulation, acid–base balance and nitrogenous waste excretion. *Arapaima gigas* is one of the most obligate extant air-breathing fishes, representing a remarkable model system to investigate (1) how the transition from aquatic to aerial respiration affects gill design and (2) the relocation of physiological processes from the gills to the kidney during the evolution of air-breathing. *Arapaima gigas* undergoes a transition from water- to air-breathing during development, resulting in striking changes in gill morphology. In small fish (10 g), the gills are qualitatively similar in appearance to another closely related water-breathing fish (*Osteoglossum bicirrhosum*); however, as fish grow (100–1000 g), the inter-lamellar spaces become filled with cells, including mitochondria-rich (MR) cells, leaving only column-shaped filaments. At this stage, there is a high density of MR cells and strong immunolocalization of Na^+/K^+-ATPase along the outer cell layer of the gill filament. Despite the greatly reduced overall gill surface area, which is typical of obligate air-breathing fish, the gills may remain an important site for ionoregulation and acid–base regulation. The kidney is greatly enlarged in *A. gigas* relative to that in *O. bicirrhosum* and may comprise a significant pathway for nitrogenous waste excretion. Quantification of the physiological role of the gill and the kidney in *A. gigas* during development and in adults will yield important insights into developmental physiology and the evolution of air-breathing.

Key words: air-breathing, gills, kidney, *Arapaima gigas*, *Osteoglossum bicirrhosum*, gas exchange, ionoregulation, acid–base balance, nitrogenous waste excretion.

Introduction

Physiological adjustments associated with the transition from aquatic to aerial respiration in vertebrates have been of interest to physiologists for centuries, with extant air-breathing fishes providing a glimpse into how aerial respiration must have arisen (Burggren and Johansen, 1986). The aquatic-to-aerial transition has obvious implications for gas transport but also has dramatic effects on the physiology of ion regulation, acid–base balance and nitrogenous waste excretion. Among fishes, air-breathing is thought to have evolved as many as 67 times (Graham, 1997), and the degree of aerial dependence among air-breathing fishes varies greatly. Facultative air-breathers depend upon air-breathing to augment gill oxygen uptake during periods of hypoxia, which may involve changes as minor as increased vascularization of the buccal cavity so that O_2 can be removed from air held in the mouth. However, at the other extreme are air-breathing fishes that are completely dependent upon aerial respiration and drown without access to the surface, even when water is well oxygenated.

One of the most impressive air-breathing fishes is *Arapaima gigas*, which is endemic to the Amazon Basin. It is one of the largest freshwater fishes in the world, reaching a length of 3 m and weighing as much as 250 kg (Salvo-Souza and Val, 1990; Graham, 1997). *Arapaima gigas* is an osteoglossid teleost that has a modified swim-bladder, which is used as an air-breathing organ, and is among the most aerially dependent of fishes. It drowns in ~10 min without access to air, despite the presence of gills (Val and Almeida-Val, 1995). The males carry the fertilized embryos and newly hatched larvae in their mouths (Salvo-Souza and Val, 1990) and, early in development, *A. gigas* is a water breather up to ~9 days post-hatch (see Graham, 1997). Thus, during development, *A. gigas* undergoes a transition from an exclusive water-breather to an air-breather,

representing an impressive model system to investigate how the transition from aquatic to aerial respiration affects gill design. Furthermore, it represents a model system to determine whether air-breathing is associated with the transition of physiological processes from the gills to the kidney; an area ripe for investigation in the evolution of air-breathing (Graham, 1997).

The first portion of this commentary will focus on how environment and development influence gill design in water-breathing fishes. This will be used to facilitate interpretation of the observed changes in the gills during development in *A. gigas*. The remainder of the commentary will focus on the evidence that exists for the relocation of physiological processes from the gills to the kidney in *A. gigas*.

