

Ontogeny and morphometrics of the gill and swim bladder of air-breathing striped catfish *Pangasianodon hypophthalmus*

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Abstract

The air-breathing fish *Pangasianodon hypophthalmus* has been shown to have highly plastic branchial surfaces whose area (SA) increases with temperature and aquatic hypoxia. This modulation occurs through development of inter-lamellar cell mass (ILCM). Paradoxically, in conditions where this fish has been shown capable of covering its entire aerobic scope from the water phase, it has been shown to have a very small branchial SA. To address this paradox, we measured the SA, harmonic mean diffusion (τ_h) and calculated the anatomic diffusion factor (ADF) of the branchial and swim bladder surfaces in fish ranging from 3 to 1900 g at 27°C in normoxia. Since the lamellae were distinguishable from the ILCM, we measured the actual SA as well as the potential SA if ILCM were lost. As a result of low τ_h , *P. hypophthalmus* has a high capacity for branchial oxygen uptake with or without ILCM. The actual and potential gill ADF being 361 and 1002 $\text{cm}^2\mu\text{m}^{-1}\text{kg}^{-1}$ respectively for 100g fish. The ADF of the swim bladder was similarly 308 $\text{cm}^2\mu\text{m}^{-1}\text{kg}^{-1}$. By swimming the fish to exhaustion at different temperatures we showed that modulation of this SA is rapid, indicating that the apparent paradox between previous studies is eliminated. Regression analysis of log-log plots of respiratory SA in relation to body mass show that the gills scale with mass similarly to active water breathing fish, whereas the swim bladder SA scales similarly to the mammalian lung. This fish presents a combination of respiratory surfaces not previously seen in air-breathing fish.

List of symbols and Abbreviations

ADF	anatomic diffusion factor
SA	surface area
τ_h	harmonic mean diffusion distance
ILCM	inter lamellar cell mass
SURS	systematic uniform random sampling
VUR	vertical uniform random sections
U_{swim}	swimming speed
U_{crit}	critical swimming speed
ABO	air-breathing organ

INTRODUCTION

Morphometric analyses have demonstrated that the gill SA in water-breathing fish scale allometrically with body mass with a median exponent of 0.8 and ranging from 0.6 to 1.0 (references listed in Table 4 for water-breathing fish). Much of the variation in this parameter correlates with the level of activity shown by the fish, with active fish possessing a much larger SA compared to sluggish fish (Gray, 1954; Hughes, 1966; Hughes and Morgan, 1973; Palzenberger and Pohla, 1992; Severi et al., 1997). Further, both gill SA and τ_h show significant plasticity and are altered by a variety of environmental factors including oxygen demand, temperature, pH, toxicants and parasites (reviewed in Nilsson et al., 2012).

In air-breathing fish there is an additional source of variation in gill morphology because, while the majority of these species start life as water-breathers in the highly oxygenated surface waters of the tropical rainy season, they undergo developmental changes in the partitioning of oxygen uptake, with reductions in gill SA as oxygen uptake transfers to a variety of air-breathing structures (Graham, 1997). Air-breathing fish are divided into obligate air-breathers (drown without access to air) where this ontogenetic change in gill structure is far advanced with very reduced adult gills, and facultative air-breathers where the gills remain the main source of oxygen uptake in normoxic water during adulthood. A good illustration of this ontogenetic change occurs in the obligate air-breathing osteoglossiformid *Arapaima gigas*, where the fish hatch with fully exposed lamellae as water breathers but following the initiation of air-breathing at 8-9 days post hatch, gradually lose lamella SA

and by 1 kg in size have completely lost these branchial respiratory surfaces and are entirely reliant on their air-breathing organ (Brauner et al., 2004).

The Pangasiidae are a group of recently evolved (Pouyard et al., 2000) air-breathing siluriform catfishes inhabiting Southeast Asia. These species are also unusual among air-breathing fish in that they undergo extreme seasonal migration, covering at least 2000 km along the Mekong River between their spawning and feeding grounds (Zalinger et al., 2002). Thus, adults migrate upstream to spawn between Kratie in Cambodia and the Khone Falls of Laos (Zalinger et al., 2002; So et al., 2006). After spawning, adults return to their feeding grounds downstream in the floodplains of central and southern Cambodia and the Vietnamese Mekong delta and their fingerlings drift passively in the water currents to the same areas for grow-out (Zalinger et al., 2002; So et al., 2006). This migration requires this species to be adapted to a wide variety of environmental variables including changes in water temperature, oxygen levels and predation risks encountered along this hazardous journey (Lucas and Baras, 2008; Li et al., 2013). While the whole migration range is tropical, water temperatures vary from around 24°C in the spawning grounds in northern Laos to a maximum of 32°C in the growth habitats of the Mekong river delta (Li et al., 2013). Recently it has been shown that this species has strongly plastic branchial surfaces affected by both water oxygenation and water temperature (Phuong et al., 2017). Also, it has been documented that the swim bladder is highly vascularized and appears well adapted for air-breathing (Browman and Kramer, 1985; Zheng and Liu, 1988; Liu, 1993; Graham, 1997). Phuong et al. (2017) showed that the respiratory SA of the gills in *P. hypophthalmus* can be very large when respiratory lamellae are fully exposed, but that when ILCM is fully developed, become very small and present a branchial SA consisting solely of the tips of the lamellae. The latter was the case with fish at 27°C in normoxia, while the former was the case in hypoxic 33°C water. This represents a paradox, because Lefevre et al. (2011, 2013) showed very clearly that this species was able to meet its entire oxygen requirements from the water-phase alone in 27°C normoxic water and concluded therefore that the gills must be very large, in itself unusual for an air-breathing fish with such a well-developed air-breathing organ.

The primary aim of this study was to elucidate this paradox. We therefore investigated the ontogeny of the respiratory SA of the gills and swim bladder of *P. hypophthalmus* from approximately 3 g to 1900 g, in addition to the harmonic mean diffusion distance (τ_h) of these organs using vertical uniform random section (VURS) - based stereology methods. Further, we used these metrics to measure the anatomic diffusion factor and compare this with the

literature. Finally we tested the hypothesis that changes in activity level would stimulate a rapid increase in branchial SA by measuring the gill metrics of a group of fish where half were allowed to swim spontaneously in a growth tank, whereas the other half underwent a swimming protocol in a swim respirometer.

MATERIALS AND METHODS

Fish

Five groups of healthy *Pangasianodon hypophthalmus* Sauvage 1878, 4.5 ± 0.8 g (n=6); 24.3 ± 0.7 g (n=6), 276 ± 19 g (n=6), 703 ± 41 g (n=4), and 1760 ± 160 g (n=2) were selected from aquariums of the College of Aquaculture and Fisheries, Can Tho University (Vietnam). These fish were maintained in a 2000 l tank at 27°C and more than 92% air saturation in clean water for one month. They were fed daily with commercial pellets and fasted for two days before their organ sampling.

2.2. Sampling and processing respiratory organs for stereological observation

The fish were anaesthetized in Benzocaine (1.5 g l^{-1}), euthanized by severing their spinal chord and their entire gills removed and immersed in 4% phosphate-buffered formalin. The swim bladders were also carefully dissected out of the fish abdomen and filled with the buffered fixative through their glottis before immersion in the same fixative for subsequent processing for stereological analysis (Fig.1A).

Gills

The left or right gill basket was randomly chosen for analysis. The gill basket was removed from the fixative and the four gill arches carefully dissected away from the cartilage. These were gently dried on filter paper and their individual masses noted. The entire filament tissue from the first gill arch was then processed for stereology. Before processing and embedding, the gill tissues were subsampled using a smooth fractionator and systematic uniform random sampling (SURS) with a sampling fraction of one third or one quarter depending on the size of the gills (Gundersen et al., 1988; Gundersen, 2002). These chosen tissues of gill filaments (after subsampling) were then dehydrated in a graded alcohol series and infiltrated with a series of methyl methacrylate Technovit®7100 solutions (Heraeus Kulzer, Germany) before final embedding. The procedures for embedding and sectioning followed the designs of de Moraes et al. (2005) and da Costa et al. (2007), applying VURS (Baddeley et al., 1986). Ten

vertical sections of 3 μ m thickness were taken from each fish. These sections were stained with Haematoxylin and Eosin solutions and analysed for stereological morphometric observation. These histological sections were then analysed using newCast stereological software VIS® (Olympus, Denmark).

