Morphometric Partioning of the Respiratory Surface Area and Diffusion Capacity of Gills in an Air Breathing Fish *Channa Gachua*

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Abstract

The gills and the respiratory swim bladders of juvenile specimens (mean body mass 100 g) of the teleost *Channa gachua* were evaluated using stereological methods in vertical sections. The surface areas, harmonic mean barrier thicknesses and morphometric diffusing capacities for oxygen and carbon dioxide were estimated. The average respiratory surface area of the swim bladder (2173 cm^2 / kg) exceeded that of the gills (780 cm^2 /kg) by a factor of 2.79. Due to the extremely thin air blood barrier in the swim bladder (harmonic mean 0.22 m) and the much thicker water blood barrier of the gills (9.61 m) the morphometric diffusing capacity for oxygen and carbon dioxide was 88 times greater in the swim bladder than in the gills. These data clearly indicate the importance of the swim bladder, even in juvenile *Channa gachua* that still engage in aquatic respiration. Because of the much greater diffusion constant of carbon dioxide than oxygen in water, the gills also remain important for carbon dioxide release.

Keywords: Bimodal respiration, surface area, water air blood barrier, thickness *Channa gachua.*

Introduction

Bimodal breathing involve dual mode of oxygen uptake from the water by gills and from the air by an air breathing organ which has evolved among teleost fishes. Within bimodal breathers, amphibious species which can breathe in or out of water can be distinguished from aquatic species; can breath while remaining in the water (Graham, 1997 and Qaisur, 2011). In addition obligate air breathers who are dependent on aerial gas exchange can be differentiated from facultative air breathers who only supplement their oxygen needs by air breathing (Hughes *et. al.,* 1974; Fernandes, 1996; Perna and Fernandes, 1996; Santos *et. al.,* 1994; Mazon *et. al.,* 1998; Moraes *et. al.,* 2005; Fernandes *et al.,* 2007; Cruz *et al.,* 2009). The accessory air breathing organs are located in the region of the head consisting of buccal and pharynx epithelia, pharyngeal pouches, modified branchial and opercular surfaces (Munshi, 1985) structures localized in the digestive tract (stomach and intestinal tract) (Carter and Beadle, 1931; Gee and Graham, 1978; Silva *et. al.,* 1997) and skin (Banerjee and Mittal, 1976; Bicudo and Johansen, 1979; Moraes *et. al.,* 2005). The teleost *Channa gachua* commonly referred to as the "garai" is an aquatic obligate air breather that uses its swim bladder to breathe atmospheric air. Its gills exhibit pronounced changes as the fish matures. Small fish have gill filaments with well defined lamellae (Brauner *et. al.,* 2004; Costa *et. al.,* 2007), while large fish have column shaped filaments that appear to have a smooth surface (Brauner *et. al.,* 2004). The *Channa gachua* swim bladder is highly vascularized and adapted for aerial respiration (Graham, 1997). Early studies reported that in 1-3 kg fish between 75% and 95% of the total oxygen uptake was derived from the air (Stevens and Holeton, 1978). Brauner and Val (1996) confirmed these values for 1.7 kg fish, showing that 79% of their total excreted CO_2 was processed through the gills. Juvenile (10–100 g) *Channa gachua* are dependent on aerial respiration than the adults (Brauner *et al.,* 2004; Brauner and Val, 2005). Recently oxygen uptake and CO_2 excretion measurements in small (67 g) and large (724 g) showed that the specific oxygen uptake rate ($molO_2$/ g/ h) of small fish is 0968-4328 matter two thirds that of the large ones, and the water fraction of oxygen uptake and

CO_2 excretion is lower in small individuals, despite the drastic morphological changes in the gills as the fish matures (Gonzalez *et. al.,* 2010). Physiological studies have documented the bimodal gas exchange in *Channa gachua* (Brauner and Val, 1996; Brauner *et. al.,* 2004; Gonzalez *et. al.,* 2010). However, morphological data on respiratory organs are scarce. Recently, the gill surface area of small *Channa gachua* was estimated to be similar to sluggish water breathing and facultative air breathing fish (Costa *et. al.,* 2007), but no data on swim bladder surface area are currently available. The present study delivers morphometrical data for the gas exchange organs of fish at a critical stage in their development where they are obligate air breathers but still engage in aquatic respiration. The main goal of the present study was to quantify the relative morphological adaptation of gill and swim bladder partitioning for gas exchange in juvenile *Channa gachua* .To this end the surface area, diffusion barrier thickness and the morphometric diffusion capacity for oxygen and CO_2 of the gills and the swim bladder were estimated using the same stereological methods in vertical sections, thus ensuring a comparison that is free of methodological differences.