Physiological constraints influencing gill design

Historically, conditions related to gas transport have been assumed to be the primary design constraint of the fish gill. In most adult water-breathing fishes, total gill surface area is greater than that of the body surface, and the blood-to-water diffusion distance is low, making the gill an ideal surface for gas exchange. Furthermore, fish that have a high total gill surface area tend to have high metabolic rates (see Brill, 1996). However, superimposed upon the architecture required for efficient respiratory gas exchange are other constraints such as those required for ionoregulation, acid–base balance and nitrogenous waste excretion. In contrast to air-breathing vertebrates, where the kidney plays a major role in these latter functions, in water-breathing fishes these processes occur in concert with gas exchange across the gills. Thus, tremendous potential exists for the interaction among these physiological processes at the gills (Randall and Brauner, 1998), and gill design will represent a compromise among these processes.

The majority of nitrogenous waste excretion in freshwater fishes appears to occur across the gills as passive NH_3 diffusion (Wood, 1993; Wilkie, 1997); therefore, conditions that facilitate respiratory gas exchange will, for the most part, facilitate NH_3 diffusion. The same is not true for ionoregulation or acid–base balance, both of which require specific cell types with specialized ion exchangers or pumps appropriately placed in the gill epithelium. In freshwater fishes, the mitochondria-rich (MR) cells (chloride cells), which are responsible for Cl^- and Ca^{2+} uptake, are generally localized to the trailing edge of the filamental epithelium at the base of the lamellae and within the inter-lamellar regions (Perry, 1997). This design permits relatively efficient ion and acid–base regulatory exchanges across the non-respiratory portion of the gill, while optimizing conditions for gas diffusion across the lamellar epithelium. This prioritization in gill design can be superseded during ionic or acid–base regulatory challenges (Goss et al., 1995), which are particularly pronounced when fish are acclimated to soft water (Greco et al., 1996). Because of the need to maintain active ion uptake under sub-optimal conditions, MR cells proliferate on both the filamental and lamellar epithelium of the gill. This acts to increase the capacity of the gill to actively take up Ca^{2+} and Cl^- from the water but results in an increase in the lamellar blood-to-water diffusion distance, which directly compromises gas exchange in the absence of compensatory measures such as hyperventilation or changes in whole-blood oxygen affinity (Perry, 1997, 1998). Thus, changes to the general pattern of gill design can be induced by environmental alterations; however, this is associated with a cost.

Physiological constraints influencing gill design in larval fishes

Gills provide a means to compensate for a reduction in body surface area-to-volume ratio that occurs as fish grow. Traditionally, it has been assumed that the gills in larval fishes develop for gas transport. Rombough (1999) has challenged this dogma, proposing that the gill may in fact develop to address the needs for ion regulation or acid–base regulation rather than gas exchange. This hypothesis is based upon the observation that gills develop in larval fishes long before they appear to be required for gas exchange (Rombough and Moroz, 1997), during which time chloride cell development is rather extensive (Rombough, 1999). Chloride cells first appear on the gills of rainbow trout (*Oncorhynchus mykiss*) 3–6 days prior to hatch at 10°C, approximately 9 days prior to the appearance of lamellae (Rombough, 1999). At hatch, 22% of all chloride cells are located on the gill, which comprises only 7% of the animals' surface area. At this time, filamental chloride cell density has already reached a similar density to that observed in the filamental epithelium of adult rainbow trout (Perry et al., 1992; Rombough, 1999). A qualitatively similar relationship has been observed in tilapia (*Oreochromis mossambicus*; Li et al., 1995). With development, the gills play a relatively larger role in gas exchange. In chinook salmon (*Oncorhynchus tshawytscha*), the gills are responsible for ~60% of oxygen uptake at yolk sac absorption (0.4 g; ~25 days post-hatch at 10°C), at a time when gill surface area represents ~24% of total surface area (Rombough and Ure, 1991). The same appears true for rainbow trout (Rombough, 1998) and is likely to be a general pattern in gill development, at least among salmonids.

Based upon morphological analyses, gills of larval fishes may develop first for ion regulation or acid–base balance and secondarily for gas exchange. As the cutaneous surface becomes incapable of satisfying the respiratory needs of the animal, due to the reduction in surface area-to-volume ratio that accompanies growth, a shift from ion or acid–base regulation to gas exchange as the principal factor influencing gill design may occur. Thus, the physiological process that is most limited under a given condition may have the greatest influence on gill design, but this may vary with developmental stage or environmental conditions. This may also be the case for the gills of air-breathing fishes, such as *A. gigas*, where different selective pressures from those observed in water-breathing fishes have a marked influence on gill design.