Swim bladder

The entire fixed swim bladder was sectioned using SURS into 5 mm slices (Fig.1B) following the procedure of de Moraes et al. (2005). The Cavalieri principle was used to estimate the volume of swim bladder (V_{SB}) following the procedure described in Gundersen et al. (1988). These slices were then subsampled using SURS to produce 10 sampling tissues in total for embedding for light microscopy. The procedures for processing, embedding, and sectioning of these sub-samples were performed using VUR principles, in the same way as with gills (described in detail in de Moraes et al., 2005).

Estimation of reference volumes, respiratory SA, and τ_h

Reference volumes of gill filaments, total volume of lamellae, volume of lamellae exposed to water, volume of swim bladder tissue, and volume of capillary bed in swim bladder, were determined using the Cavalieri principle (Gundersen et al., 1988; Howard and Reed, 1998; Michel and Cruz Orive, 1988). Potential lamellae SA (including parts of lamellae embedded by ILCM as well as parts exposed to water), lamellae SA (only parts exposed to water), and capillary bed SA in swim bladder were estimated using point and intersection counts of lines hitting the outer surface of the lamellae and capillary bed in swim bladder (Gundersen et al., 1988; Howard and Reed, 1998). The τ_h on lamellae and swim bladder capillary bed were determined from orthogonal intercepts based on Jensen et al. (1979). The total gill SA was calculated by multiplying the 1st gill arch SA by the total mass of all 4 gill arches and dividing by the 1st gill arch mass. This value was then multiplied by 2 for the whole fish. Phuong et al., (2017) showed that gill arch mass and gill SA (without ILCM) correlates well (see Fig. S1). Further, it was found that there was no systematic difference between gill arches with respect to ILCM growth or τ_h .

Swimming experiment

Two size groups of 20 healthy fish (50.1 \pm 1.0 g; 18.6 \pm 0.2 cm) were also selected from aquariums of the College of Aquaculture and Fisheries, Can Tho University and acclimated in two 1500 l tanks at 27°C and 33°C under normoxic conditions (more than 92% air

saturation) for one month. Gills of six fish (control groups) in each tank were sampled. The gill samples were fixed and processed for stereology as described above.

The remaining fish were prepared for the swimming experiment. Each fish from the two treatments (n=6) was placed in the swimming respirometer (Loligo Systems, Tjele, Denmark; described in details in Lefevre et al., 2013) for 16 hours. During this period, the U_{swim} was maintained at 1 body length per second (1 BL s^{-1}) after which, water speed was increased by 1 BL every hour following the procedure of Lefevre et al. (2013). Water and air in the swimming chamber were renewed automatically; In addition, temperature and oxygen levels in the respirometer were controlled at the same level as the acclimation conditions. The experiment was ended when the fish was unable to remove itself from the grid for 5 seconds. The fish was then removed from the swimming respirometer and its gills sampled and processed for stereology as described above.

Calculations

Equations

Total volumes of gill filaments from the first gill arch– V_{F1st} ; Volume of swim bladder tissue– V_{TSB} ;
Volume of lamella which exposed to water– $V_{L(1st)}$;
Volume of capillary bed in swim bladder– V_{CB} ;
Volume of air-space in swim bladder– V_{air}

$$V_{F(1st),TSB} \text{ (mm}^3\text{)} = \frac{1}{TSF} \times \frac{1}{SSF} \times \frac{1}{ASF} \times \bar{t} \times \left(\frac{A}{P}\right) \times \sum P_{F(1st),TSB}$$

$$V_{L(1st),CB} \text{ (mm}^3\text{)} = \frac{1}{TSF} \times \frac{1}{SSF} \times \frac{1}{ASF} \times \bar{t} \times \left(\frac{a}{p}\right) \times \sum P_{L(1st),CB}$$

Total volumes of gill filaments from 8 gill arches–
 $V_{F(Total)}$ (mm³)

$$V_{F(Total)} \text{ (mm}^3\text{)} = 2 \times \frac{V_{F(1st)} \times \sum \text{gill arch mass (1-4)}}{1st \text{ gill arch mass}}$$

$$V_{air} = V_{SB} - V_{TSB}$$

Lamellar SA of the first gill arch and swim bladder
 $SA_{L(1st),SB}$

$$SA_{L(1st),SB} \text{ (mm}^2\text{)} = 2 \times \frac{\sum I_i}{\left(\frac{l}{p}\right) \times \sum P_i} \times V_{L(1st)}$$

Total lamellar SA of 8 gill arches– $SA_{L(Total)}$

$$SA_{L(Total)} \text{ (mm}^2\text{)} = 2 \times \frac{SA_{L(1st)} \times \sum \text{gill arch mass (1-4)}}{1st \text{ gill arch mass}}$$

Where ...

TSF is tissue sampling fraction;
 SSF is section sampling fraction;
 ASF is area sampling fraction;
 t is section thickness;
 A/P is area per point;
 a/p is area per point;
 $\sum P_{F(1st),TSB}$ are total number of points hitting gill filaments/tissue of swim bladder, respectively;
 $\sum P_{L(1st),CB}$ are total number of points hitting lamellae/capillary bed, respectively;

V_{SB} is total volume of swim bladder (including tissue and air inside)

$\sum I_i$ are total number of intersections between cycloids and lamellae/capillary bed;

$\sum P_i$ are total number of points hitting lamellae/capillary bed;

l/p is length per point;

V_{SB} is volume of swim bladder;

τ_h of the lamellae/capillary bed

$$\tau_h = 3/8 \times \pi \times l_h$$

l_h is harmonic mean of orthogonal intercept length of the lamellae/capillary bed

ADF of gill and swim bladder

$$ADF = SA_{L(Total),SB} / \tau_h$$

The precision of the Cavalieri reference volume was estimated using the coefficient of error (CE)

$$CE(\Sigma P) = \frac{\sqrt{\text{Total variance of } \Sigma P}}{\Sigma P}$$

Total variance of $\Sigma P = \text{Noise} + \text{Var}_{\text{SURS}}(\Sigma \text{area})$;

$$\text{Noise} = 0.0724 \times (b/\sqrt{a}) \times \sqrt{n \times \Sigma P}$$

$$\text{Var}_{\text{SURS}}(\Sigma \text{area}) = \frac{3(A - \text{Noise}) - 4B + C}{240}$$

$$\text{with } A = P_i \times P_i; B = P_i \times P_{i+1}; C = P_i \times P_{i+2}$$

$$\text{Mean CE} = [(CE_1^2 + CE_2^2 + \dots + CE_n^2)/n]^{1/2}$$

$$CV = SD/\text{Mean}$$

Noise is the point counting error variance;

b/\sqrt{a} is the average profile shape of the observed tissues according to the nomogram of Gundersen and Jensen (1987);

n is the number of examined sections;

ΣP is the total number of points hitting the observed tissue;

$\text{Var}_{\text{SURS}}(\Sigma \text{area})$ is variance of the total SA;

CV is Coefficient of variance

Statistics

Data are presented as means \pm standard error of the mean (means \pm s.e.m). One-way ANOVA and Turkey's post hoc test were applied to compare means between the gill arch masses. Two-way ANOVA was applied to test whether swimming and/or temperatures significantly affected gill SA, τ_h , and ADF; Turkey's post hoc test was then used to identify significant differences between fish gill SA across treatments. A probability (P) value at less than 0.05 levels was considered as significant. A linear regression analysis was applied to determine the relationships between logarithm data of morphometric parameters and fish body mass. These relationships are presented according to the allometric equation $Y = aW^b$ where Y is the morphometric parameter, W is body mass, and b is the allometric exponent (the slope of the regression line in a $\log_{10}(Y)$ vs. $\log_{10}(W)$ plot). Thus, a represents the value of the morphometric parameter at one body mass unit. These statistics were performed using PASW Statistic 18.0.

