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Acute effects of temperature and hypercarbia on cutaneous and branchial gas exchange in the South American lungfish, *Lepidosiren paradoxa*

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ABSTRACT

The South American lungfish, *Lepidosiren paradoxa* inhabits seasonal environments in the Central Amazon and Paraná-Paraguay basins that undergo significant oscillations in temperature throughout the year. They rely on different gas exchange organs, such as gills and skin for aquatic gas exchange while their truly bilateral lungs are responsible for aerial gas exchange; however, there are no data available on the individual contributions of the skin and the gills to total aquatic gas exchange in *L. paradoxa*. Thus, in the present study we quantify the relative contributions of skin and gills on total aquatic gas exchange during warm (35 °C) and cold exposure (20 °C) in addition to the effects of aerial and aquatic hypercarbia on aquatic gas exchange and gill ventilation rate (f_G; 25 °C), respectively. Elevated temperature (35 °C) caused a significant increase in the contribution of cutaneous (from 0.61 ± 0.13 to 1.34 ± 0.26 ml. STPD.h^{-1} kg^{-1}) and branchial (from 0.54 ± 0.17 to 1.73 ± 0.53 ml. STPD.h^{-1} kg^{-1}) gas exchange for $\dot{V}CO_2$ relative to the lower temperature (20 °C), while $\dot{V}O_2$ remained relatively unchanged. *L. paradoxa* exhibited a greater branchial contribution in relation to total aquatic gas exchange at lower temperatures (20 and 25 °C) for oxygen uptake. Aerial hypercarbia decreased branchial $\dot{V}O_2$ whereas branchial $\dot{V}CO_2$ was significantly increased. Progressive increases in aquatic hypercarbia did not affect f_G. This response is in contrast to increases in pulmonary ventilation that may offset any increase in arterial partial pressure of CO_2 owing to CO_2 loading through the animals' branchial surface. Thus, despite their reduced contribution to total gas exchange, cutaneous and branchial gas exchange in *L. paradoxa* can be significantly affected by temperature and aerial hypercarbia.

1. Introduction

The South American lungfish, *Lepidosiren paradoxa*, along with African (four species), and Australian lungfish (one species) are living representatives of the Dipnoi group (Carrol, 1987; Romer, 1970). Dipnoi is considered to be a sister-group to land vertebrates (Tetrapoda) (Brinkmann et al., 2004a, 2004b; Meyer and Zardoya, 2003; Tohyama et al., 2000; Zardoya et al., 1998) and, thus, is an important group regarding the transition from aquatic to air-breathing.

South American lungfish inhabit temporary hydric systems like the Central Amazon and Paraná-Paraguay basins (Paraná, Paraguay and Amazon Rivers), and they experience changes in environmental gases due to high rates of organic vegetal decomposition in temporary lakes (Chellappa et al., 2005; Neiff, et al., 2009). They also experience phases of dryness (aestivation during dry winters); changes in water pH (ranging from 6.5 to nearly 9.0); and large temperature oscillations (18−34 °C) (Harder et al., 1999; da Silva et al., 2013) with the highest temperatures recorded mainly between September and December (Chellappa et al., 2005).

All living species of lungfishes (Lepidosirenidae, Protopteridae and Ceratodontidae) show both aquatic and aerial respiration (Amin-Naves et al., 2004; Burggren and Johansen, 1986; Graham, 1997; Johansen, et al., 1967; Johansen and Lenfant, 1968; McMahon, 1970) with the predominant role of gas exchange organs varying among species and throughout development (de Moraes et al., 2005; Johansen and Lenfant, 1967; Babiker, 1979; Perry, et al., 2005). While pulmonary

(aerial) gas exchange has been preferentially examined in lungfishes (Amin-Naves et al., 2004; Johansen and Lenfant, 1967, 1968), skin-gill (aquatic) gas exchange contributions have gained less attention probably because branchial surface area in full-grown animals is reduced and may not be enough to provide adequate oxygen supply to the whole organism especially during high metabolic demands imposed by high temperature challenges (de Moraes et al., 2005; Amin-Naves et al., 2004). Few studies have compared the roles of aerial and aquatic respiratory gas exchange in lungfish (Amin-Naves et al., 2004; Babiker, 1979; Johansen et al., 1976; McMahon, 1970; Perry et al., 2005). Studies in *L. paradoxa* have assessed the role of aerial and total aquatic gas exchanges at different temperatures and demonstrated that *L. paradoxa* is capable of increasing oxygen consumption at high temperature (35 °C) relying mainly on their lungs (Amin-Naves et al., 2004). In addition, combined hypercarbia, *i.e.* in the air and aquatic phases, evokes acid-base perturbations that are defended by large and transient increases in pulmonary ventilation in *L. paradoxa* (Sanchez and Glass, 2001; Sanchez et al., 2005).

Besides studies that have emphasized the primary role of the lungs in total gas exchange, morphometric measurements of all gas exchange organs (lungs, skin and gills) in *L. paradoxa* have found that the skin is a potentially important respiratory surface for CO₂ elimination, and the gills have a negligible role for oxygen uptake (de Moraes, et al., 2005). However, no studies have quantified the partitioning of aquatic gas exchange between skin and gills nor investigated their possible differential control facing acute warm and cold exposure or high CO₂ levels that may alter cutaneous and branchial gas exchange. Therefore, we quantified the relative contributions of skin and gills to total aquatic gas exchange of adult specimens of the South American lungfish, *Lepidosiren paradoxa,* at different acute temperatures (20, 25 or 35 °C) and during exposure to aerial hypercarbia at 25 °C.

2. Material and methods

2.1. Animals

Specimens of *Lepidosiren paradoxa* Fitzinger 1837 (585 ± 28 g body weight; $N = 9$) were collected in the region of the Pantanal close to Cuiabá, Mato Grosso, Brazil. Animals were transported to the University of São Paulo, Ribeirão Preto, São Paulo State, where they were kept in 250 l tanks containing PVC tubes for hiding and filled with de-chlorinated tap water under constant temperature (25 °C). They were fed to satiety with chicken liver three times a week; food was withheld at least 48 h before surgeries and experiments. All experimental protocols were carried out during the animals' active season (September 2005 to April 2006). Animal collection was approved by the environmental agency SISBIO/IBAMA (Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis/IBAMA/MMA – nº 02027.0021722005-68 and n° 46160-1), and animal use was approved by the Ethics Committee of University of São Paulo, Faculty of Medicine of Ribeirão Preto (CETEA - Process #1712005).