Changes in gill structure during development in *Arapaima gigas*

The gills of *A. gigas* are greatly reduced in mass relative to

a closely related water-breathing osteoglossid teleost (*Osteoglossum bicirrhosum*; Hulbert et al., 1978), and the gills of 1–2 kg *A. gigas* are only responsible for 5–25% of whole-body oxygen uptake (Sawaya, 1946; Stevens and Holeton, 1978; Brauner and Val, 1996). Consequently, it is likely that gill design in *A. gigas* is less strongly influenced by conditions for oxygen transport relative to water-breathing fishes. Larval *A. gigas* are water-breathers but, within 8–9 days post-hatch (about 18 mm), they become air-breathers (see Graham, 1997). Assuming a similar mass–length ratio between *A. gigas* and salmonids, the size at which *A. gigas* begins air-breathing is beyond that at which gills would be required for gas exchange. Based upon the arguments put forth by Rombough (1999), initial gill development in larval *A. gigas* may be largely influenced by the need for ionoregulation or acid–base balance. However, shortly thereafter, constraints associated with aquatic gas exchange may be expected to influence gill design, as has been hypothesized for larval fish. With further growth, constraints for gas exchange may wane as *A. gigas* becomes progressively more dependent upon aerial respiration, and selective pressures for gill design may revert back to those for ionoregulation or acid–base regulation. Gill design in *A. gigas* may be subjected to different selective pressures at different stages of development, making the ontogeny of the gills in this species a very interesting model for developmental physiology.

Three different sizes of *A. gigas* (10 g, 100 g and 1 kg) were obtained to investigate changes in gill morphology, ultrastructure and immunohistochemistry during development. The smallest available size (10 g) is larger than that at which the transition from water- to air-breathing occurs, but fish of this size are still less dependent upon aerial respiration relative to 1 kg *A. gigas* and can survive for twice as long without access to air (20 min *vs* 10 min, respectively; C. J. Brauner and A. L. Val, unpublished). Scanning electron microscopy (SEM) of gills from 10 g fish revealed that they possess well-developed lamellae, typical of water-breathing fish gills (Fig. 1A). While the lamellae are compact relative to those of other water-breathers such as trout (*O. mykiss*), they are more similar to those of *O. bicirrhosum* (Fig. 2). *Arapaima gigas* grows very quickly, and the next largest group (100 g) is only ~45 days older than the 10 g fish; however, changes in gill structure have taken place, and the lamellae have become less discernible (Fig. 1B). Within 4–5 months, fish have reached 1 kg, and the changes in gill morphology are striking. The lamellae are no longer visible by SEM, and the gills consist of what appear to be smooth, column-shaped filaments (Fig. 1C). The filaments of 1 kg *A. gigas* appear qualitatively similar to the recent SEM images obtained for the crucian carp (*Carassius auratus*) in normoxia (Sollid et al., 2003). However, in carp, several days exposure to hypoxia results in pronounced lamellar protrusion from the filaments that is associated with the disappearance of the interlamellar cell mass, indicating that environmental conditions exert reversible effects on gross gill morphology in carp. The filling of the interlamellar space during development in *A. gigas* is likely to be non-reversible; however, this remains to be investigated.