RESULTS

General structure of respiratory organs

Gills

The gills of striped catfish *P. hypophthalmus* are similar in layout to other active water-breathing teleosts, with 4 gill arches on each side of the head. Each gill arch includes anterior and posterior hemibranches connected with a gill raker. Lamellae are distributed along the length of the filaments from their both sides, and consist of two epithelial layers separated by pillar cells forming a sheet through which the blood flows.

Swim bladder

The swim bladder of *P. hypophthalmus* extends along the body ventral cavity from the posterior part of the head to the tail region beyond the anus. It is a single chamber, wide from the anterior part but gradually tapering to a point at the end of the coelom (Fig.1A,B). There is a strong correlation between swim bladder length and body mass; their log-log plot gave a straight line with the slope (b) of 0.5 (r^2 , 0.98) (Table 1). The supporting structure of the swim bladder consists of collagenic fibrous walls forming subdivisions, which contain air (Fig.1C,D). The surfaces of the walls inside the swim bladder are covered by two types of epithelium. The first covering most of the surface is a thin respiratory type (thickness: 0.7-2.3 μ m) and highly vascularized epithelium identified by the presence of red blood cells, making a capillary network where the gas exchange between blood and air in the swim bladder occurs (Fig. 1D); The second type is thick (5-15 μ m) with a brush boarder (Fig.1E). These two surface types are spread throughout the ABO and no discernible spatial organisation was evident.

Morphometrics of respiratory organs and relationship between these morphometrics and body mass

The morphometrics of the gills and swim bladders at different growth stages of *P. hypophthalmus* and the relationship between these parameters to fish mass are summarised in Table 1 and Table 2.

Gills

The total potential respiratory SA of the lamellae and the SA of lamellae unblocked by ILCM are shown in Table 2. The mass of gill filament from each gill arch correlated strongly with fish mass (r^2 , 0.99), and scaled with body size with slopes from 1.068 to 1.098 (Table 1). In all fish, the 1st and the 3rd gill arches were slightly larger than those of the gill arches 2 and 4 (Fig.2; $P < 0.05$). Regression analysis of log-log plots of total respiratory lamellar SA to body mass revealed slopes of 0.83 and 0.92 respectively, for actual and potential values (Fig. 3; Table 1). The τ_h was between 1.5 and 2.2 μm (Table 2). The ADF value of potential lamellar SA for a 100 g fish was $1002.4 \text{ cm}^2\mu\text{m}^{-1}\text{kg}^{-1}$, which is 3 times higher than that of the actual lamellar SA at $361.3 \text{ cm}^2\mu\text{m}^{-1}\text{kg}^{-1}$ for the same size fish (Table 3).

Swim bladder

There was a strong correlation between respiratory SA of swim bladder and fish mass. On a log-log plot the scaling coefficient was 0.71 (r^2 , 0.94) (Fig.4A; Table 1). The total volume of swim bladders ranged from 9 to 85 $\text{mm}^3 \text{ g}^{-1}$ where air accounted for between 50 to 94% of this volume and a tendency for the air space fraction to increase with fish body mass (Table 2). Both the total ABO volume and the air volume of the ABO were highly correlated with body mass; the r^2 values exceeded 0.97 and the slope b gained from the regression lines were 1.4 and 1.5 respectively (Fig.4B; Table 1). The τ_h of the swim bladder was in the range of 0.7 to 2.3 μm (Table 2). The ADF was $308 \text{ cm}^2\mu\text{m}^{-1}\text{kg}^{-1}$ so the apparent contribution of this organ to the total diffusing capacity in *P. hypophthalmus* at 27°C and in normoxia is approximately 46% (Table 3). The resistance to diffusion in the boundary layers of respiratory surfaces is, however, much more significant in water than in air and, indeed, mathematical models point out that this resistance contributes substantially to the total resistance to diffusion in the fish gill (Scheid and Piiper, 1971). Therefore the apparent,

relative contribution of the ABO to the total diffusing capacity of the animal is an underestimate.

Gill SA in relation to swimming performance

There were significant differences in gill morphology between fish sampled before and after swimming in Figure 5. The fish sampled before swimming showed gills predominantly filled with ILCM in line with expectations from normoxic conditions (Fig. 5A). Temperature alone caused a small increase in ADF mediated by a small change in SA and little change in τ_h (Fig. 5C). Swimming on the other hand caused a large change in τ_h and in combination with elevated temperature a very large change in SA where the gills were almost emptied of ILCM (Fig. 5B,C). Thus the ADF and SA were significantly affected by both temperature and swimming and the interaction between temperature and swimming ($P < 0.001$ for all three). The τ_h on the other hand was only significantly affected by swimming ($P < 0.001$) and with no significant interaction ($P = 0.184$).

DISCUSSION

Gill plasticity and Anatomic diffusion factor (ADF)

Lefevre et al. (2011, 2013) showed that in normoxic water at 27°C, *P. hypophthalmus* did not require the air phase to cover its oxygen requirements either in a resting state or when swimming at maximum sustainable speed. On this basis, and as a result of superficial low magnification examination of the branchial SA, she argued that the gills of this species are unusually large for an air-breathing fish. In contrast to this, Phuong et al. (2017) showed using stereological methods that in the conditions of the Lefevre studies, *P. hypophthalmus* gills are in fact very small because the inter-lamella spaces are entirely filled with cell mass, presenting a difficult paradox of how branchial oxygen uptake can be achieved in such an active tropical fish. Gill SA are important in gas exchange, but examining gill SA alone is insufficient for evaluation of oxygen uptake capacity. The τ_h is equally important because diffusion capacity is proportional to the respiratory gill area and inversely proportional to τ_h (Hughes, 1972; Hughes and Morgan, 1973). These two parameters can be conveniently combined to calculate the ADF, which represents the anatomical component of the diffusion capacity of the particular respiratory SA (Perry, 1978). This allows an evaluation of the relative importance of the respiratory SA in this species under different conditions as well as a more informed comparison with other species. The gill SA in 27°C normoxic water in the

present study was strongly reduced by ILCM (Table 2), which is in agreement Phuong et al. (2017). However, both studies show that this species presents an unusually thin diffusion barrier between the water and the blood (Table 3). The result of this is that even with ILCM, the gills still present a relatively large ADF ($361.3 \text{ cm}^2\mu\text{m}^{-1}\text{kg}^{-1}$ for a 100 g fish), which is similar to active water breathers such as trout. When fully exposed by removal of ILCM, the gills show a higher ADF ($1002 \text{ cm}^2\mu\text{m}^{-1}\text{kg}^{-1}$ for a 100 g fish) than most water breathers and significantly higher than for other air-breathing fish (Table 3). In addition, at 27°C *P. hypophthalmus* haemoglobin has a rather strong oxygen affinity with a P_{50} of 4.6 torr (calculated from Damsgaard et al., 2015a). At higher oxygen demands, through elevated temperature or through swimming, *P. hypophthalmus* can increase the diffusion capacity of its branchial SA by reduction of ILCM. It should be noted when making this inter species comparison that the habitat temperature for pangasius is significantly higher than most species in this branchial ADF comparison. However, the present study strongly indicates that even with ILCM this species should probably be able to sustain a standard metabolic rate from the water alone. Should the need for more oxygen arise, for example as a result of exhaustive swimming as in Lefevre et al. (2013), the present study shows that the SA can change rapidly (Fig. 5). Hence, the paradox presented in this fish is resolved.