Materials and Methods

Live specimens of *Channa gachua* were procured from local fish dealers at Hazaribag (Latitude 25º 59'N and Longitude 85º 22'E) and maintained in large glass aquaria size (90x60x60cm) with continuous flow of water. The specimens were fed on chopped goat liver daily during a minimum acclimation period of 15 days in the laboratory. Six juvenile *Channa gachua* (body mass (MB) = 85–110 g; mean = 100 ± 9 g; body length (LB) = 20–26 cm; mean = 24 ± 2 cm) were used in the present investigation. The gills were immediately removed and fixed by immersion in 2.5% phosphate buffered glutaraldehyde solution with a pH of 7.8 and an osmolality of approximately 300 mosmol. The specimen was then opened ventrally, and the swim bladder was exposed and fixed with fixative solution. Next, the entire fish was immersed in the above mentioned fixative at 4^{o}C. Sampling and processing for light (LM) and transmission electron (TEM) microscopy. The sampling and embedding procedures were designed to combine the Cavalieri principle for determining the reference volume with the stereological vertical sectioning method for measuring surface area, as described by Moraes *et. al., (*2005) for the swim bladder and by Costa *et al.,* (2007) for gills. Briefly, the rakers and bone of the epibranchial and cerato branchial elements of each gill arch were removed in the way that the gill filaments from anterior and posterior hemibranchs were kept attached in the gill arch tissue. The epi and ceratobranchial portions were separated, and the latter portion was cut in half yielding three samples from each gill arch. The samples were dehydrated by graded ethanol series and embedded in historesin. Random numbers were assigned to the samples and then the samples were placed with the opercular side (horizontal plane) down. The samples were embedded in methacrylate in stacks of 3 one atop the other with each sample rotated sequentially ± 15^{o}C relative to the previous one around the vertical axis. Historesin was used as the embedding methacrylate due to the negligible shrinkage of section and the fish tissue (Cruz *et al.,* 2009 b). The entire gill from one side of the animal was contained in four blocks, properly oriented for stereological vertical sectioning. Ten equidistantly spaced vertical sections of a 3 µm thickness that had been stained with toluidine blue and acid fuchsin were used to estimate the gill volume using the Cavalieri method as well as the surface area in vertical uniform random (VUR) sections (Michel and Cruz Orive, 1988). Surface area and volume of the gills were determined using stereological point and intersection counting methods (Howard and Reed, 2007; Costa *et. al.,* 2007) under a BX51 Olympus microscope with a 20×/0.80 oil immersion lens. A new angle was selected at random for each histological section. The harmonic mean thickness of the air blood barrier was then measured as 2/3 the harmonic mean intercept length (Weibel and Knight, 1964). The fixed swim bladder was removed and transected by 10 equidistantly spaced slices, beginning at a random starting point within the first interval (Moraes *et al.,* 2005). A square lattice grid was placed on the anterior surface of each section and point counts of the projected surfaces of the parenchyma, central lumen and ventral membrane