Light microscopy provides insight into the developmental changes of the gills. Again, 10 g fish possess gills similar to those of other water-breathers, with MR cells localized to the interlamellar region of the filament (Fig. 3A). In 100 g fish, the interlamellar region of the gill becomes partially filled with developing cells, including MR cells. Consequently, the

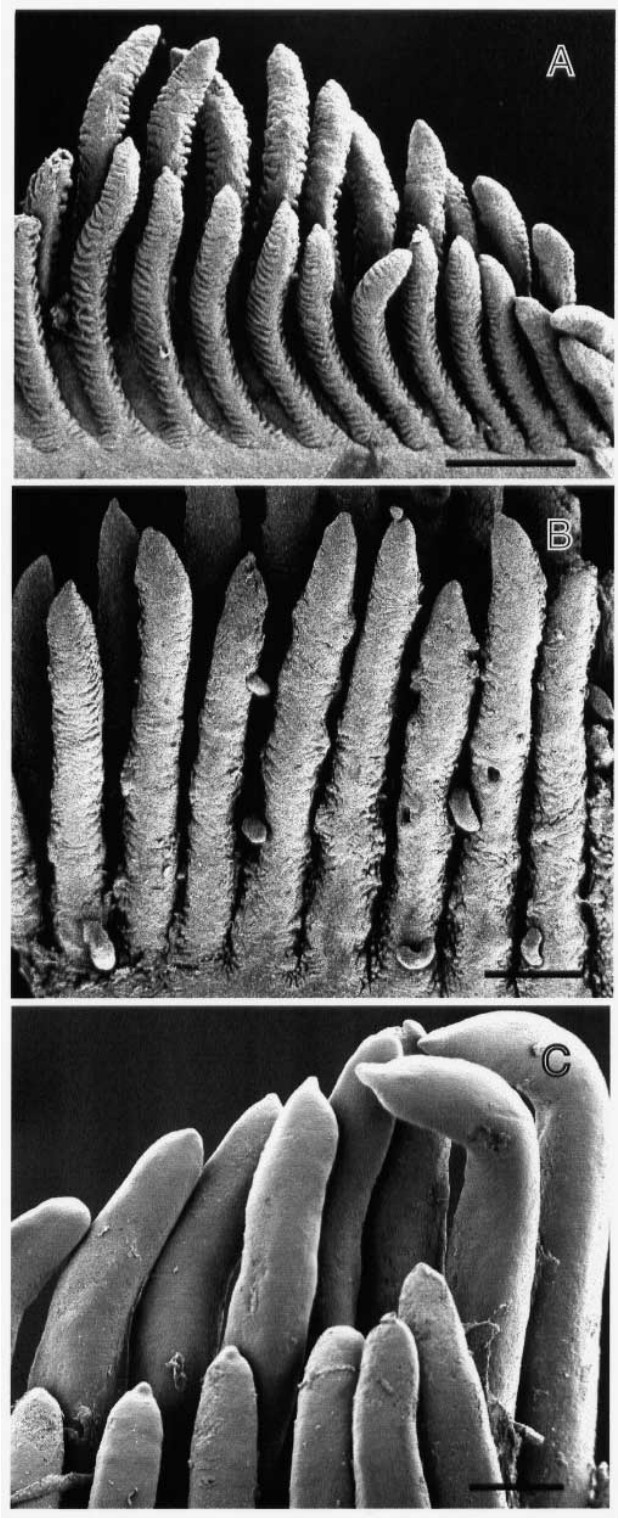


Fig. 1. Scanning electron micrographs (SEM) of the gills from three different sizes of the obligate air-breathing teleost *Arapaima gigas*: (A) 10 g, (B) 100 g and (C) 1 kg body mass. Scale bars, 500 μm.

Fig. 2. Scanning electron micrographs (SEM) of gills from a closely related water-breathing osteoglossid, *Osteoglossum bicirrhosum* (300 g). Scale bar, 500 μm.

lamellae expand laterally and become stubby in appearance, consistent with that observed under SEM. MR cells at this stage are even observed on the tips of the lamellae (Fig. 3B). In gills from 1 kg fish, interlamellar regions become completely filled due to cellular proliferation, predominantly with MR cells, again consistent with gross anatomical changes observed under SEM. The MR cells are large and extensive on the outer layer of the filament. Immunohistochemistry reveals strong immunofluorescence for Na^+/K^+-ATPase in the outer epithelium of the filament of the 1 kg fish, consistent with the location of the MR cells in the light micrographs. Immunolabelling of the Cl^-/HCO_3^- anion exchanger 1 (AE1) is restricted to the red blood cells (Fig. 4), and preliminary experiments indicate that Na^+/H^+ exchanger 2 (NHE2) may be co-localized in Na^+/K^+-ATPase-immunoreactive cells in the apical region. Further studies are required to verify the presence of NHE2, which is rare among freshwater fishes; however, in acid-tolerant dace (*Tribolodon hakonensis*), an apical NHE has been found associated with MR cells (Hirata et al., 2003).