Relative importance of the ABO and the osmo-respiratory compromise

The ABO of a 100 g *P. hypophthalmus* accounts for between 50% of the animals total ADF, when gills are filled with ILCM, decreasing to $<25\%$ when the gills are fully exposed to the water (Table 3). Examination of data in table 3 reveals that this value is typical of the facultative air-breathing fish examined to date. Interestingly, while the ABO accounts for only 25-50% of the total, this organ can support this at the industrial scale production in deeply hypoxic Vietnamese growth ponds (Damsgaard et al., 2015b, supplementary data), although the available growth data from controlled laboratory environments show improved growth in normoxic water (Lefevre et al., 2014; Phuong et al., 2017). The ability to obtain oxygen from both aquatic and aerial source has surely conferred an adaptive advantage in organically rich tropical water where hypoxia is a regularly recurring event (Johansen, 1968; Johansen et al., 1970). However, the question remains as to why a fish reduces its branchial ADF in normoxic water. It has previously been argued that branchial reductions might serve to reduce branchial loss of oxygen obtained from the surface in hypoxic water (Randall et al., 1981), but in pangasius this does not seem to hold true with the largest branchial SA in high temperature hypoxia and the smallest in low temperature normoxia. Perhaps a more likely

explanation is that this fish provides positive evidence of the trade-off between the requirement for a thin respiratory epithelium to facilitate gas exchange whilst at the same time maximising the diffusion barrier for water and passive ion loss, termed the osmo-respiratory compromise (Wood and Randall, 1973a, 1973b; Nilsson, 1986; Gonzalez and McDonald, 1992, 1994; Nilsson, 2007). Reducing the respiratory SA by adding the ILCM barrier may help prevent ion loss or unwanted water uptake and therefore reduce ion-regulatory energy consumption. This is an area in need of further research as we could see no evidence of mitochondrial rich cells on the lamellae in the present study, though fluorescence microscopy will be necessary to confirm this. Also, the speed with which ILCM can be increased or reduced should be examined on a finer time scale and whether there are vascular shunts within the gills; or whether there is some other mechanism modulating the leakiness of lamellar epithelial membrane in this species.

Relationships between morphometrics of gills and body size

It has long been known that fish gill SA in relation to body mass show a good fit with the equation $Y=aW^b$. This relationship has been confirmed for numerous water-breathing fish and several air-breathing fish (summarized in Table 4). Much of the variation in the exponent (b values), has been argued to be correlated with the activity level of the species and its habitat choice (Gray, 1954; Hughes, 1966; Palzenberger and Pohla, 1992; Fernandes et al., 1994; Severi et al., 1997). In the present study, the slopes of the regression lines for the actual and potential respiratory lamellar area against body mass were 0.83 and 0.92, respectively. These values are within the range of 0.8 to 0.9 found in active water-breathing fish species, but significantly greater than found in other air-breathing fish, such as *Anabas testudineus* (0.615), *Saccobranchnus fossilis* (0.746), *Channa punctata* (0.592) (Table 4). The high value of the slopes in *P. hypophthalmus* also correlates with the active habits of this species with its strenuous migratory pattern between Laos and the Mekong river delta, a distance of more than 2000 Km (Zalinge et al., 2002; So et al., 2006).

It has previously been pointed out that the scaling relationship between respiratory SA and body size was similar to the relationship between oxygen consumption rates and body size in a variety of fish species, which log-log plot slopes also in the range of 0.8 to 0.9 (reviewed in Schmidt – Nielsen, 1984). However, it has been noted that these two slopes do not always match (Singh and Munshi, 1985). The argument for this mismatch has typically been that the functional SA changes dramatically with oxygen demand as a result of recruitment of lamellae (Jones and Randall, 1978; Booth, 1978; Farrell et al., 1980; Wood and Perry, 1985;

Perry and Wood, 1989). In addition to lamellar recruitment changing SA, it has also been shown that ILCM can change the functional SA. Thus, crucian carp (*Carassius carassius*) exposed to hypoxia in a closed respirometer at 20°C significantly reduced their ILCM within 6h (Sollid et al., 2005). This species also reduced ILCM volume by 65% within 8h of an increase in swimming speed (Brauner et al., 2011). Similarly, the closely related goldfish (*C. auratus*) lost 18% of ILCM within 30 min of exposure to hypoxia at 7°C (Tzaneva et al., 2011), and Fu et al. (2011) found that 48h exposure to hypoxia or sustained exercise (70% of U_{crit}) induced a significant increase in lamellar SA. Perry et al. (2012) found that while the gills of goldfish acclimated to low temperature (7°C) were filled by ILCM, the SA increased by 45% after approximately 3h of swimming by reducing the ILCM. Similarly in the present study we found that *P. hypophthalmus* at 27°C & normoxia have their gills partly embedded by ILCM, making their respiratory gill SA less 30% of their maximal area. Swimming the fish for 20h caused an increase in SA, with the effect most pronounced at 33°C where oxygen demand is highest (Fig. 5C). The mechanisms involved in these changes in ILCM are at present unclear but Nilsson et al. (2012) suggested two possible routes of ILCM removal: a slow route through increased apoptosis and decreased mitosis (Sollid et al., 2003) and a rapid route through simple shedding of ILCM cells into the water. Given the rapidity of the changes incurred during swimming in both *P. hypophthalmus* and goldfish it is likely that simple shedding of cells occurs during these urgent increases on oxygen demand.

It is clear that the main mediator of gill ADF is the oxygen demand of the fish. Swimming has a large effect on τ_h in our study, which might be expected through increased lamella perfusion causing distension of the lamellae (Sovio and Tuurala, 1981). The temperature change alone in our study had no effect on gill diffusion distance τ_h but did cause a minor significant increase in gill SA. This is in line with our previous findings in this species where the same temperature increase caused only a minor effect on SA unless combined with hypoxia (Phuong et al., 2017). In the eel (*Anguilla anguilla*), a larger temperature change (7 to 25°C) was associated with a halving of the diffusion distance τ_h (Tuurala et al., 1998). The combined oxygen demand caused by both swimming and elevated temperature then led to both distension and hence thinning of τ_h and a massive increase in SA as a result of shedding of the entire ILCM (Fig. 5). It would be interesting to further investigate the time scale of modulation of ILCM to determine how it might match the dynamics of a shifting oxygen environment (for example in river eddies) and the energetic needs of the animal. The role of ILCM in offsetting negative costs of osmo-respiratory

compromise has been discussed on numerous occasions (Nilsson et al., 2012; Perry et al., 2012; Phuong et al., 2017), but the costs of building and shedding of cell mass are presently unknown.

Air breathing organ and body size

The air-breathing organ volumes in the present study are between 1 to 8.5 % of fish volume, which is in the range found in other fish species where it has been quantified for its role in providing positive buoyancy (Alexander, 1959a,b). However, a note of caution should be raised here because of the difficulties of maintaining ABO volume during fixation. In the large fish (groups c-e; Table 2), the ABO was filled with fixative and the trachea tied off to prevent deformation of the lung type structure during fixation. It was not possible to tie the trachea in the two smallest groups (groups a,b; Table 2) hence their unusually low volumes may well be a result of some deformation during fixation. Thus the scaling coefficients for the ABO with body mass must be viewed with caution. Information on the relationship between ABO dimensions and body mass is available on very few fish species, but this has been extensively investigated in mammals and birds (reviewed in Schmidt-Nielsen, 1984). The regression line from the log-log plot of swim bladder respiratory SA against body mass gave a slope of 0.714, which is smaller than that for gill SA (0.83 and 0.92, respectively for actual and potential SA), but similar to the scaling of the mammalian lung (Schmidt-Nielsen, 1984).

Summary

P. hypophthalmus is in several respects unusual among air-breathing fish. It is a fast swimmer capable of the sustained exertion necessary for the >2000 Km upstream migration for spawning, in itself unusual in this group. Whereas, all other air-breathing fish examined previously have strongly reduced gills, this species has adopted the alternative of maintaining large gills with thin respiratory epithelia, instead modulating the SA using ILCM, while at the same time developing a large ABO through which it can sustain itself during periods of hypoxia. This combination is at present unique among air breathing fish. Pouyard et al. (2000) showed that speciation in the Pangasiidae is very recent, occurring in the mid Miocene, and it is tempting to speculate that the respiratory set up in *P. hypophthalmus* represents an early evolutionary step in the transition from water to air breathing.

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Competing interests

No competing interests declared.

Author contributions

MB, JRN and LMP designed the study. LMP performed all experiments and data analyses. LMP drafted the manuscript in close collaboration with MB, HM, JRN and DTTH.