2.2. Surgeries

Animals were anaesthetized by immersion in benzocaine solution (1 g L⁻¹) for 10 min until postural reflexes disappeared. To partition measurements of aquatic gas exchange an elastic ring (65% polyester and 35% elastodien) of variable diameter (dependent on the animal's size) and 15 mm of width was sutured in place behind the gills. A ring made of a vinyl glove cuff was sutured to the elastic to create a septum that filled the entire diameter of the recording chamber partitioning the head from the rest of the body (Fig. 1). After this procedure, lungfishes were returned to water at 25 °C and allowed to recover from the anesthesia for one hour. All experiments were performed in unanesthetized animals > 24 h after the surgical procedure.

The elastic ring was removed after the experiments for partitioning
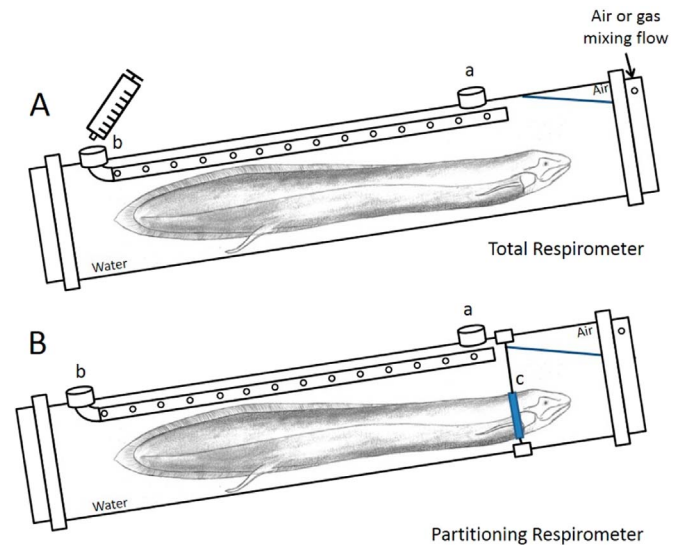
Fig. 1. Drawings of experimental apparatus (respirometer) constructed with a PVC tube show how aquatic and aerial gas exchange was measured. **A.** A total respirometer was used to measure whole body gas exchange (gills + skin); **B.** A partitioning respirometer was used to measure skin gas exchange. In A and B, *a* and *b* represent holes in PVC tube for respirometer water renewal. *b* also represents the hole for water samples to quantify P_wO_2 and P_wCO_2. *c* represents the combination between an elastic and a vinyl ring (a septum) sutured to the animal skin just caudal to the gills openings (for details see material and methods). Source: Drawn by L.A. Zena.

of aquatic gas exchange, and animals were allowed to recovery from the above-mentioned procedures for at least one month. A group of six animals were again anaesthetized (as described above), and a polyethylene cannula (PE-50; Clay Adams, Parsippany, NJ, USA) filled with water was inserted into the right gill opening and sutured in place for gill ventilation rate (f_G) measurements. Gill ventilation experiments were performed > 24 h after the cannula insertion.

2.3. Measurements of gas exchange and gill ventilation rate

Partial pressure of oxygen in water (P_wO_2) was analyzed with O₂ electrodes (FAC Instruments, São Carlos, SP, Brazil) connected to an O₂ analyzer (FAC 204 A; FAC Instruments, São Carlos, SP, Brazil). The electrodes were calibrated using water equilibrated with pure N₂ and with room air. Partial pressure of CO₂ in water (P_wCO_2) was measured by a CO₂ electrode (BGM 200-Cameron Instruments, Port Aransas, TX, USA) calibrated with 3% and 6% CO₂ in 21% O₂ and balance N₂ in the gas mixture using a gas mixing flowmeter (GF-3/MP, Cameron Instrument, Port Aransas, TX, USA). Electrodes were calibrated and maintained at specific temperatures of the experimental procedures (20, 25, and 35 °C). Oxygen uptake from the water ($\dot{V}O_2$) was measured as the decrease of O₂ (P_wO_2 initial − P_wO_2 final):

$$Aquatic\,\dot{V}O_2(mL.\,STPD.\,h^{-1}kg^{-1}) = V_{tot} \cdot \alpha O_2 \times \frac{P_wO_{2\;initial} - P_wO_{2\;final}}{\Delta time \times bodymass} \quad (A1)$$

where V_{tot} = total water volume in the experimental apparatus; αO_2 = solubility of O₂ in distilled water (Dejours, 1981); $P_wO_{2\;initial}$ - $P_wO_{2\;final}$ = difference in final O₂ partial pressure in relation to the initial values; $\Delta time$ = time difference between final and initial reading.

Carbon dioxide elimination ($\dot{V}CO_2$) was calculated (P_wCO_2 final − P_wCO_2 initial) as:

$$Aquatic\,\dot{V}CO_2(mL.\,STPD.\,h^{-1}kg^{-1}) = V_{tot} \cdot \alpha CO_2 \times \frac{P_wCO_{2\;final} - P_wCO_{2\;initial}}{\Delta time \times bodymass} \quad (A2)$$

where αCO_2 = solubility of CO₂ in distilled water; $P_wCO_{2\;final}$ - P_wCO_2

$_{initial}$ = difference in final partial pressure of CO_2 in relation to the initial values; Δtime = time difference between final and initial reading. All values for $\dot{V}O_2$ and $\dot{V}CO_2$ are expressed as mass-specific, *i.e.* ml. STPD.h^{-1} kg^{-1}.

Gill ventilation rate was obtained using a pressure transducer (MLTO380/D, ADInstruments, Sydney, Australia) connected to a catheter implanted in the animal's right gill opening. The pressure signals were recorded with a data acquisition system (MML110 Power Lab, ADInstruments, Sydney, Australia) and analyzed using Labchart software (PowerLab, ADInstruments, Sydney, Australia).

2.4. Experimental protocols

2.4.1. Aquatic gas exchange

On the day of the experiment, an unanaesthetized, intact lungfish was placed in an aquarium with dechlorinated water and exposed to a specific temperature (20, 25 or 35 ºC) for one hour. Different temperatures were performed with at least a 24 h interval between challenges. After this period, the animal was transferred to the experimental apparatus (respirometer) constructed with a PVC tube containing water equilibrated with room air at the same temperature to which it had been exposed in the temperature challenge. The tube was maintained slightly inclined in an aquarium with dechlorinated water (Fig. 1A), permitting adequate movement for voluntary aerial respiration. The experimental apparatus had two holes (anterior and posterior, see Fig. 1A) to permit flushing of fresh water and to take water samples for analysis with O_2 and CO_2 electrodes. Water samples were extracted from the respirometer with a 1 ml syringe connected to a perforated catheter extending along the PVC tube (Fig. 1A). Water control samples (1 ml) were extracted immediately after the animal transference to the experimental apparatus (initial reading) and one additional water sample was obtained two hours later (final reading).

