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The breathing pattern and the ventilatory response to aquatic and aerial hypoxia and hypercarbia in the frog *Pipa carvalhoi*

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ABSTRACT

Anuran amphibians are known to exhibit an intermittent pattern of pulmonary ventilation and to exhibit an increased ventilatory response to hypoxia and hypercarbia. However, only a few species have been studied to date. The aquatic frog *Pipa carvalhoi* inhabits lakes, ponds and marshes that are rich in nutrients but low in O_2. There are no studies of the respiratory pattern of this species and its ventilation during hypoxia or hypercarbia. Accordingly, the aim of the present study was to characterize the breathing pattern and the ventilatory response to aquatic and aerial hypoxia and hypercarbia in this species. With this purpose, pulmonary ventilation (V_I) was directly measured by the pneumotachograph method during normocapnic normoxia to determine the basal respiratory pattern and during aerial and aquatic hypercarbia (5% CO_2) and hypoxia (5% O_2). Our data demonstrate that *P. carvalhoi* exhibits a periodic breathing pattern composed of single events (*single breaths*) of pulmonary ventilation separated by periods of apnea. The animals had an enhanced V_I during aerial hypoxia, but not during aquatic hypoxia. This increase was strictly the result of an increase in the breathing frequency. A pronounced increase in V_I was observed if the animals were simultaneously exposed to aerial and aquatic hypercarbia, whereas small or no ventilatory responses were observed during separately administered aerial or aquatic hypercarbia. *P. carvalhoi* primarily inhabits an aquatic environment. Nevertheless, it does not respond to low O_2 levels in water, although it does so in air. The observed ventilatory responses to hypercarbia may indicate that this species is similar to other anurans in possessing central chemoreceptors.

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1. Introduction

Amphibians are extensively used in physiological studies aimed at generating new insights in evolutionary biology. Although all modern amphibians are highly specialized and represent a significant departure in morphology, ecology and behavior from the stem group that gave rise to the later tetrapods (Kardong, 2005; Gargaglioni and Milsom, 2007), studies using modern amphibians are very useful for revealing a number of characteristics associated with the evolution of air breathing. The form and function of the respiratory system of amphibians reflects the diversity of adaptations imposed by the different properties of water and air (Gargaglioni and Milsom, 2007).

In general, anuran amphibians display an intermittent breathing pattern in which lung ventilation occurs either in single breaths separated by periods of breath holding or in episodes of consecutive lung ventilation followed by long non-ventilatory periods of variable duration (Milsom, 1991). Hypoxia elicits an increase in ventilation in amphibians due to the activation of peripheral chemoreceptors located in the aortic arch and carotid labyrinth (Van Vliet and West, 1992). The carotid labyrinth can also detect changes in arterial blood pressure of CO_2 and pH (Van Vliet and West, 1992). Chemoreceptors sensitive to hypoxia may also be present on the pulmocutaneous trunk (Hoffmann and de Souza, 1982) and central nervous system (Winmill et al., 2005). Hypercarbia is also a powerful stimulus of the respiratory control system. In amphibians, the presence of central respiratory chemoreceptors has been clearly established (Smatresk and Smits, 1991; Branco et al., 1992; Noronha-de-Souza et al., 2006). Central chemoreceptors responding to changes in PCO_2/pH are an important source of respiratory drive. Furthermore, olfactory receptors sensitive to CO_2 strongly inhibit breathing in unanesthetized bullfrogs (Kinkead and Milsom, 1996). In adult anurans, pulmonary stretch receptors are also CO_2 sensitive, and their firing rates decrease with increasing pulmonary CO_2 concentrations (Milsom and Jones, 1977).

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The species *Pipa carvalhoi* is an aquatic anuran of the family Pipidae. Regarding the phylogenetic relationships in the anuran group, Pipidae is considered to be one of the most basal families (Pyron and Wiens, 2011). Thus, Pipidae anurans may provide special insight into the evolution of air breathing.

P. carvalhoi is found in eastern and northeastern Brazil in wetlands, marshes, ponds and lakes (environments in which the O_2 level may vary; Weygoldt, 1976). Although *Pipa* is primarily aquatic, it can move over land during heavy rain. For this reason, it can also be found occasionally in dry and moist savannas. *P. carvalhoi* occurs in the Atlantic forest and also in the Brazilian Cerrado, which are important areas for conservation in Brazil. Little is known about the natural history, life cycles, physiology and ecology of this anuran. To our knowledge, no previous studies have addressed respiratory function in this species or the ability of the animal to respond to stimuli such as hypoxia and hypercarbia. Accordingly, the aim of the present study was to characterize the breathing pattern and to assess the ventilatory responses to aerial and aquatic hypercarbia and hypoxia in *P. carvalhoi*.