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Figures

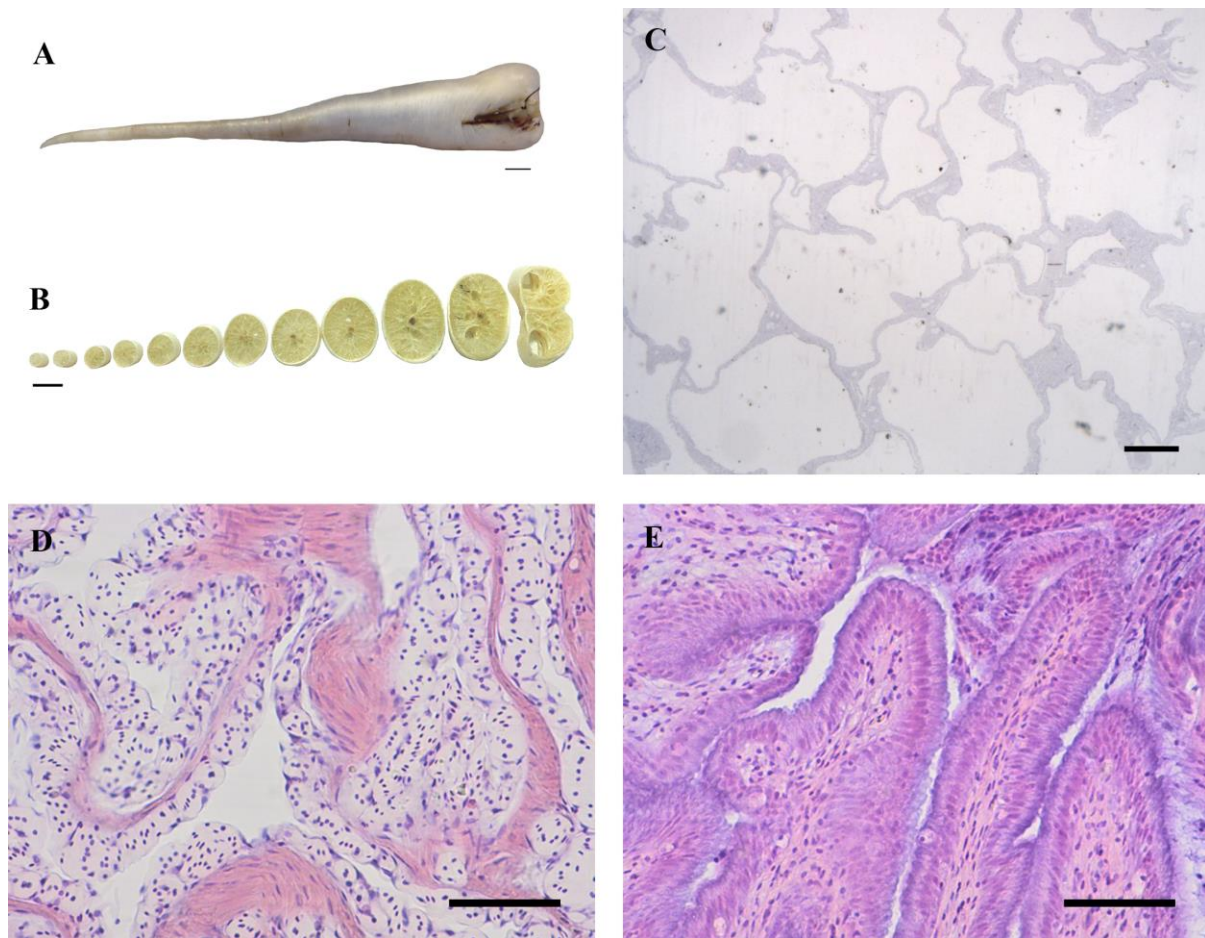


Fig.1. Swim bladder processed for stereology and histological sections of swim bladder of *Pangasianodon hypophthalmus*. (A) shows an entire fixed swim bladder; (B) shows swim bladder slices (5 mm thickness) prepared for SURS; (C) shows parenchyma of the swim bladder; (D) shows a highly vascularized respiratory epithelium and (E) shows a brush boarder epithelium. Scale bars shown for (A) and (B) are 1 cm, for (C) 500 μm , for (D) and (E) 50 μm .

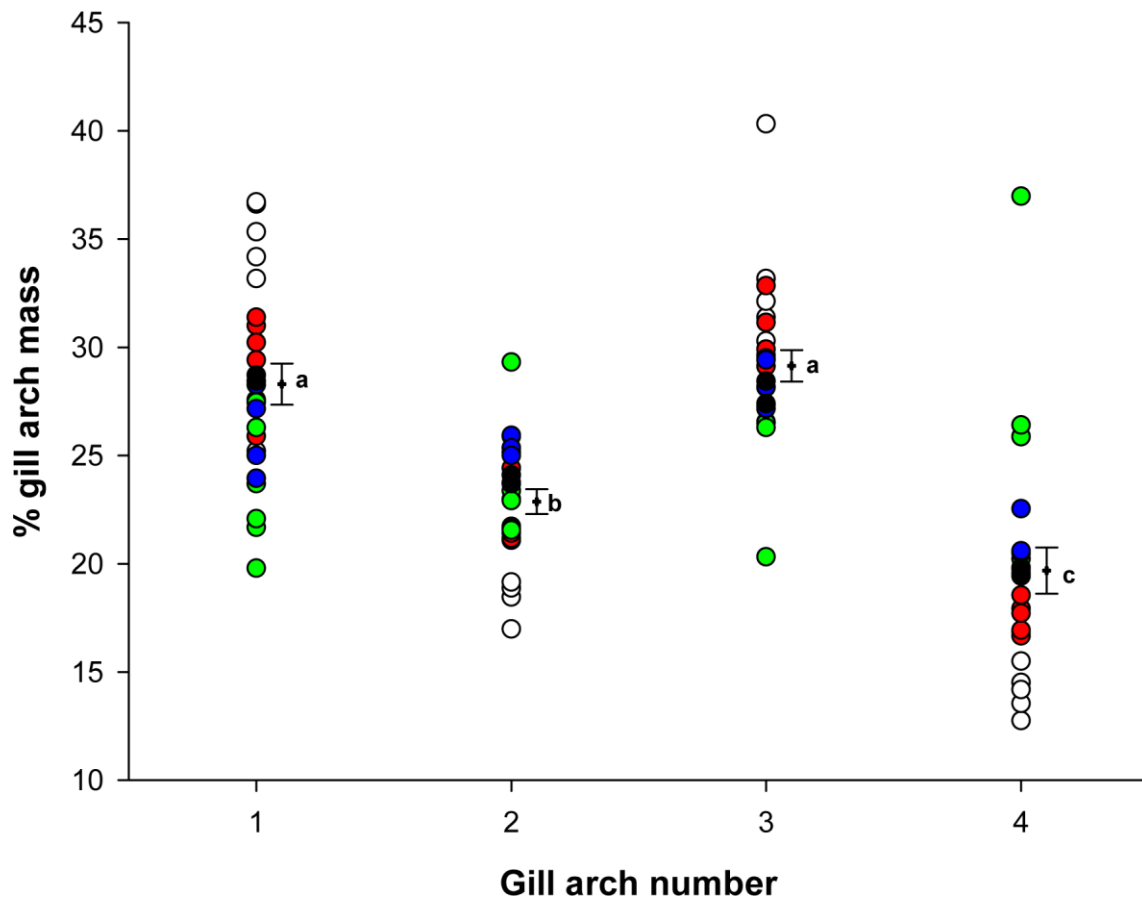


Fig.2. Relative mass of the 4 gill arches expressed as percentage of total. The four gill arches were broadly similar in size, although arches 2 and 4 were significantly smaller than 1 and 3. Mean and s.e.m. for each arch are shown next to the individual data from different size groups. Different letters indicate significant differences. Empty circles 3-5g fish, Red circles 20-30g, Green circles 200-300g, Blue circles 600-800g, and Black circles 1.6-1.9 Kg.