were performed. The volume of these three components was estimated using the Cavalieri method (Michel and Cruz-Orive, 1988), and 10 tissue samples from the parenchyma of each fish were then taken by systematic random sampling and processed for light microcopy to estimate the differential tissue volume. The samples were rotated ± 18^0 C relative to the previous one and embedded in historesin (Leica) with the adventitial side down, defining the horizontal plane. Tissue was sectioned vertically (3µm thickness) and stained with toluidine blue and acid fuchsin. To measure differential tissue volumes and the respiratory surface area, stereological point and intersection counting methods were employed using the CAST System software. To measure the air blood distance, 10 systematic random samples were taken from the posterior surface of swim bladder slices of each fish and processed for transmission electron microscopy. The samples were embedded in epon 812 (EMS, Hatfield, USA) sectioned at 60 nm in thickness and contrasted on 300 mesh grids using standard uranyl acetate and lead citrate procedures. The exact magnification was calculated for each series of electron micrographs with the aid of a calibration grid. The intersections were used as starting points to estimate the air blood diffusion distance (Fig. 4A). The harmonic mean thickness of the air blood barrier was measured as 2/3 the harmonic mean intercept length (Weibel and Knight, 1964). Anatomical diffusion factor and the morphometric diffusion capacity of the water blood or air blood barrier. The morphometric diffusion capacity (Dm) was calculated as the product of the ADF and the weighted mean of Krogh's diffusion coefficient (K) for the respective cell layers (epithelium/endothelium/pillar cells) or basal membrane/connective tissue (for gills and swim bladder) of the diffusion barrier. The volume proportion of the different components was estimated separately by point counting (Fig. 3B). Each gill or swim bladder element was multiplied by the appropriate K value and this weighed numerical ratio yielded a K value for oxygen (KO_2) or CO_2 (KCO_2) in the water (air) blood barrier corrected to 25 ^oC. The relative and absolute variables were first calculated for each animal (Howard and Reed, 2007) to determine the precision of the estimates of volume, area and barrier thickness. To evaluate the variability between animals, the mean values were accompanied by the respective standard errors (SEM) for the gills and swim bladder (Howard and Reed, 2007).

Results

The gills of *Channa gachua* had the same basic structure as those seen in most teleost fish four gill arches, each bearing two rows of filaments. Lamellae, the gas exchange units of the gills, projected from both sides of the filaments. The lamellae consisted of the pillar cell system covered by basement membrane and two or three epithelial cell layers. In general, the cells of the inner most epithelial cell layer were flat and those of the outermost cell layer were cuboidal. Mucous and chloride cells were distributed throughout the filament epithelium, which was stratified and contained in 5–7 cell layers. The swim bladder lay ventral to the vertebral column and was fused to dorsal muscles of the body wall and ribs. It extended the full length of the body cavity from the posterior part of the head and kidneys to the posterior end of the intestine. The trunk kidneys projected dorsally as a median ridge into the lumen of the swim bladder. The highly vascularized, dorsolateral wall of *Channa gachua* swim bladder constituted the parenchymal layer that also enveloped the kidneys. The parenchyma was contiguous with a tough, translucent membrane that delimited the central lumen of the organ ventrally. This membrane contained some capillaries close to the mucosal surface, and their potential respiratory function could not be completely disregarded. The parenchyma was irregularly subdivided by numerous septa to form compartments (ediculae) of highly variable size that were supported by smooth muscular trabeculae, which consisted of a connective tissue matrix with numerous large and small blood vessels. The parenchyma tissue consisted of numerous small vessels and a dense capillary layer just below the respiratory epithelium. The respiratory epithelium covered all surfaces of the ediculae and consisted of pavement and columnar epithelial cells. The pavement cells had a low number of mitochondria very small lamellar bodies and, in general were underlined by the capillaries and separated from