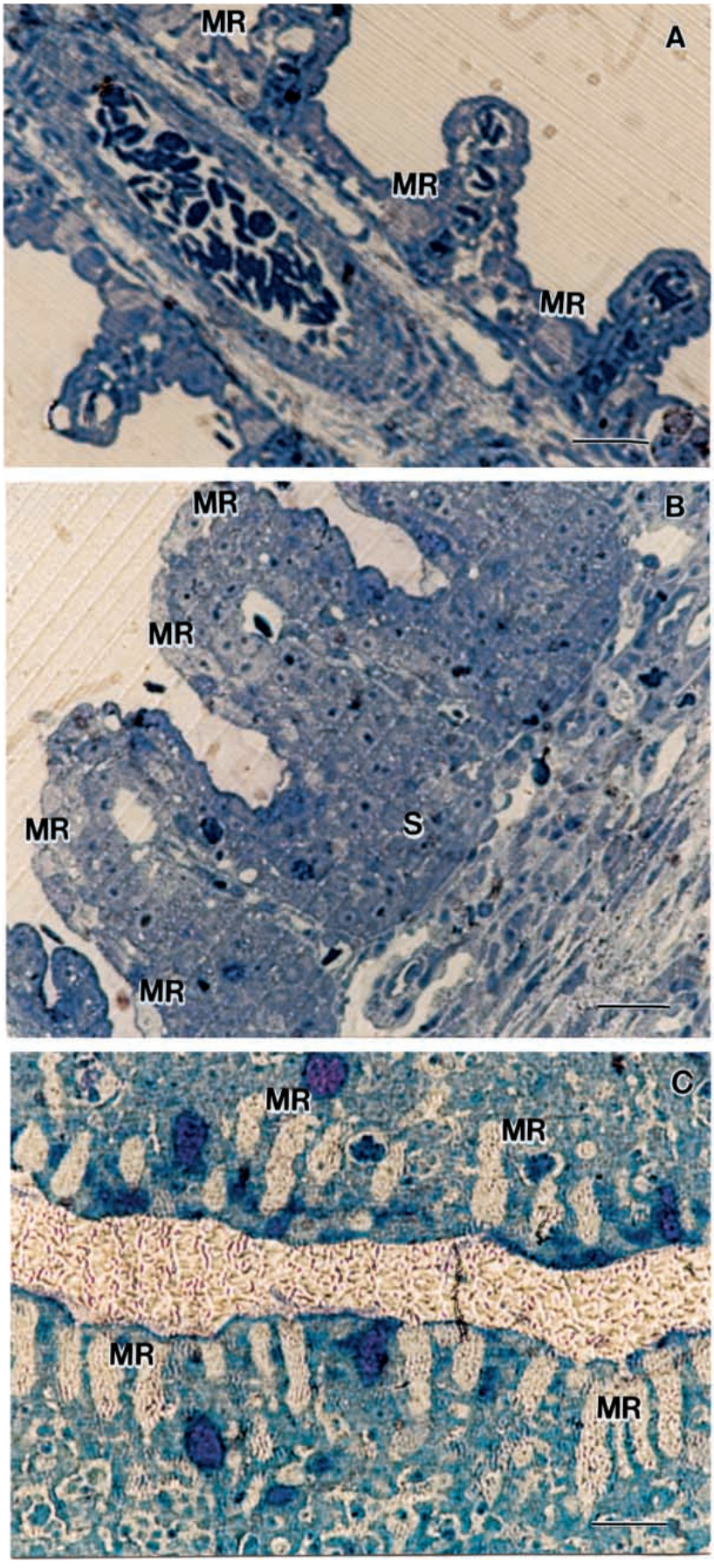
In both the light micrographs and immunohistochemistry, it is clear that the diffusion distance between the blood and the water in the 1 kg fish is very large relative to the 10 g *A. gigas* and other water-breathers. It is not surprising that so little oxygen uptake occurs across the gills at this stage. Despite the large diffusion gradient, the majority of CO_2 is excreted into the water (Randall et al., 1978), with up to 79% presumably excreted across the gills (Brauner and Val, 1996). Such significant CO_2 excretion across the gills is accomplished in part by the large blood–water CO_2 partial pressure (P_{CO_2}) gradient. Blood P_{CO_2} values are 26–30 mmHg (1 mmHg=133.3 Pa) in similarly sized *A. gigas* (Randall et al., 1978), which is far greater than the 2–3 mmHg measured in most water-breathing fishes. The high P_{CO_2} values in *A. gigas* result from the reduced total gill surface area (characteristic of obligate air-breathers in general; see Graham, 1997) and the increased diffusion distance (Figs 3, 4), both of which limit CO_2 excretion across the gills. It is intriguing to think that *A. gigas* lives in a continuous state of compensated respiratory acidosis, which is supported by the large difference between plasma Na^+ and Cl^- concentrations (150 mmol l^{-1} and 60 mmol l^{-1}, respectively; R. Gonzalez and C. J. Brauner, unpublished data). Given the large dependence of the gills for CO_2 removal, it is clear that conditions for CO_2 excretion do not impose large selection pressures to optimize gill design for gas exchange at this stage, and the gills appear to be more designed for ionoregulation or acid–base balance.