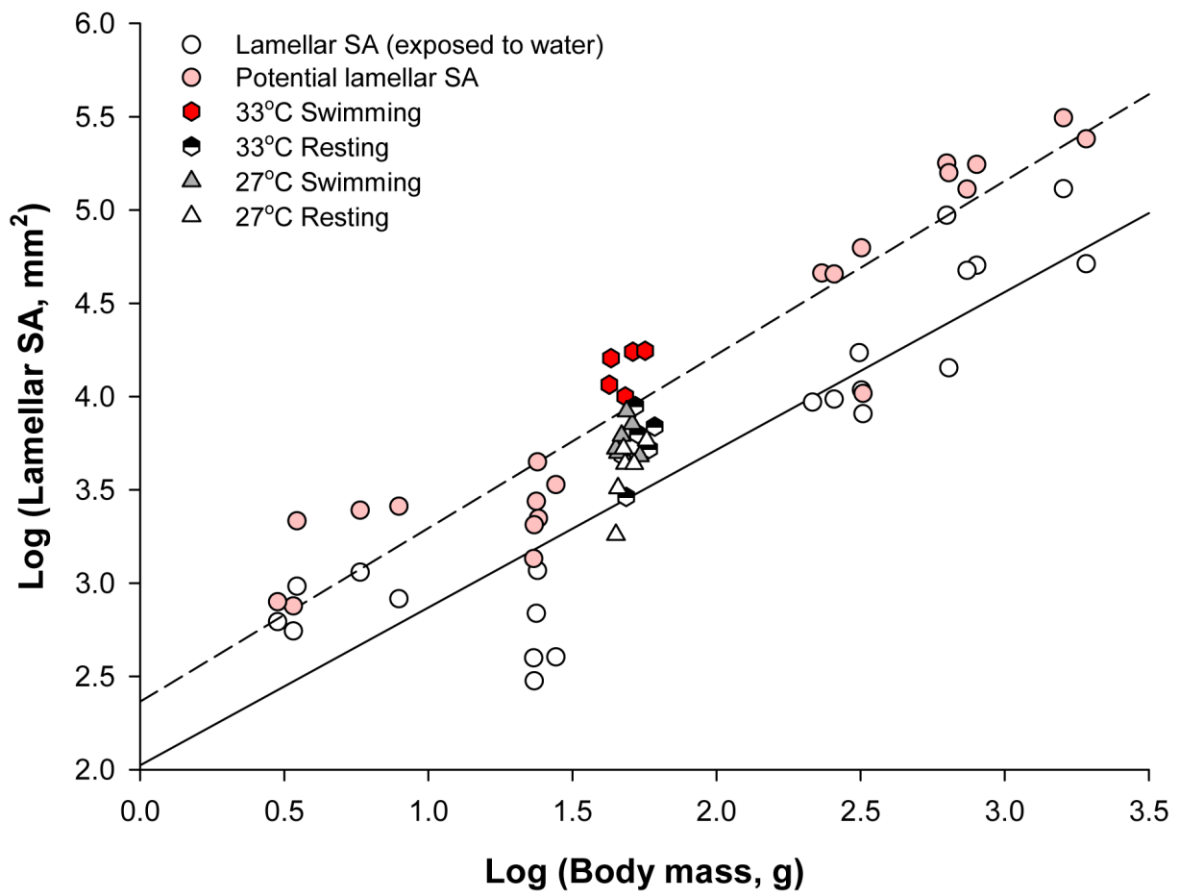


Fig.3. Bilogarithmic plots of lamellar SA in relation to body mass of *Pangasianodon hypophthalmus*. The solid regression line shows the relationship between the respiratory lamellar SA (parts of lamellae exposed to water) and body mass, with $Y = 0.833X + 1.999$ and $R^2 = 0.776$; the dashed regression line shows the relationships between the potential respiratory lamellar SA and body mass, with $Y = 0.919X + 2.333$ and $R^2 = 0.873$.

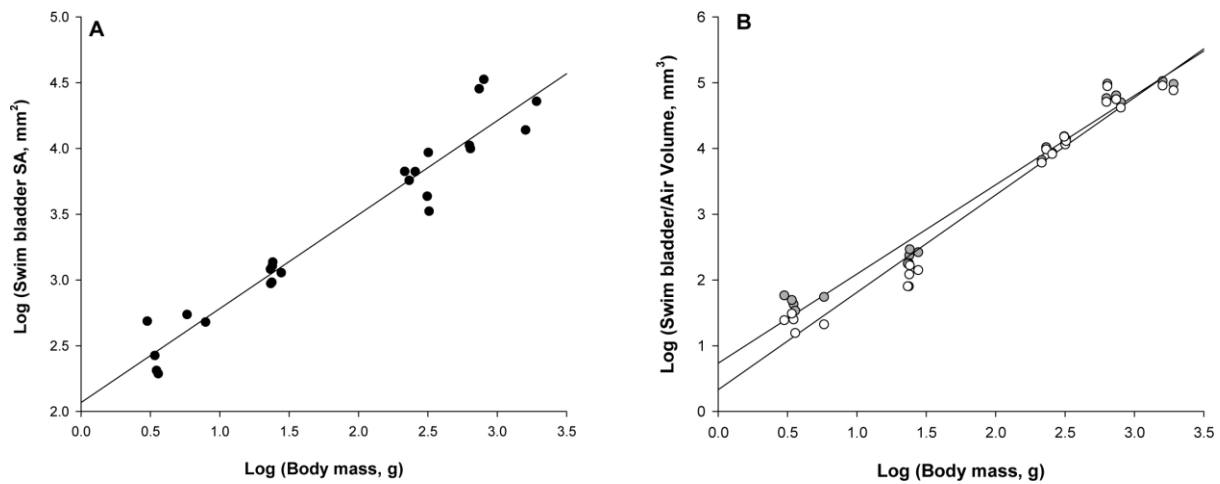


Fig.4. Bilogarithmic plots of swim bladder dimensions in relation to body mass of *Pangasianodon hypophthalmus*. (A) shows the relationship between swim bladder respiratory SA and body mass, with the equation of regression line being $Y = 0.714X + 2.069$ and $R^2 = 0.940$; (B) shows the relationships between total swim bladder volume (grey circles) and air-space volume in swim bladder (white circles) against body mass, with the equations of regression lines $Y = 1.357X + 0.736$ and $R^2 = 0.973$, and $Y = 1.482X + 0.331$ and $R^2 = 0.967$, respectively.