2.4.2. Skin gas exchange

This protocol was designed to quantify the skin's contribution in the total aquatic gas exchange. To this end, one day after combined elastic and vinyl glove ring (the septum) attachment to the animal's skin, the lungfish was transferred to the experimental apparatus (partitioning respirometer; Fig. 1B) at the same temperature to which it was exposed in the temperature challenge (20, 25 or 35 ºC). In this apparatus the region posterior to the gills was inserted into the PVC tube so that the water surrounding the animal's head was separated from the water surrounding its body by the septum into another extension of the PVC tube (Fig. 1B). This septum did not prevent the animal's voluntary movement to breathe air. Water samples were extracted from the respirometer with a 1 ml syringe connected to a perforated catheter extending along the PVC tube (Fig. 1B). Control water samples (1 ml) were extracted immediately after the animal's transference to the experimental apparatus (initial reading) and the additional samples were obtained two hours later (final reading). By subtracting the skin gas exchange (Fig. 1B) from total aquatic gas exchange (described above; Fig. 1A) it was possible to estimate branchial contribution of total aquatic gas exchange.

2.4.3. Aquatic gas exchange during aerial hypercarbia

Aquatic gas exchange during aerial hypercarbia (5% CO_2 in 21% O_2 and balance N_2) at 25 ºC was measured after aerial normocarbia at 25 ºC for both protocols 2.4.1 and 2.4.2 where partitioning of cutaneous and branchial gas exchange ($\dot{V}O_2$ and $\dot{V}CO_2$) were repeated during aerial hypercarbia through two-hours of exposure after which a final water sample (1 ml) was obtained and compared to the initial water sample taken at the beginning of the experiment.

2.4.4. Gill ventilation rate under aquatic normocarbia and hypercarbia

One month after experiments performed for aquatic gas exchange measurements (protocols 2.4.1; 2.4.2 and 2.4.3) in which the vinyl glove ring had already been removed and the animal had fully recovered from the previous experimental protocol (aquatic gas exchange), a group of six intact *L. paradoxa* had gill ventilation rate measured under aquatic normocarbia followed by serial increases in hypercarbia. After the surgical procedure for catheter implantation into the right gill opening, animals were maintained for 24 h in a rectangular glass chamber (40 cm×20 cm×22 cm) filled with water (at 25 ºC; no PVC tube) before starting the experiments. Subsequent experiments were performed in the glass chamber which had its four corners bubbled with room air (using four aquarium aerators) with a flow rate of 5000 ml min^{-1} split between the four aerators for the first 30 min. Then the water was bubbled with serial increasing hypercarbic mixtures (0.5%, 1.0%, 3.0% and 5.0% CO_2 in 21% O_2 and N_2 balance), at the same flow rate (5000 ml min^{-1}), lasting 30 min per mixture. At the end of each normocarbic and hypercarbic exposure, a water sample was analyzed with a PCO_2 electrode to ensure that equilibrium was reached. Gill ventilation rate was recorded throughout the 30 min period for each condition.

2.5. Statistics

Results are presented as means ± S.E.M. Statistical analysis was performed using a two-way ANOVA (factors: respiratory organ × temperature or hypercarbia). A one-way RM ANOVA was used to analyze the effect of graded aquatic hypercarbia on gill ventilation rate. Whenever an ANOVA resulted in significant main or interaction effects, a Holm–Šidák *post hoc* test was performed to identify the differences. Data were tested for unequal variance and normality, and a log transformation was carried out when necessary. Data for relative contribution of skin and gill gas exchange (percentage data) for total $\dot{V}O_2$ and $\dot{V}CO_2$ were calculated as proportions, and an arcsin-square root transformation was applied for subsequent ANOVA analysis. Significance levels were accepted where $P < 0.05$.

3. Results

3.1. Aquatic gas exchange at different temperatures

Aquatic O_2 uptake and CO_2 excretion of *L. paradoxa* through the skin and gills are shown in Fig. 2. Acute changes in temperature did not modify gas exchange for either the cutaneous or branchial $\dot{V}O_2$ (Fig. 2A and B). In contrast, cutaneous and branchial $\dot{V}CO_2$ were significantly affected by temperature (P=0.002; $F_{2,48}$ =7.391) where a decrease from 25 to 20 ºC did not affect cutaneous $\dot{V}CO_2$ significantly (20 ºC: 0.61 ± 0.13 *vs.* 25 ºC: 0.60 ± 0.10 ml. STPD.h^{-1} kg^{-1}; Fig. 2B), while an increase from 25 to 35 ºC augmented cutaneous $\dot{V}CO_2$ by 123% (25 ºC: 0.60 ± 0.10 *vs.* 35 ºC: 1.34 ± 0.26 ml. STPD.h^{-1} kg^{-1}; Fig. 2B). Branchial $\dot{V}CO_2$ exhibited a decrease of 80% when cooled from 25 to 20 ºC (20 ºC: 0.54 ± 0.17 *vs.* 25 ºC: 0.98 ± 0.21 ml. STPD.h^{-1} kg^{-1}; Fig. 2B), while acute exposure to 35 ºC elevated $\dot{V}CO_2$ 80% (25 ºC: 0.98 ± 0.21 *vs.* 35 ºC: 1.73 ± 0.53 ml. STPD.h^{-1} kg^{-1}; Fig. 2B). There was no difference between cutaneous and branchial O_2-uptake (P=0.23) and CO_2 production (P=0.42) nor interaction between factors for both $\dot{V}O_2$ and $\dot{V}CO_2$ across temperatures (P=0.06 and 0.30, respectively).