them by the basal lamina. The apical surface of pavement cells was smooth with short microvilli distributed at the cell border. Columnar cells were distributed among the pavement cells. They were characterized by microvilli at the cell surface and numerous mitochondria and lamellar bodies of different sizes. The lamellar bodies reached up to 1.5 um in diameter. Tight junctions characterized the junction complex between pavement and columnar cells. Numerous neutrophils were found in the capillaries and blood vessels. The parenchyma tissue was rich in collagen fibers consisting of a layer between the capillaries and the small and large blood vessels in the inner tissue. The air blood barrier was composed of the endothelial cells of capillaries (23%), basal lamina of the swim bladder epithelium (24%) and the pavement epithelial cells themselves (57%) respectively.The gills of juvenile *Channa gachua* had a volume of 7.5 ± 0.3 cm^3 including rakers, epi and ceratobranchial bones, filaments and lamella. Most of total gill volume (80%) consisted of the long rakers and branchial bones. The gill filaments made up only 15% of the total gill volume, while 5% was ascribed to the lamellae (3.6% epithelium, 1.1% blood spaces and 0.3% pillar cells. The swim bladder length was approximately 0.7 of the total body length with a total volume of 9.5 ± 0.3 cm^3. Most of this volume (7.5 cm^3) represented the air in the central lumen and correspond to 78.6% of total swim bladder volume. The volume of ventral membrane and the parenchyma of swim bladder were 0.5 cm^3 and 1.6 cm^3 which corresponded to 5.4% and 16% of the total swim bladder volume, respectively. The tissue of the respiratory portion of the swim bladder (parenchyma excluding the trabeculae and trabecula air) consisted of 0.1% epithelium, 1.7% capillaries and 1.8% large vessels and approximately 4.4% of other tissues, such as connective tissue and nerves. The mean respiratory surface area of the protruded lamella (portion of the lamellae that contacts water) was 780.21 ± 31.23 $cm^2\ kg^{-1}$ ranging from 690.06 to 880.96 $cm^2\ kg^{-1}$. This represented approximately 70% of filament and lamellar surface areas taken together. The potential respiratory surface area of the lamellae was 5.1 times greater than the filament surface area. The surface area of the ediculae tissue of swim bladder was 2173.05 ± 118.89 cm^2 kg−1. Of this area, 1912.39 $cm^2\ kg^{-1}$ (94%) was potentially respiratory, while 167.97 $cm^2\ kg^{-1}$ (6%) was not potentially respiratory. The latter consisted of large trabeculae that lacked capillaries under the epithelium. Thus, the potentially respiratory surface area of parenchyma of swim bladder was 2.8 times greater than that of gill lamellae in these juvenile specimens. The gill lamellar epithelium had an arithmetic mean thickness of 18.15 ± 1.34 um and ranged from 14.76 to 22.72 µm. The harmonic mean was 9.61 (range 8.1–12.2 µm). The gas exchange barrier of the swim bladder had an arithmetic mean of 1.56 ± 0.44 µm with a range of 1.08–2.71 µm, and its harmonic mean was 0.22 um, ranging from 0.16 to 0.43 µm. In the large trabeculae regions the harmonic mean is up to 5–7 um higher as there are not capillaries just under the epithelium. The anatomical diffusion factor (ADF) of the swim bladder (8650.74 ± 752.16 cm^2 µm−1 kg^{-1}) was thus 107.3 times greater than that of the gills (80.65 $cm^2\ um^{-1}\ kg^{-1}$. calculated as the mean of the ADF values for the individual fish. The morphometric diffusion capacity for oxygen of gills was 0.021 ± 0.002 cm3 $min^{-1}\ mmHg^{-1}\ kg^{-1}$, while that of the swim bladder was 1.86 ± 0.19 ($cm^3\ min^{-}1\ mmHg^{-1}\ kg^{-1}$). The morphometric diffusion capacity for CO_2 was 0.403 ± 0.035 and 35.545 ± 3.69 $cm^3\ min^{-1}\ mmHg^{-1}\ kg^{-1}$ for the gill lamella and the swim bladder, respectively. The morphometric DO_2 and DCO_2 values of the swim bladder were approximately 88 times greater than those for the gills.

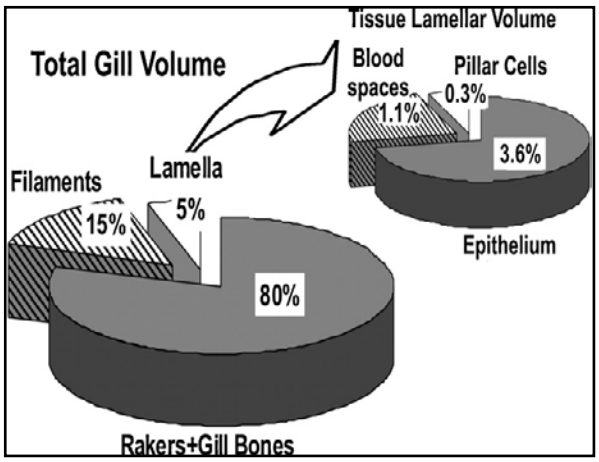

Fig. 1. ***Channa gachua*** **gills, Percentage volumes of the gill structural elements including rakers, gill arch bones, filaments and lamellae.**