The gills in 1 kg fish possess a twofold-higher density of MR cells for a given distance along the filament relative to the 10 g animals (V. Matey and C. J. Brauner, unpublished data). Interestingly, the unidirectional Na^+ uptake rate in resting undisturbed *A. gigas* (1 kg) is quite low (70 nmol g^{-1} h^{-1}; R. Gonzalez, personal communication) relative to other freshwater fishes, seemingly paradoxical given the high density of MR cells. An overall reduction in gill surface area and an increase in blood–water diffusion distance of the gill of *A. gigas* will greatly reduce diffusive ion loss across the gills, which in water-breathing fishes is the primary surface for ion efflux. In fish that possess low ion efflux, unidirectional uptake of ions is also low (McDonald et al., 1991) and, thus, the low unidirectional Na^+ uptake rate in *A. gigas* may be a reflection of low gill ion permeability more than anything else. The role of the gills *in vivo* will be best evaluated by analyzing ion transport during an ionoregulatory or acid–base regulatory challenge, where the full potential of the gills may be revealed.

The architecture of the gill appears to vary dramatically with development in *A. gigas*. Shortly after becoming an air-breather (i.e. 10 g), secondary lamellae are evident and the appearance of the gills is similar to that of a closely related water-breather where conditions for efficient gas transfer have a large influence on gill design. With development, the gills of *A. gigas* appear to become better designed for ionoregulation or acid–base balance, particularly by the time *A. gigas* reaches 1 kg. This is similar to that observed in larval fishes (but chronologically reversed), where pressures related to ionoregulation and acid–base balance appear to have a greater influence on gill design than those for gas exchange early in development. The role of the gills in whole-body ionoregulation and acid–base balance in ≥100 g *A. gigas* remains to be investigated.

Transition of branchial physiological function to the kidney in *Arapaima gigas*

The large reduction in gill to body total surface area of air-breathing fishes (Graham, 1997) may imply a shift in physiological function away from the gills to the kidney during the evolution of air-breathing. While this hypothesis has been investigated in facultative (Cameron and Wood, 1978) and