Owing to the absence of differences for $\dot{V}O_2$ and $\dot{V}CO_2$ between skin and gills within each temperature (Fig. 2), we calculated cutaneous and branchial $\dot{V}O_2$ and $\dot{V}CO_2$ as a percentage of the total aquatic gas exchange in order to distinguish the relative contribution between gas exchange organs, cutaneous and branchial, at 25 ºC and during exposure to low (20 ºC) and high (35 ºC) temperatures (Fig. 3; see Amin-Naves et al., 2004). Gills contributed more than skin to $\dot{V}O_2$ at lower temperatures, 20 and 25 ºC (interaction effect: $P < 0.05$; $F_{2,47}$ =5.282). In contrast, regardless of temperature, there were no differences in the contributions to $\dot{V}CO_2$ of gills and skin (P=0.63; Fig. 3B), *i.e.* they equally contributed to aquatic CO_2 excretion. CO_2 was fourfold
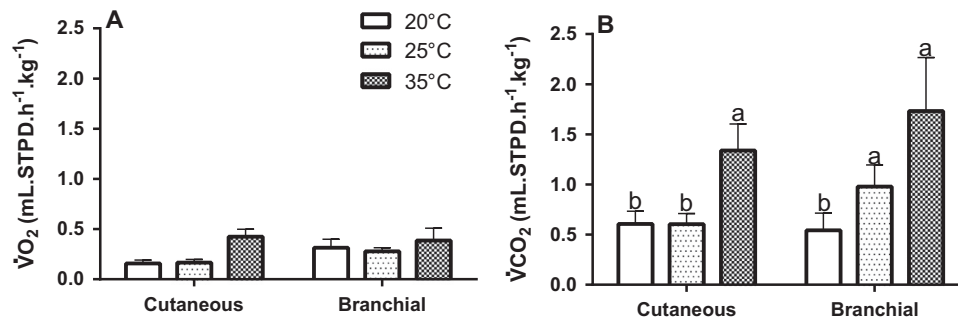
Fig. 2. Cutaneous and branchial O_2 uptake ($\dot{V}O_2$; **A**) and CO_2 excretion ($\dot{V}CO_2$; **B**) in the lungfish *Lepidosiren paradoxa* across temperatures. **A.** Across changes in temperature (20–35 °C), cutaneous and branchial $\dot{V}O_2$ remained unchanged; **B.** In contrast, temperature evoked significant increases in cutaneous and branchial $\dot{V}CO_2$ ($P < 0.01$). Different letters (**a** *vs.* **b**) mean significant effect of temperature within cutaneous or branchial ($P < 0.05$; Holm–Šidák *post hoc* test). Data are means ± S.E.M. N =8–9 animals.
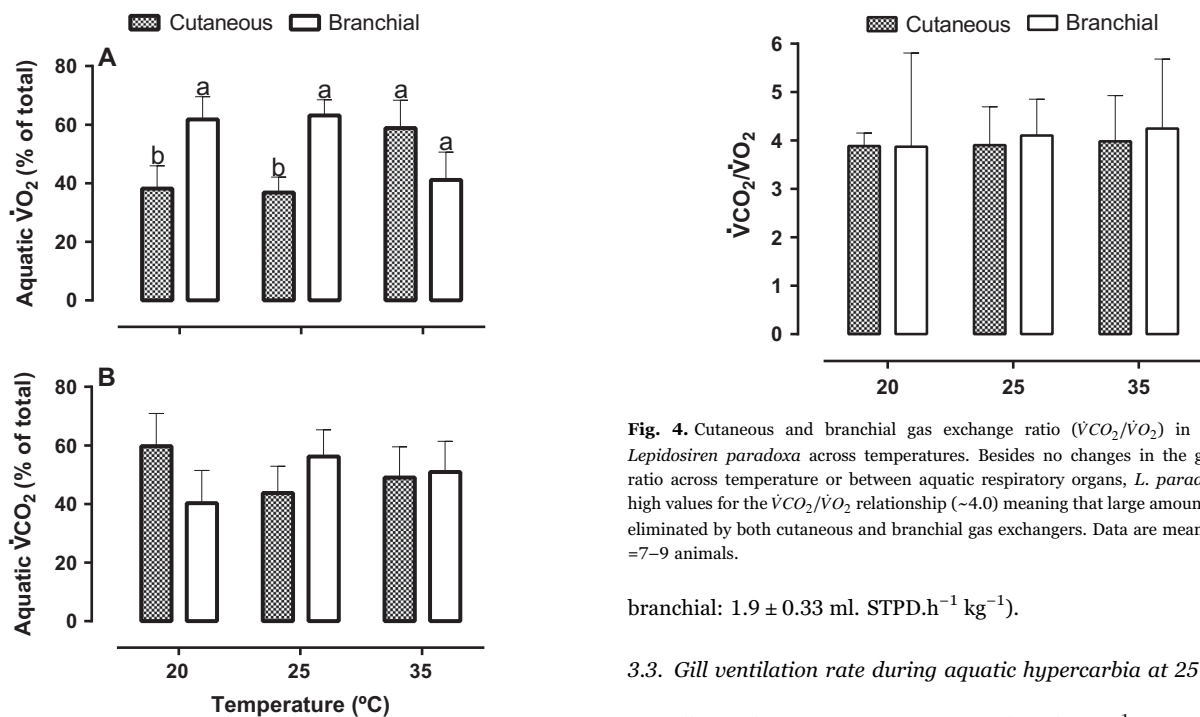
**Fig. 3.** Relative contribution to aquatic O_2 ($\dot{V}O_2$; **A**) and CO_2 ($\dot{V}CO_2$; **B**) gas exchange through the skin and gills across temperature in the lungfish *Lepidosiren paradoxa*. **A.** Effect of temperature in the aquatic $\dot{V}O_2$ contribution from skin and gills as a percentage relative to total aquatic gas exchange; **B.** Effect of temperature in the aquatic $\dot{V}CO_2$ contribution from skin and gills as a percentage relative to total aquatic gas exchange. Different letters (**a** *vs.***b**) mean significant effect of branchial over cutaneous gas exchange within each temperature ($P < 0.05$; Holm–Šidák *post hoc* test). Data are means ± S.E.M. N =8–9 animals.

higher than O_2, as expressed by the respiratory exchange ratio ($\dot{V}CO_2/\dot{V}O_2$) for aquatic gas exchange for skin (3.9 ± 0.5) and gills (4.1 ± 0.75), and aquatic respiratory exchange ratio did not change with acute changes in temperature (P=0.98; Fig. 4).