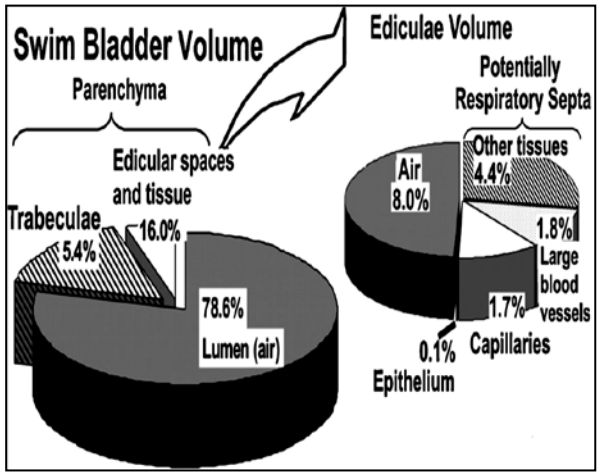

Fig. 2. ***Channa gachua*** **swim bladder, Volume percentages of elements in the entire swim bladder.**

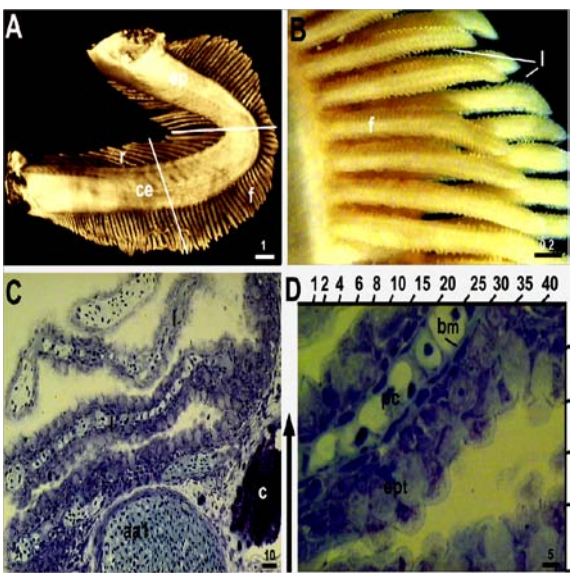

Fig. 3. ***Channa gachua*** **representation of gill arches**

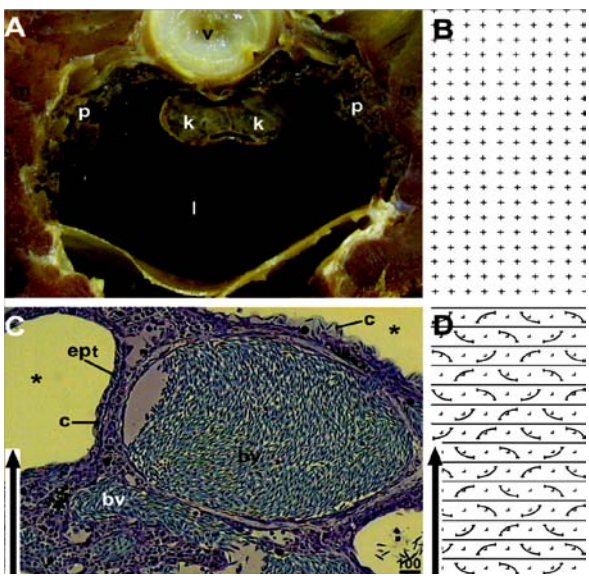

Fig. 4. ***Channa gachua*** **swim bladder Cross section of swim bladder showing the parenchyma**