obligate (reviewed by Graham, 1997) air-breathers with mixed findings, the greatest support has been obtained in comparisons among *A. gigas* and *O. bicirrhosum*. Estimates of ion-regulatory capacity, based upon measurements of gill and kidney masses and their respective homogenate Na^+/K^+-ATPase and Ca^{2+}-ATPase activities (Hochachka et al., 1978; Hulbert et al., 1978), indicate that the kidney may play a much greater role in *A. gigas* than in *O. bicirrhosum*; however, no *in vivo* physiological data presently exist to support or refute this hypothesis. The Na^+/NH_4^+ exchanger was found in the kidney of *A. gigas* but not *O. bicirrhosum*, implying a potential role for the kidney in nitrogenous waste excretion. Total ammonia levels in the blood of 1 kg *A. gigas* are ~1 mmol l^{-1} and those of the urine are ~10 mmol l^{-1} (Y. Wang; personal communication), the latter in particular being very high for a teleost fish. Assuming that ammonia production is ~5–10% of oxygen consumption rate, with urine flow rates of almost 6 ml kg^{-1} h^{-1} (Brauner and Val, 1996), the kidney could account for 20–40% of whole body nitrogenous waste excretion, a topic clearly worthy of further investigation.

Conclusions

The morphological, biochemical and physiological alterations discussed here shed insight into how the transition from aquatic to aerial respiration affects gill design and the degree to which evolution of air-breathing is associated with the transition of physiological processes from the gills to the kidney. While the transition in organ function of the gills and kidney observed in *A. gigas*, relative to *O. bicirrhosum*, is not universal among facultative and obligate air-breathers (Graham, 1997), this may not be surprising. Air-breathing may have evolved independently as many as 67 times, so the patterns observed among these events will undoubtedly differ to some degree. While it is intuitively appealing that an interspecific continuum may exist for the transition of gill and kidney function among water-breathers, facultative air-breathers and obligate air-breathers, this may not occur because of the degree to which the animal must commit to these changes, and the physiological costs that these changes impose. Consequently, the interspecific transition in gill and kidney function during different degrees of aerial dependence in fish may be more punctuated than

Fig. 3. Light micrographs of the gills from three different sizes of the obligate air-breathing teleost *Arapaima gigas*: (A) 10 g, (B) 100 g and (C) 1 kg body mass. C is a micrograph of two neighbouring filaments; note the absence of lamellae and extensive proliferation of mitochondria-rich (MR) cells. Scale bars, 20 μm.

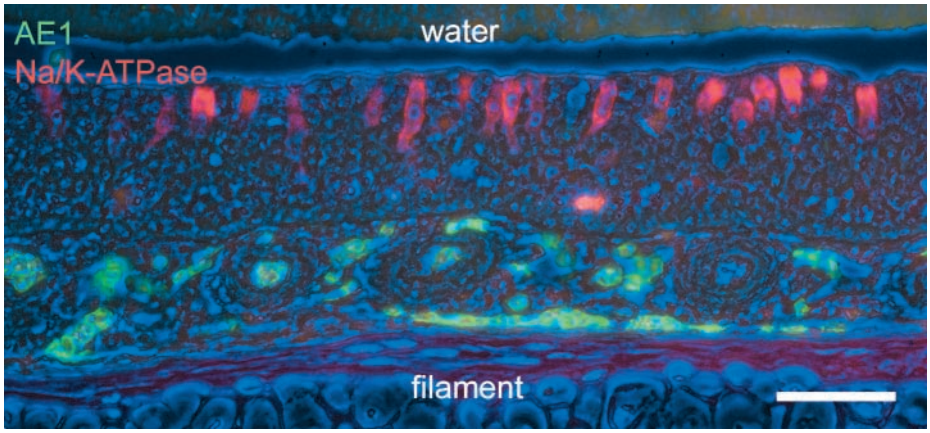


Fig. 4. Immunohistochemistry of the gills of 1 kg *Arapaima gigas*. The red fluorescence indicates Na^+/K^+-ATPase immunoreactivity, while the green fluorescence indicates anion exchange 1 (AE1) immunoreactivity predominantly localized to red blood cells. Scale bar, 50 μm.

gradual. Because *A. gigas* is one of the most aerially dependent obligate air-breathing fishes, it can commit to truly large and irreversible changes, as discussed here. The existence of such a closely related obligate water-breather, *O. bicirrhosum*, makes this species comparison very powerful. While it is clearly not the only model for the transition in the physiological function of organs during the evolution of air-breathing, it certainly is a wonderful one!

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