3.2. Aquatic gas exchange during aerial hypercarbia at 25 °C

Aquatic $\dot{V}O_2$ and $\dot{V}CO_2$ responses were measured during hypercarbia in the gas phase (Fig. 5). Hypercarbia did not change cutaneous O_2-uptake, but it decreased branchial $\dot{V}O_2$ from 0.44 ± 0.05 ml. STPD.h^{-1} kg^{-1} (normocarbia) to 0.06 ± 0.06 ml. STPD.h^{-1} kg^{-1} (interaction effect: $P < 0.001$, $F_{1,34}$=23.940; Fig. 5A). Hypercarbia caused an increase in branchial $\dot{V}CO_2$ ($P < 0.05$; $F_{1,34}$=5.036; Fig. 5B), but did not change cutaneous CO_2 excretion. CO_2 excretion was significantly higher across the branchial surface compared with the cutaneous surface ($P < 0.01$; $F_{1,34}$ =9.446; Fig. 5B) during hypercarbia (skin: 0.85 ± 0.23 *vs.*
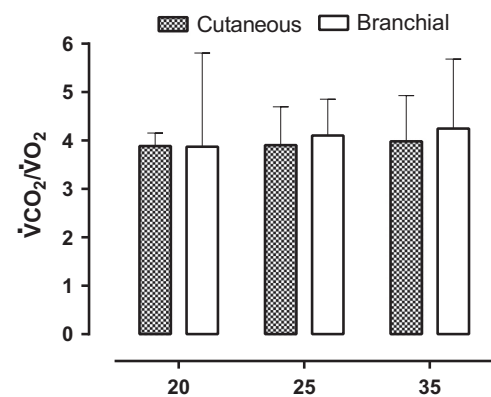
Fig. 4. Cutaneous and branchial gas exchange ratio ($\dot{V}CO_2/\dot{V}O_2$) in the lungfish *Lepidosiren paradoxa* across temperatures. Besides no changes in the gas exchange ratio across temperature or between aquatic respiratory organs, *L. paradoxa* exhibits high values for the $\dot{V}CO_2/\dot{V}O_2$ relationship (~4.0) meaning that large amounts of CO_2 are eliminated by both cutaneous and branchial gas exchangers. Data are means ± S.E.M. N =7–9 animals.

branchial: 1.9 ± 0.33 ml. STPD.h^{-1} kg^{-1}).

### 3.3. Gill ventilation rate during aquatic hypercarbia at 25 °C

Gill ventilation rate was 1.0 ± 0.2 breath min^{-1} in normocarbia. Gradual increases in aquatic partial pressure of CO_2 did not significantly affect this variable in *L. paradoxa*, compared with the normocarbic condition (Fig. 6).

4. Discussion

The present study describes the contributions of branchial and cutaneous O_2 and CO_2 gas exchange to aquatic respiration of *L. paradoxa*. We report for the first time in adult specimens of South American lungfish an estimation of contributions for each component involved in the aquatic gas exchange, *i.e.* skin and gills, and we describe how acute changes in temperature and hypercarbia affect them. Temperature affected metabolic rate of lungfishes whereby CO_2 excretion from branchial tissue decreased and increased during acute reduction (20 °C) and elevation (35 °C) in temperature, respectively, in contrast to no thermal effect on cutaneous and branchial $\dot{V}O_2$ (Fig. 2). Regardless of changes in temperature, the gills and skin of *L. paradoxa* relative contributions remained about the same for aquatic $\dot{V}CO_2$, and conversely, gills exhibited a relative higher percent contribution compared to cutaneous aquatic $\dot{V}O_2$ at 25 °C and when exposed to 20 °C, but not 35 °C (Fig. 3).

The predominance of a particular type of breathing - aquatic or aerial - is related, among other factors, to the respiratory organ surface area (Hughes, 1980) which varies among species and developmental
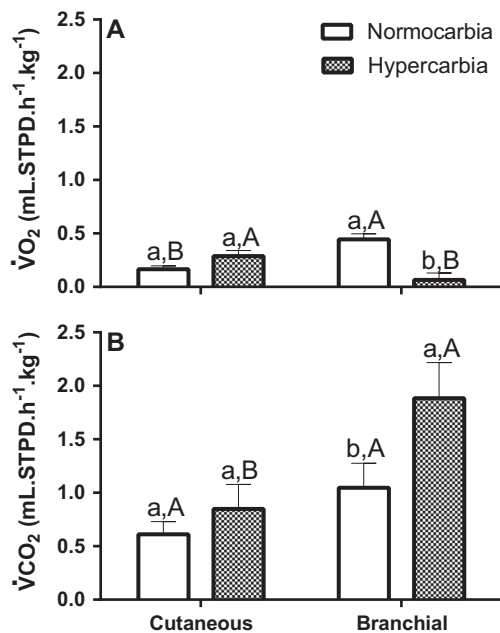
Fig. 5. Cutaneous and branchial O_2 uptake ($\dot{V}O_2$; **A**) and CO_2 excretion ($\dot{V}CO_2$; **B**) in the lungfish *Lepidosiren paradoxa,* at 25 °C, during aerial normocarbia and hypercarbia (5% CO_2). **A**. Effect of aerial normocarbia and hypercarbia on cutaneous and branchial $\dot{V}O_2$. **B**. Effect of aerial normocarbia and hypercarbia on cutaneous and branchial $\dot{V}CO_2$. Different lowercase letters (**a** *vs.***b**) mean significant effect of hypercarbia within cutaneous or branchial, and different capital letters (**A** *vs.***B**) mean significant difference between cutaneous and branchial within normocarbia or hypercarbia ($P < 0.05$; Holm–Šidák *post hoc* test). Data are means ± S.E.M. $N = 9$ animals.
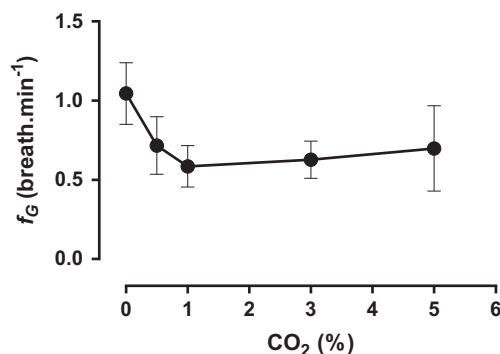
Fig. 6. Effect of increasing levels of aquatic hypercarbia on gill ventilation rate (f_G) in the lungfish *Lepidosiren paradoxa*. Although there appears to be a trend of decreasing f_G during 1% exposure to high aquatic CO_2, *L. paradoxa* maintained f_G relatively constant across all levels of aquatic hypercarbia (no significant differences). $N = 6$ animals.

stages (McMahon, 1970). African lungfishes, for example, possess true lungs, and gills are also present permitting bimodal breathing (reviewed by Burggren and Johansen, 1986). For instance, i) high branchial ventilation occurs in juvenile *Protopterus annectens*, but in mature fishes (> 400 g) 80–85% of O_2-uptake occurs in the lungs (Babiker, 1979); ii) in adult *Protopterus dolloi*, approximately 9% of O_2 uptake and 24% of CO_2 excretion are aquatic (skin and gills) (Perry et al., 2005); iii) in *Protopterus amphibius*, the juvenile fishes are able to supply 70% of O_2-uptake by their aquatic breathing route compared with only 10–15% in older fishes that also exhibit external gills (Johansen et al., 1976); and iv) in adults *P. aethiopicus*, 60% of CO_2 excretion is aquatic (gills) while 90% of oxygen consumption is acquired by the lungs (McMahon, 1970).