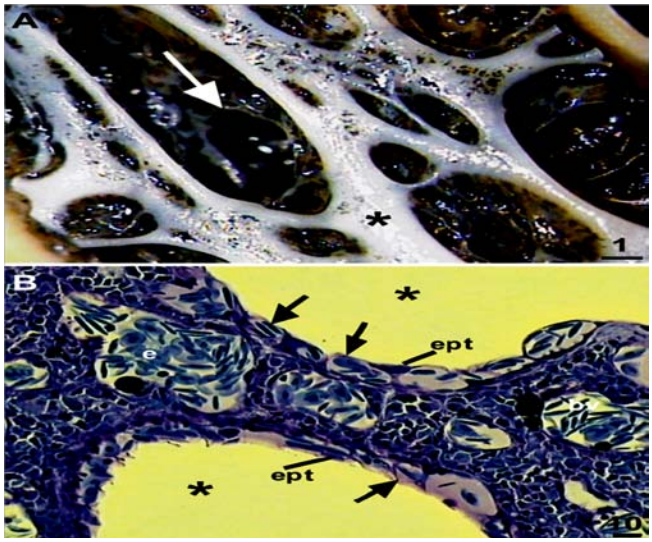

Fig. 5. *Channa gachua* swim bladder (A) Respiratory region (parenchyma) of the swim bladder ing trabecula (B) Respiratory tissue of an inter radicular septum, showing capillaries (arrow) underlying the epithelium.

Table 1. Dimension (means ± SEM) of the respiratory organs of the *Channa gachua.* Body weight (100 ± 9 g)

Parameter	**Gills**	**Swim bladder**
Total respiratory surface area (cm^2)	76.36 ± 2.27	157.01
Respiratory surface area ($cm^2\ kg^{-1}$)	780.21 ± 31.23	2173.05 ± 118.89
Arithmetic mean of water/blood or air/blood barrier (µm)	18.15 ± 1.34	1.56 ± 0.44
Harmonic mean of water/blood or air/blood barrier (µm)	9.61 Range 8.10–2.16	0.22 Range 0.16–0.43
ADF (cm2 µm)−1 k	80.62 ± 5.38	8424.68 ± 752.16
DmO_2 (cm3 min−1 mmHg−1 kg−1)	0.021 ± 0.002	1.86 ± 0.19
$DmCO_2$ (cm3 min−1 mmHg−1 kg−1)	0.403 ± 0.035	35.545 ± 3.69

Discussion

This study clearly demonstrates the importance of the swim bladder for gas exchange in *Channa gachua* even at a size where branchial water breathing is still possible. Although the basic gill structure of *Channa gachua* did not differ from that of other species, its respiratory surface area is lower, and its water blood diffusion barrier is very thick compared to some water breathing species and similar to those of air breathing fish (excepting the lungfish which has a non respiratory functional gills. The extensive surface area and the very thin air blood diffusion barrier