2. Materials and methods

2.1. Animals

Pipa carvalhoi (Miranda-Ribeiro, 1937) of undetermined sex weighing 25.2 ± 2.9 g were collected in the city of Buararema in the state of Bahia (Brazil). The animals were captured and transported in agreement with the "Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis" — IBAMA (Animal License no. 22696–1) and were maintained for several weeks prior to experiments in black 50 L tanks containing dechlorinated, aerated tap water at 25 °C. The water was continuously renewed, and a 12:12 h light:dark cycle was maintained. The animals were fed beef liver twice weekly and did not receive any food 48 h prior to experimentation. The present study was conducted in compliance with the guidelines set by SBCAL (Sociedade Brasileira de Ciência em Animais de Laboratório) and with the approval of the São Paulo State University Animal Care and Use Committee (Protocol no. 000229–09). All experiments were performed between September and December.

2.2. Measurement of ventilation

Pulmonary ventilation (V_I), tidal volume (V_T) and respiratory frequency (fR) were measured with the pneumotachography method for diving animals (Glass et al., 1983). One animal at a time was placed within the thermostatically controlled experimental apparatus. The animal was able to move freely in the apparatus. The experimental set-up consisted of a perforated plastic box positioned within a second box. The inner box was perforated to minimize water movement and hence prevent volume artifacts. An inverted funnel with an attached pneumotachograph was placed on the top of the animal box. The animal was allowed to breathe inside the box (for details and an illustration see Glass et al., 1983). The apparatus allowed inspirations and expirations to be measured continuously. The method is based on laminar gas flow according to the principle of Poiseuille. A differential pressure transducer (Biopac Systems, model TSD160A) was connected to a data acquisition system that included specific applications software (AcqKnowledge MP 100, Biopac Systems, Inc., Santa Barbara, CA, USA). The withdrawal or injection of a known volume of air was used to calibrate the system. All measurements of pulmonary ventilation were made under constant temperature conditions (25 °C) and atmospheric pressure of 716 mmHg.

2.3. Measurements of aerial and aquatic oxygen uptake (VO2)

The values of the oxygen uptake from air and water were measured in separate experiments because the animal chamber used to measure

pulmonary ventilation was too large to allow an accurate determination of oxygen uptake. The measurements of aerial and aquatic VO_2 were made using a closed system. Each animal was placed individually in a small Plexiglas chamber adapted to allow O_2 measurements in the gas and water phases (aerial and aquatic VO_2). The temperature was stabilized by placing the chamber in a water bath fitted with a cooling/heating system (VWR Scientific, 1160A, Niles, IL, USA) that maintained the temperature at 25 °C. For the water measurements, a peristaltic pump (Harvard App., Millis, MA, USA) was used to provide the flow to the electrode. The water PO_2 was analyzed with an O_2 electrode (FAC Instruments, São Carlos, SP, Brazil) connected to an O_2 analyzer (FAC 204 A). The electrode was calibrated using water equilibrated with pure N_2 (zero) and with room air and was maintained at the temperature used in the experiment. For the aerial measurements, the O_2 levels were monitored using an O_2 analyzer with a built-in flow control pump (Adinstruments, ML206). To remove water vapor, a desiccant (Adinstruments, DM-060-24) was used before the introduction of the analyzer gas.

The aerial and aquatic VO_2 values were then calculated as follows:

$$\text{Aerial } VO_2 \left(\text{mL STPD g}^{-1} \text{min}^{-1} \right) = V_{tot} \cdot (\Delta O_2 / \Delta t) / \text{body mass(g)} \quad (1)$$

$$\text{Aquatic } VO_2 \left(\text{mL STPD g}^{-1} \text{min}^{-1} \right) = V_{tot} \cdot \alpha O_2 (\Delta O_2 / \Delta t) / \text{body mass(g)} \quad (2)$$

where V_{tot} = total water or air volume; αO_2 = water solubility of O_2 (Lomholt and Johansen, 1974; Dejours, 1981); and $\Delta O_2 / \Delta t$ = best-fit regression for the decrease of PO_2 over time.