In the South American lungfish the gills are important organs for aquatic gas exchange during larval and juvenile phases (Babiker, 1979; Johansen et al., 1976; Kerr, 1899). *L. paradoxa* embryos hatch with 4 large pairs of gill arches that continue to grow in length. About six

weeks after hatching, the larvae undergo changes, and their circulation to the external gills becomes sluggish which then start to regress (Kerr, 1899). Hence, the gills of adult South American lungfish are short and reduced to a few lobe-like filaments (de Moraes et al., 2005).

Using morphometric measurements to determine the respiratory surface area and thickness of the water-blood barrier of gills, skin and lungs of adult *L. paradoxa*, de Moraes et al. (2005) reported a lower morphometric diffusing capacity in the aquatic gas exchange organs (skin and gill) compared to the lungs, which is in agreement with a higher aerial compared to aquatic (skin+gill) gas exchange in the adult L. *paradoxa* (Amin-Naves et al., 2004). Based on the skin and gills $\dot{V}O_2$ and $\dot{V}CO_2$ obtained in the present study as well as data reported by Bassi et al. (2005) for arterial O_2 and CO_2 partial pressures at 25 and 35 °C, we estimated values for physiological gas diffusing capacity for cutaneous (D_cO_2 or D_cCO_2) and branchial (D_bO_2 or D_bCO_2) respiratory organs (Glass, 2010) of *L. paradoxa*. Thus, changes in temperature affected D_cO_2 which decreased to 4.5×10^{-5} (20 °C) and increased to 13×10^{-5} (35 °C) ml STPD kg^{-1} min^{-1} $mmHg^{-1}$. These values are lower than the cutaneous morphological diffusing capacity previously reported for O_2 (94.3×10^{-5} ml STPD kg^{-1} min^{-1} $mmHg^{-1}$; de Moraes et al., 2005). Morphological diffusing capacity values always exceeded the value obtained by physiological methods (Glass, 2010); however, our estimated values for D_bO_2 ranged between 8.9×10^{-5} (20 °C) to 12×10^{-5} (35 °C) ml STPD kg^{-1} min^{-1} $mmHg^{-1}$, which represent at least sixty times higher values than morphological data (0.15×10^{-5} ml STPD kg^{-1} min^{-1} $mmHg^{-1}$; de Moraes et al., 2005) predicted. This is intriguing, but it has to be taken into consideration that besides numerous capillaries located close to the epithelium of the gill lamellae, gill arches also have numerous capillaries near the epithelium, and their possible respiratory function in aquatic gas exchange cannot be neglected (de Moraes et al., 2005). Another possible explanation for those differences accounts for gas exchange from the head's integument comprising ~10% of total skin that was included in the anterior chamber of the respirometer, in addition to any gas exchange from the buccal cavity. For CO_2, physiological diffusing capacity values estimated for all tested temperatures ranged between 5.1×10^{-4} (20 °C) to 8.1×10^{-4} (35 °C) ml STPD kg^{-1} min^{-1} $mmHg^{-1}$ for skin, and between 4.5×10^{-4} (20 °C) to 7.8×10^{-4} (35 °C) ml STPD kg^{-1} min^{-1} $mmHg^{-1}$ for gills, which fell below morphological values for either skin (18,000 ml STPD kg^{-1} min^{-1} $mmHg^{-1}$) or gills (28 ml STPD kg^{-1} min^{-1} $mmHg^{-1}$) reported by De Moraes and coworkers (2005).

At least for the experimental conditions in the present study, gills exhibited a higher relative contribution than skin for O_2-uptake, a pattern that was not affected by acute exposure to 20 °C, but was disrupted once temperature reached 35 °C. Besides having a higher relative contribution at lower temperatures, branchial $\dot{V}O_2$ Q_{10} (1.1) did not follow a typical Q_{10} effect (*i.e.* 2.0–3.0) for physiological and biochemical processes (Rome, et al., 1992), while thermal influences on $\dot{V}CO_2$ fell within typical Q_{10} values ($Q_{10} = 2.2$). As pointed out by Amin-Naves et al. (2004), total aquatic O_2 uptake (gills and skin) is important at lower temperatures (15 °C) likely because lungs are playing a smaller role, but still higher than aquatic gas exchange, at cooler temperature when they are not solely needed. Metabolic functions are at least partially sufficed by gill ventilation plus any gas exchange occurring through the animals' head/buccal cavity. Furthermore, besides minimal gill surface area (0.0013%), *L. paradoxa* exhibits larger numbers of mitochondria and ribosomes in all epithelial cells of the gills, suggesting a highly active metabolism, at least for ion transport (Wright, 1974). A lower morphological diffusing capacity for aquatic organs, less than 1% (de Moraes, et al., 2005), does not impair arterial PO_2 from reaching similar values when aquatic hyperoxia (plus aerial normoxia), or aerial hyperoxia (plus aquatic normoxia) is applied (146 *vs.* 114 mmHg, respectively). Although lung ventilation slightly drops during aerial hyperoxia, it is only during aquatic hyperoxia that *L. paradoxa* markedly reduces lung ventilation (Bassi, et al., 2010).

It is noteworthy that the early literature reported that before the

breeding season, papillae in the pelvic limb of male *L. paradoxa* grow into bright-red vascular filaments that persist while the males nurse the eggs and the developing larvae (Kerr, 1899). Although its function remains to be determined, one suggested hypothesis is that the developed filaments during breeding season serves as an auxiliary (or even major) organ for aquatic gas exchange, allowing the male to stay and guard the nest for longer periods without surfacing to breath air (Cunningham and Reid, 1933). This may be an interesting example of a physiological adaption that importantly increases the role of aquatic gas exchange. Development of vascular filaments and their respiratory function remains to be further investigated especially in breeding males.