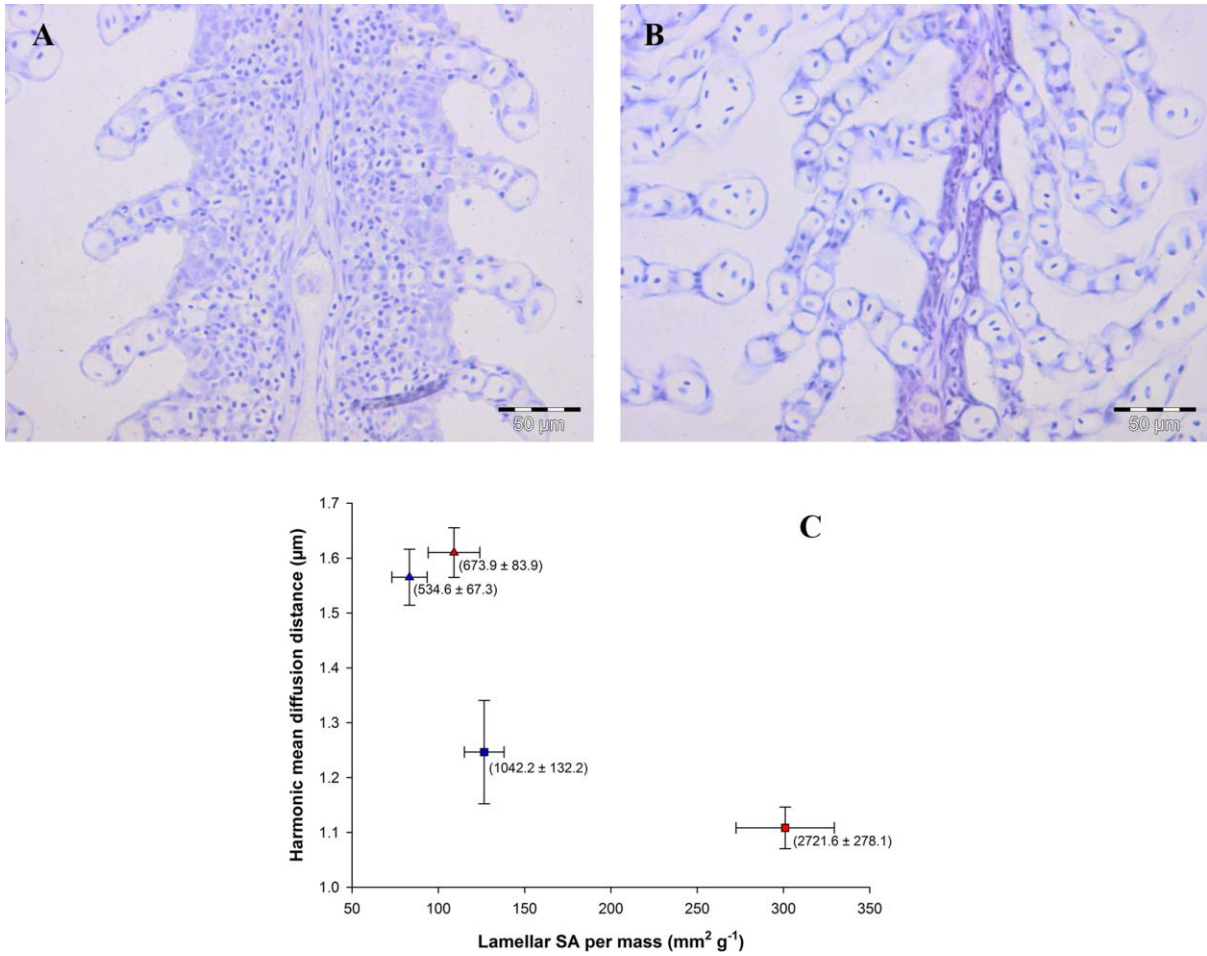


Fig.5. Histological cross sections of gill filaments of *Pangasianodon hypophthalmus*. (A) 27°C before swimming; (B) 33°C fish after swimming; (C) Respiratory lamellar SA and τ_h of *P. hypophthalmus* induced by different temperature and swimming conditions: 27°C Resting (Blue triangle), 33°C Resting (Red triangle), 27°C Swimming (Blue square), 33°C Swimming (Red square). Numbers brackets indicate associated gill ADF values ($\text{cm}^2 \mu\text{m}^{-1} \text{kg}^{-1}$). n=6 in all groups.

Tables

Table 1. Relationship between body mass (M_b , g) and morphometrics (Y) of respiratory organs of *Pangasianodon hypophthalmus* according to the allometric equation $Y = aM_b^b$.

Morphometric parameters (Y)		Equation	Correlation coefficient (r)
<i>Gills</i>			
Respiratory lamellar SA	mm ²	$Y=99.98M_b^{0.833}$	0.78
Potential respiratory lamellar SA	mm ²	$Y=215.43M_b^{0.919}$	0.87
Gill filament volume	mm ³	$Y=6.39M_b^{1.0416}$	0.94
Gill filament mass from the 1 st gill arch	g	$Y=0.0016M_b^{1.097}$	0.98
Gill filament mass from the 2 nd gill arch	g	$Y=0.0013M_b^{1.098}$	0.99
Gill filament mass from the 3 rd gill arch	g	$Y=0.0016M_b^{1.098}$	0.99
Gill filament mass from the 4 th gill arch	g	$Y=0.0012M_b^{1.068}$	0.99
<i>Swim bladder</i>			
Respiratory swim bladder SA	mm ²	$Y=117.17M_b^{0.714}$	0.94
Volume of swim bladder	mm ³	$Y=5.441M_b^{1.357}$	0.97
Volume of air in swim bladder	mm ³	$Y=2.141M_b^{1.482}$	0.97
Volume of capillary bed	mm ³	$Y=1.924M_b^{0.7192}$	0.90
Swim bladder length	mm	$Y=6.99M_b^{0.496}$	0.98

Table 2. Total respiratory SA of lamellae (actual and potential) and swim bladder ($\text{mm}^2 \text{g}^{-1}$), τ_h (μm), total volumes of gill filaments and of swim bladder ($\text{mm}^3 \text{g}^{-1}$); percentages of lamellar volume and air volume in swim bladders at different developmental stages of *Pangasianodon hypophthalmus*. Data are presented as mean \pm s.e.m.

Groups	Body Mass (g)	Gills							Swim bladder						
		Respiratory Lamellar SA/mass ($\text{mm}^2 \text{g}^{-1}$)	Potential Respiratory Lamellar SA/mass ($\text{mm}^2 \text{g}^{-1}$)	τ_h (μm)	CV	Gill Filament volume/mass ($\text{mm}^3 \text{g}^{-1}$)	Mean CE (%)	% Secondary lamellae Volume /Gill filament Volume	Respiratory SA/mass ($\text{mm}^2 \text{g}^{-1}$)	τ_h (μm)	CV	Σ SB Volume /mass ($\text{mm}^3 \text{g}^{-1}$)	%SB volume /mass	Mean CE (%)	% Air Volume / Σ SB volume
A (n=6)	4.5 \pm 0.8	201.2 \pm 32.1	391.4 \pm 71.6	2.10 \pm 0.14	0.15	10.0 \pm 1.6	0.25	15.2 \pm 2.1	84.5 \pm 16.7	1.17 \pm 0.10	0.21	13.0 \pm 1.9	1.1 \pm 0.3	0.01	49.4 \pm 4.7
B (n=6)	24.3 \pm 0.7	20.0 \pm 7.4	87.5 \pm 20.8	2.16 \pm 0.07	0.08	6.3 \pm 1.3	0.08	2.2 \pm 0.6	47.3 \pm 3.1	0.82 \pm 0.03	0.08	9.3 \pm 0.8	1.0 \pm 0.1	0.01	50.3 \pm 2.4
C (n=5)	276 \pm 19	30.2 \pm 5.7	113.9 \pm 28.0	2.11 \pm 0.08	0.10	5.2 \pm 0.9	0.05	8.5 \pm 1.8	22.5 \pm 3.5	0.72 \pm 0.01	0.03	40 \pm 2.8	4.0 \pm 0.3	0.01	94.2 \pm 1
D (n=4)	703 \pm 41	73.3 \pm 27.1	221.2 \pm 21.8	1.53 \pm 0.20	0.26	12.2 \pm 2.2	0.01	11.0 \pm 1.0	28.1 \pm 7.0	1.85 \pm 0.52	0.57	84.6 \pm 14.7	8.5 \pm 1.5	0.03	87.5 \pm 1.7
E (n=2)	1760 \pm 160	47.4 \pm 24.0	140.0 \pm 31.1	1.65 \pm 0.37	0.32	15.8 \pm 2.7	0.01	5.7 \pm 3.1	10.2 \pm 1.6	2.26 \pm 0.45	0.28	57.8 \pm 8.1	5.8 \pm 0.8	0.01	82.7 \pm 2.7

Table 3. Anatomic diffusion factor (ADF) of gills and ABO of different fish species at the same size (100 g)

Species (100g fish)	Gills			ABO				References
	τ_h (μm)	ADF ($\text{cm}^2\mu\text{m}^{-1}\text{kg}^{-1}$)	% of total ADF	Type	τ_h (μm)	ADF ($\text{cm}^2\mu\text{m}^{-1}\text{kg}^{-1}$)	% of total ADF	
<i>Chaenocephalus aceratus</i>	6	200.00	100.00					Hughes (1972)
<i>Hoplias malabaricus</i>	3.16	759.49	100.00					Fernandes et al. (1994)
<i>Oncorhynchus mykiss</i>	6	400.00	100.00					Hughes (1972)
<i>Tinca tinca</i>	3	833.33	100.00					Hughes (1972)
<i>Anabas testudineus</i>	10	47.20	2.43	Sc	0.21	371.43	19.12	Hughes et al. (1973)
				Lo	0.21	1523.81	78.45	
<i>Arapaima gigas</i>	9.61	81.17	0.82	Sb	0.22	9863.64	99.18	Fernandes et al. (2012)
<i>Channa punctatus</i>	2.03	354.68	41.37	As	0.78	502.56	58.63	Hughes and Munshi (1973)
<i>Boleophthalmus boddarti</i>	1.43	517.48	90.80	Oc	1.22	52.46	9.20	Niva and Ojha (1981)
<i>Heteropneustes fossilis</i>	3.58	156.42	42.44	Sk	98	20.27	5.50	Hughes and Munshi (1973)
				As	1.6	191.88	52.06	
<i>Lepidosiren paradoxa</i>	80	0.05	0.00	L	1.56	3357.05	99.80	de Moraes et al. (2005)
				Sk	161.7	6.83	0.20	
<i>Pangasianodon hypophthalmus (Actual)</i>	1.97	361.27	53.97	Sb	1.35	308.10	46.03	Present study
<i>P. hypophthalmus (Potential)</i>	1.97	1002.38	76.49*	Sb	1.35	308.10	23.51*	Present study

As air sac; Sk skin; Sc suprabranchial chamber; Sb swim bladder; L lung; St stomach; Oc opercular chamber; Gf gill fans; Ao Arborescent organ; Lo labyrinth organ

* ADF of swim bladder was assumed not to be changed.