of its modified swim bladder shows 2.8 times greater surface area and 43 times thinner gas diffusion distance than in the gills, respectively. Based on these values alone, it is clear whether the swim bladder is the major respiratory organ. Compared to the accessory organs for respiration of other air breathing fish, the *Channa gachua* swim bladder has a larger surface area and a similar air blood diffusion barrier that favor air respiration. Physiological data have shown that in general three quarters of all oxygen needs are taken up by the swim bladder (Brauner *et al.,* 2004; Gonzalez *et al.,* 2010). Assuming that oxygen is 30 times less soluble than carbon dioxide in water, and that approximately equal molar equivalents of oxygen and CO_2 are taken up and given off, the gills would be able to excrete 30 mol. of CO_2 for each mole of oxygen taken up in the swim bladder. Additionally, the diffusion capacity expresses the rate of gas exchange per unit of driving pressure. As the driving pressure for oxygen falls between breaths, the rate of oxygen uptake in the swim bladder will fall accordingly, while it will remain at constant, albeit low, levels in the gills. Earlier physiological studies suggested that a spatial uncoupling between oxygen uptake in the swim bladder and CO_2 excretion in the gills might occur (Brauner and Val, 1996). In 2-3 kg fish, at least 78% of total oxygen uptake is from air, but only 37% of total CO_2 is excreted into the swim bladder. Slightly different data were found by Brauner and Val (1996), reporting an oxygen uptake of 78% by the swim bladder with only a 15% CO_2 excretion into this organ. Thus, 79% of CO_2 must have been excreted by the gills with an additional 6% via the kidney in the urine. Recently, Gonzalez *et al.,* (2010) showed that gill morphology changes do not place limitations on oxygen uptake in large fish and that the CO_2 excretion through the gills is similar (85–90%) in small (67 g) and large (724 g) fishes. They also showed that at lower blood pH, PCO_2 and concentrations of HCO_3^- in small fish suggest a possible diffusion limitation for CO_2. Ammonia excretion is mainly conducted through the gills. Our morphometric data support this functional partitioning, which may be less pronounced in our juvenile specimens than in large fish. The respiratory surface area of gills (78 cm^2) of *Channa gachua* is smaller than in typical water breathing fish and the facultative air breathers such as *Lepisosteus osseus* (83 cm^2) (Landolt and Hill, 1975), *Amia calva* 191 cm^2 (Crawford, 1971) and *Hoplerythrinus unitaeniatus* (125 cm^2) (Fernandes *et al.,* 1994). However, compared with the obligate air breathers such as *Anabas testudineus (*94 cm^2) (Hughes *et al.,* 1973) or the American lungfish *Lepidosiren paradoxa* 0.38 cm^2 (Moraes *et al.,* 2005), the gill surface areas of small *A. gigas* are relatively well developed (Costa *et al*., 2007). Low gill surface area in air-breathing fish has been considered an adaptation to reduce the loss of oxygen in hypoxic waters (Graham, 1997). Low surface area and a large water blood distance reduce the gill diffusion capacity for oxygen uptake, but it may favor the air-breathing fish living in hypoxic and stagnant waters by reducing the oxygen loss into water through the air breathing organ via the gills. It may also solve problems related to osmoregulation by reducing water influx and ion losses. The reversible protrusion of lamellae in the water breathing fish (*Carassius carassius*) when the oxygen supplying meets the species needs (Sollid *et al*., 2003; Sollid and Nilsson, 2006) supports the hypothesis that the low gill surface area of *Channa gachua* is able to support its ability to live in the hypoxic and ion poor waters of the rivers because the swim bladder is the main respiratory organ in this species. The compromise between gas exchange and other gill functions such as osmoregulation, acid base balance and nitrogen excretion also have to be considered. In general, both swim bladder types have low blood supply, excepting in air breathing fish that has a modified swim bladder for atmospheric air respiration. In this case, the swim bladder has a highly vascularized extense trabeculae region which varies among species. In *A. gigas* volume percentage of respiratory region of swim bladder in relation to body mass is the smallest among fish that use the swim bladder as both a respiratory organ and a hydrostatic organ (Graham, 1997). Just the reverse is seen in the parenchyma of *L. paradoxa* weighing approximately 600 g (Moraes *et al.,* 2005), where the lung volume (25 ml kg^{-1}) is too small to achieve neutral buoyancy, but the parenchyma makes up 43% of the total lung volume and has a surface to volume ratio of only 129 cm^{-1} (Moraes *et al.,* 2005). The swim bladder surface area of *Channa gachua* is between 5-33 fold that of the accessory organs of several air breathing fish taking oxygen directly from the air.

Conversely, the thin air blood barrier of the swim bladder, with a harmonic mean ranging from 0.16 to 0.43 um, is lower than that of other air breathing organs. The air breathing *A. gigas* exhale first and, then inhale the atmospheric air. The air inhalation is the result of the action of a buccal pump combined with the swim bladder aspiration by Farrell and Randall (1978) and Qaisur Rahman (2011) in *Channa gachua* reported that the ventral membrane of the swim bladder may act as a diaphragm like septum that stretches down wards between the body flanks, creating suction and filling the air breathing organ. It needs to rise in the water surface to gulp air. Its gills surface, although similar to some facultative air breathing fish, and function efficiently enough to fulfill all oxygen requirements. The lower morphometric and DO_2 of the gills in addition to low oxygen solubility in the natural environment of *Channa gachua* restricts oxygen uptake by the gills compared to swim bladder oxygen uptake from the air. Energy requiring physiological mechanisms however results in greater CO_2 excretion through the gills despite a larger morphometric DCO_2 for the air breathing organ.

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