Acute thermal effects on both cutaneous and branchial $\dot{V}CO_2$ (Q_{10} =2.0) reflects an alternative route for CO_2 release when metabolic rate is heat challenged. The relative contribution of skin and gills for $\dot{V}CO_2$ was not affected by changes in temperature (Fig. 3B), and both contributed about the same for CO_2 excretion. Higher capacitance of the water for CO_2 relative to O_2 (Dejours, 1981; Pittman, 2011) in addition to a large temperature-induced metabolic acidosis (Amin-Naves et al., 2004; Bassi et al., 2005) contributed to high rates of CO_2 excretion and hence to a high aquatic $\dot{V}CO_2/\dot{V}O_2$ relationship observed at all temperatures tested (Fig. 4). In the African lungfish *P. aethiopicus* an elevated aquatic gas exchange ratio of 5.0 (McMahon, 1970) is similar to values obtained for *L. paradoxa* $\dot{V}CO_2/\dot{V}O_2$ relationship (~4.0). In contrast, pulmonary gas exchange ratio for *L. paradoxa* exhibits smaller values compared to skin/gills gas exchange ratio (from 0.11 to 0.62; Amin-Naves et al., 2004), showing that more O_2 is taken up through the lungs and large amounts of CO_2 are excreted by skin/ gills.

L. paradoxa exhibits large increases of pulmonary ventilation when exposed to ambient hypercarbia in the gas phase (Amin-Naves et al., 2007; Sanchez et al., 2005, 2001). Part of that response involves central chemoreceptors since stepwise pH reductions of the mock cerebrospinal fluid increases the ventilatory response in *L. paradoxa* (Sanchez et al., 2001; Amin-Naves et al., 2007). In addition to that response, the present study shows that aerial hypercarbia also increases branchial CO_2 excretion in this species. Such increase in CO_2 excretion through the gills may reflect a compensatory response due to a respiratory acidosis expected to occur during high inspired CO_2 through the gas phase with the aquatic gas exchange organs, *i.e.* gills, playing an important role in CO_2 excretion (Perry et al., 2005).

Aerial hypercarbia also reduced branchial O_2 uptake in *L. paradoxa* (Fig. 5). It is not clear why reductions in water oxygen uptake take place when the animal is inspiring hypercapnic gas through their lungs. A possible explanation for that response may involve blood shunting to avoid respiratory branchial arches. In these animals the branchial artery provides a ventral to dorsal shunt in gill arches 3 and 4 that provides an alternative route for blood flow instead of passing through the filaments (branchial shunt) that may reduce the capacity for gas exchange (Laurent et al., 1978; Sandblom and Axelsson, 2011; Morgan and Wright, 1989). In contrast, serial increases in percent aquatic hypercarbia exposure did not affect f_G beyond a trend to decrease it once aquatic hypercarbia reached 1% CO_2 (Fig. 6). After that CO_2 level, any further increase in aquatic hypercarbia did not further modify f_G in our animals. Besides insignificant decreases in f_G, previous studies have shown that aquatic hypercarbia evokes rises in arterial PCO_2 and, consequently, decreases in pH of *L. paradoxa* as well as increases in pulmonary ventilation (Sanchez et al., 2005). Overall, it seems that exposure to aquatic hypercarbia while animals have access to normocarbic air may induce increases in CO_2 loading through animals' aquatic organs surfaces. Previous reports on African lungfish, *P. dolloi*, have shown a decrease in branchial ventilation that might help to prevent CO_2 loading from the environment across their gills. Conversely, an increase in pulmonary ventilation would permit the fish to eliminate the excess CO_2 gained through the aquatic route (Perry et al., 2008).

5. Conclusions

In conclusion, the relative contribution of each component involved in the aquatic gas exchange, cutaneous and branchial, in *L. paradoxa* is affected by acute changes in temperature and also hypercarbia. The South American lungfish lives in a seasonally changing environment, inhabiting stagnant and temporary water systems in Central Amazon and Paraná-Paraguay basins in Brazil, and it may undergo considerable temperature oscillations on a daily and seasonal basis affecting aquatic and aerial gas exchange (Chellappa et al., 2005; Amin-Naves et al., 2004). While branchial O_2-uptake has a higher relative role at lower temperatures, *L. paradoxa* lungs are the crucial respiratory organ for supplying metabolic demands, especially at high temperatures (Amin-Naves, et al., 2004). In contrast, regardless of temperature, both the gills and skin had relatively the same contribution for CO_2 unloading across changes in temperature in water.

References

Amin-Naves, J., Giusti, H., Glass, M.L., 2004. Effects of acute temperature changes on aerial and aquatic gas exchange, pulmonary ventilation and blood gas status in the South American lungfish, *Lepidosiren paradoxa*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 138, 133–139.

Amin-Naves, J., Giusti, H., Hoffmann, A., Glass, M.L., 2007. Central ventilatory control in the South American lungfish, *Lepidosiren paradoxa*: contributions of pH and CO(2). J. Comp. Physiol. B 177, 529–534.

Babiker, M.M., 1979. Respiratory behaviour, oxygen consumption and relative dependence on aerial respiration in the African lungfish (*Protopterus annectens*, Owen) and an air-breathing teleost (Clarias lazera, C.). Hydrobiologia 65, 177–187.

Bassi, M., Klein, W., Fernandes, M.N., Perry, S.F., Glass, M.L., 2005. Pulmonary oxygen diffusing capacity of the South American lungfish *Lepidosiren paradoxa*: physiological values by the Bohr method. Physiol. Biochem. Zool. 78, 560–569.

Bassi, M., Giusti, H., da Silva, G.S., Amin-Naves, J., Glass, M.L., 2010. Blood gases and cardiovascular shunt in the South American lungfish (Lepidosiren paradoxa) during normoxia and hyperoxia. Respir. Physiol. Neurobiol. 173, 47–50.

Brinkmann, H., Denk, A., Zitzler, J., Joss, J.J., Meyer, A., 2004a. Complete mitochondrial genome sequences of the South american and the Australian lungfish: testing of the phylogenetic performance of mitochondrial data sets for phylogenetic problems in tetrapod relationships. J. Mol. Evol. 59, 834–848.

Brinkmann, H., Venkatesh, B., Brenner, S., Meyer, A., 2004b. Nuclear protein-coding genes support lungfish and not the coelacanth as the closest living relatives of land vertebrates. Proc. Natl. Acad. Sci. USA. 101, 4900–4905.

Burggren, W.W., Johansen, K., 1986. Circulation and respiration in lungfishes (Dipnoi). In: Bemis, W.E., Burggren, W.W., Kemp, N.E. (Eds.), Biology and Evolution of Lungfishe. Alan R. Liss, New York, 217–236.

Carrol, R.L., 1987. Vertebrate Palaeontology and Evolution first ed.. W. H. Freeman & Company, New York.