Table 4. Results of regression analysis from dimensions of respiratory organs (Y) in relation to body mass (M_b) in different fish species

Fish species	Respiratory mode	Gill SA (Y=aM _b ^b)	Type of ABO	ABO (SA)	ABO (Volume)	References
<i>Thunnus albacares</i>	w	3151M _b ^{0.875}				Muir and Hughes (1969)
<i>Coryphaena hippurus</i>	w	5208M _b ^{0.713}				Hughes (1969)
<i>Scomber scombrus</i>	w	424.1M _b ^{0.997}				Hughes (1970)
<i>Salmo gairdneri</i>	w	314.8M _b ^{0.932}				Hughes (1980)
<i>Tinca tinca</i>	w	867.2M _b ^{0.698}				Hughes (1970)
<i>Opsanus tau</i>	w	560.7M _b ^{0.790}				Hughes (1969)
<i>Scyliorhinus canicula</i>	w	262.3M _b ^{0.961}				Hughes (1970)
<i>Torpedo marmorata</i>	w	117.5M _b ^{0.937}				Hughes (1978)
<i>Blennius pholis</i>	w	424.1M _b ^{0.850}				Milton (1971)
<i>Piaractus mesopotamicus</i>	w	565.04M _b ^{0.769}				Severi et al 1997
<i>Thunnus thynnus</i>	w	2443.43M _b ^{0.901}				De Jager and Dekkers (1975)
<i>Hoplias malabaricus</i>	w	125.1M _b ^{1.140}				Fernandes et al. (1994)
<i>Micropterus dolomieu</i>	w	731.14M _b ^{0.820}				De Jager and Dekkers (1975)
<i>Oncorhynchus mykiss</i>	w	389.94M _b ^{0.900}				De Jager and Dekkers (1975)
<i>Hoplias lacerdae</i>	w	491.7M _b ^{0.810}				Fernandes et al. (1994)
<i>Botia lohachata</i>	w	913.3M _b ^{0.699}				Sharma et al. (1982)
<i>Oreochromis niloticus</i>	w	515.16M _b ^{0.750}				Fernandes and Rantin (1986)
<i>Ictalurus nebulosus</i>	w	264.85M _b ^{0.845}				De Jager and Dekkers (1975)
<i>Catostomus commersonii</i>	w	797.99M _b ^{0.639}				De Jager and Dekkers (1975)
<i>Macrogathus aculeatum</i>	w	217.3M _b ^{0.733}				Ojha and Munshi (1974)
<i>Amphipnous cuchia</i>	w	888.4M _b ^{0.709}				Hughes et al. (1974)
<i>Anabas testudineus</i>	a	556M _b ^{0.615}	-	-	-	Hughes and Munshi (1973)
<i>Channa punctata</i>	a	470.4M _b ^{0.592}	Sc	1.591M _b ^{0.696}	0.024M _b ^{0.855}	Hakim et al. (1978)
<i>Saccobranchus fossilis</i>	a	186.1M _b ^{0.746}	As	145M _b ^{0.662}	0.004M _b ^{0.999}	Hughes et al. (1974)
			Sk	851.1M _b ^{0.684}		
<i>Pangasianodon hypophthalmus</i>	a	99.98M _b ^{0.833}	Sb	117.17M _b ^{0.714}	5.441M _b ^{1.357}	Present study
<i>P. hypophthalmus</i> (potential)	a	215.43M _b ^{0.920}				

Sc suprabranchial chamber; As air sac; Sk skin; Sb swim bladder; w water breathing; a air-breathing

Supplementary Material

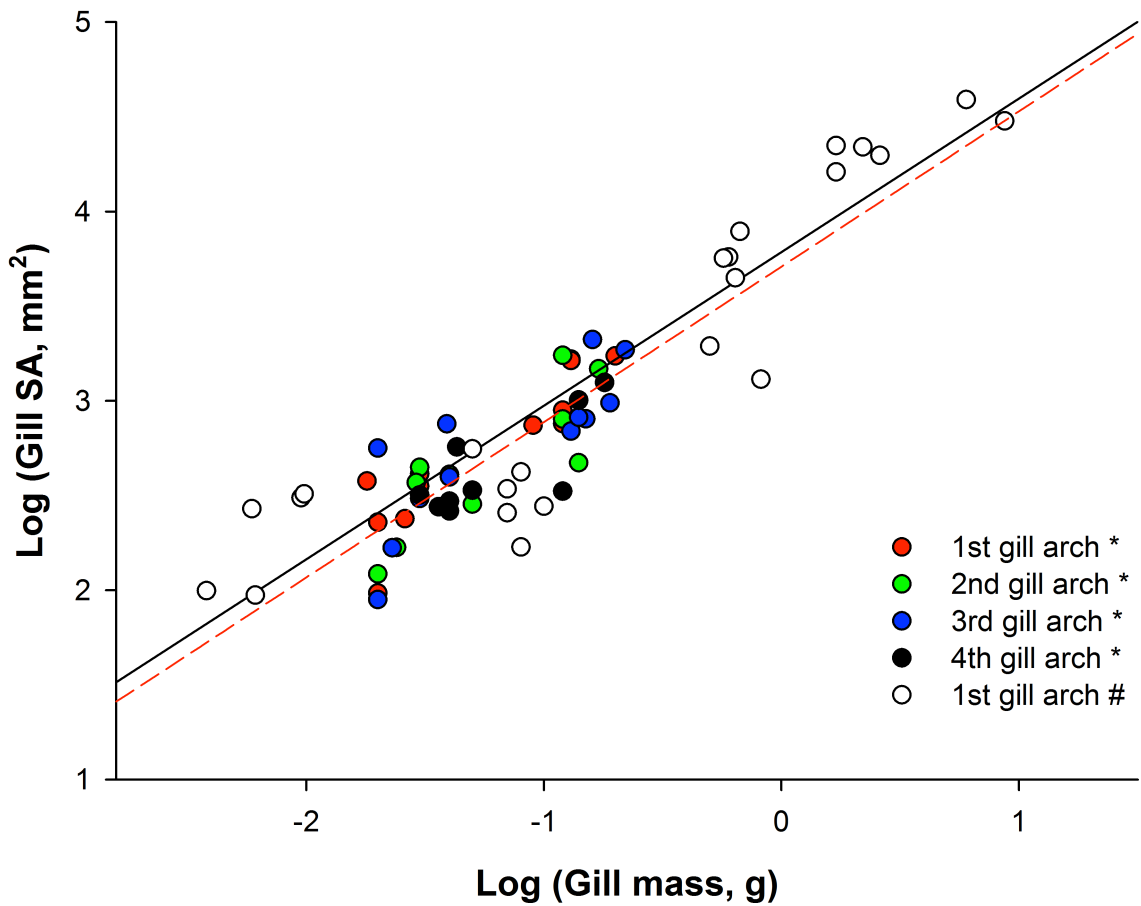


Fig. S1. Bilogarithmic plots of lamellar SA in relation to gill arch mass of *Pangasianodon hypophthalmus*. * indicates data from Phuong et al. (2017) of fish with no ILCM; # indicates potential lamella SA from first gill arch in this study. The equation for the regression line (Black line) for the whole data set is $Y=0.834X + 3.75$ and $R^2=0.845$. For the data from Phuong *et al.*, (2017) the regression line (red dashed line) is given by $Y=0.820X+3.71$ and $R^2=0.705$.