Chellappa, S., Sá-Oliveira, J.C., Chellappa, N.T., 2005. Fish fauna of a temporary lake in an Amazonian conservation area. Acta Limnol. Bras. 17, 283–289.

Cunningham, J.T., Reid, D.M., 1933. Pelvic filaments of Lepidosiren. Nature 131, (913-913).

Dejours, P., 1981. Principles of Comparative Respiratory Physiology second ed.. North Holland Publishing, Amsterdam.

Glass, M., 2010. Respiratory function in lungfish (Dipnoi) and a comparison to land vertebrates. In: Jørgensen, J.M., Joss, J. (Eds.), The Biology of Lungfishes. Science Publishers, New Hampshire, 265–282.

Graham, J.B., 1997. Air-Breathing Fishes: Evolution, Diversity and Adaptation first ed.. Academic Press, San Diego.

Harder, V., Souza, R.H.S., Severi, W., Rantin, F.T., Bridges, C.R., 1999. Biology of tropical fishes. In: Val, A.L., Almeida-Val, V.M.F. (Eds.), The South American Lungfish – Adaptations to an Extreme Habitat. INPA, Manaus, 99–110.

Hughes, G.M., 1980. Morphometry of fish gas exchange organs in relation to their respiratory function. In: Ali, M.A. (Ed.), Environmental Physiology of Fishes. Plenum Press, New York, 33–56.

Johansen, K., Lenfant, C., 1967. Respiratory function in the South American lungfish,

Lepidosiren paradoxa (Fitz). J. Exp. Biol. 46, 205–218.

Johansen, K., Lenfant, C., 1968. Respiration in the African lungfish *Protopterus aethiopicus*. II. control of breathing. J. Exp. Biol. 49, 453–468.

Johansen, K., Lomholt, J.P., Maloiy, G.M., 1976. Importance of air and water breathing in relation to size of the African lungfish *Protopterus amphibius* Peters. J. Exp. Biol. 65, 395–399.

Kerr, J., 1899. The external features in the development of *Lepidosiren paradoxa*. Fitz. Philos. Trans. R. Soc. Lond. B Biol. Sci. 65, 160–161.

Laurent, P., DeLaney, R.G., Fishman, A.P., 1978. The vasculature of the gill in the aquatic and aestivating lungfish (*Protopterus aethiopicus*). J. Morphol. 156, 173–208.

McMahon, B.R., 1970. The relative efficiency of gaseous exchange across the lungs and gills of an African lungfish, *Protopterus aethiopicus*. J. Exp. Biol. 52, 1–15.

Meyer, A., Zardoya, R., 2003. Recent advances in the (molecular) phylogeny of vertebrates. Annu. Rev. Ecol. Evol. Syst. 34, 311–338.

de Moraes, M.F., Holler, S., da Costa, O.T., Glass, M.L., Fernandes, M.N., Perry, S.F., 2005. Morphometric comparison of the respiratory organs in the South American lungfish *Lepidosiren paradoxa* (Dipnoi). Physiol. Biochem. Zool. 78, 546–559.

Morgan, M., Wright, D.E., 1989. Morphology of central compartment and vasculature of the gills of *Lepidosiren paradoxa* (Fitzinger). J. Fish. Biol. 34, 875–888.

Neiff, J.J., Neiff, A.P., Verón, M.B.C., 2009. The role of vegetated areas on fish assemblage of the Paraná River floodplain: effects of different hydrological conditions. Neotrop. Ichthyol. 7, 39–48.

Perry, S.F., Gilmour, K.M., Swenson, E.R., Vulesevic, B., Chew, S.F., Ip, Y.K., 2005. An investigation of the role of carbonic anhydrase in aquatic and aerial gas transfer in the African lungfish *Protopterus dolloi*. . J. Exp. Biol. 208, 3805–3815.

Perry, S.F., Euverman, R., Wang, T., Loong, A.M., Chew, S.F., Ip, Y.K., Gilmour, K.M., 2008. Control of breathing in African lungfish (*Protopterus dolloi*): a comparison of aquatic and cocooned (terrestrialized) animals. Respir. Physiol. Neurobiol. 160, 8–17.

Pittman, R.N., 2011. Regulation of Tissue Oxygenation – Colloquium Series on Integrated Systems Physiology: From Molecule to Function first ed.. Morgan & Claypool Publishers, San Rafael.

Rome, L.C., Stevens, E.D., John-Alder, H.B., 1992. The influence of temperature and thermal acclimation on physiological function. In: Feder, M.E., Burggren, W.W. (Eds.), Environmental Physiology of the Amphibia. University of Chicago Press, Chicago, 183–205.

Romer, A.S., 1970. The Vertebrate Body first ed.. W.B. Saunders Company, Philadelphia.

Sanchez, A.P., Glass, M.L., 2001. Effects of environmental hypercapnia on pulmonary ventilation of the South American lungfish. J. Fish. Biol. 58, 1181–1189.

Sanchez, A.P., Hoffmann, A., Rantin, F.T., Glass, M.L., 2001. Relationship between cerebro-spinal fluid pH and pulmonary ventilation of the South American lungfish, *Lepidosiren paradoxa* (Fitz.). . J. Exp. Zool. 290, 421–425.

Sanchez, A.P., Giusti, H., Bassi, M., Glass, M.L., 2005. Acid-base regulation in the South American lungfish *Lepidosiren paradoxa*: effects of prolonged hypercarbia on blood gases and pulmonary ventilation. . Physiol. Biochem. Zool. 78, 908–915.

Sandblom, E., Axelsson, M., 2011. Autonomic control of circulation in fish: a comparative view. Auton. Neurosci. 165, 127–139.

da Silva, G.S.F., Glass, M.L., Branco, L.G.S., 2013. Temperature and respiratory function in ectothermic vertebrates. J. Therm. Biol. 38, 55–63.

Tohyama, Y., Ichimiya, T., Kasama-Yoshida, H., Cao, Y., Hasegawa, M., Kojima, H., Tamai, Y., Kurihara, T., 2000. Phylogenetic relation of lungfish indicated by the amino acid sequence of myelin DM20. Brain Res. Mol. Brain Res. 80, 256–259.

Wright, D.E., 1974. Morphology of the gill epithelium of the lungfish, *Lepidosiren paradoxa*. Cell Tissue Res. 153, 365–381.

Zardoya, R., Cao, Y., Hasegawa, M., Meyer, A., 1998. Searching for the closest living relative(s) of tetrapods through evolutionary analyses of mitochondrial and nuclear data. Mol. Biol. Evol. 15, 506–517.