2.4. Experimental protocols

The animals were placed in the experimental apparatus for measuring pulmonary ventilation at least 24 hours before the beginning of the experiment. The chamber was constantly ventilated with humidified air, and the ambient temperature was controlled and maintained at 25 °C throughout the experiments.

2.4.1. Characterization of the breathing pattern of P. carvalhoi
After 24 hours of acclimation, the respiratory measurements were performed during a 2 hour period under aerial or aquatic normoxic-normocarbic conditions. The inverted funnel in the animal apparatus was flushed with room air at a constant flow rate (0.5 L/min) and monitored with a flowmeter (Sierra Instruments, model 822-13-OV1-PV2-V4). The normoxic condition of the water was maintained with an air pump throughout the experimental protocol.

2.4.2. Effect of aquatic hypoxia or hypercarbia
This experimental protocol was designed to evaluate the ventilatory responses of *P. carvalhoi* to aquatic hypoxia or hypercarbia in a normoxic-normocarbic aerial condition. After 24 h of acclimation, a control measurement of ventilation was performed. The animals were then exposed in random order to aquatic hypoxic (5% O_2) or hypercarbic (5% CO_2) gas mixture. Both conditions were maintained with a gas mixing flowmeter (GF3/MP, Cameron Instrument, Port Aransas, TX, USA) for 2 h each with an interval of 1 h between hypoxia and hypercarbia. The water PO_2 and PCO_2 were measured throughout the experiment (from a 1 mL water sample). Aquatic hypoxia or hypercarbia did not affect the aerial PO_2 or PCO_2.

2.4.3. Effect of aerial hypoxia and hypercarbia
This experimental protocol was designed to evaluate the ventilatory responses of *P. carvalhoi* to aerial hypoxia or hypercarbia in a normoxic/normocarbic aquatic condition. After 24 h of acclimation, a control measurement of ventilation was made. The animals were then

exposed in random order to aerial hypoxia (5% O_2) or hypercarbia (5% CO_2). Both conditions were produced with a gas mixing flowmeter (GF3/MP, Cameron) for 2 hours each, with an interval of 1 hour between hypoxia and hypercarbia. The same animals were used for both aquatic and aerial measurements; however an interval of 3 hours was given between aerial and aquatic exposures. We performed the protocols in random orders; in half of animals, aquatic hypoxia and hypercarbia were applied first and in other half aerial treatments.

The gas phase was flushed at a flow rate of 100 mL/min through an air inlet of the inverted funnel. The normoxic condition of the water was maintained with an air pump throughout the experimental protocol. PO_2 and PCO_2 were monitored throughout experiments and revealed that there was insignificant "cross talk" between water and air.

2.4.4. Effect of combined aerial and aquatic hypercarbia

This experimental protocol was designed to evaluate the ventilatory responses of *P. carvalhoi* to combined aerial and aquatic hypercarbia. After 24 h of acclimation, a control measurement of ventilation was made. The animals were then exposed simultaneously to aerial and aquatic hypercarbia (5% CO_2). Both conditions were produced with a gas mixing flowmeter (GF3/MP, Cameron) for a period of 2 h. The gas phase was flushed at a flow rate of 100 mL/min through an air inlet of the inverted funnel. Measurements of the water PO_2 and PCO_2 were made before the beginning of each protocol.

2.4.5. Aerial and aquatic VO_2 during normoxic normocarbia

Each animal was placed individually in a small Plexiglas chamber at a constant temperature of 25 °C. The water in the chamber was equilibrated with room air for a minimum of 2 h. The gas phase was simultaneously flushed with room air. The water aeration was then stopped, the system was closed and the PO_2 of the water and air were continuously measured for 2 h. As previously mentioned, the decrease of O_2 in the gas and water phase was used to calculate the aerial and aquatic O_2 uptake, respectively. After the 2 h interval measurements, the system was opened, and the animal was gently removed.

2.5. Data analysis

The respiratory frequency (fR) was quantified by analyzing the number of respiratory events (lung breaths) per minute. The tidal volume (V_T) was obtained from the integrated area of the inspired flow signal. Buccal ventilations were identified by small positive and negative pressures, whereas lung ventilations were identified by the greater pressure changes involved. The V_I ($V_I = V_T \times fR$) was expressed as mL BTPS $kg^{-1} \cdot min^{-1}$. All the statistical analysis was performed using a software program (Statistical Analysis System — SAS®; Littell et al., 2002). The effects of hypercarbia and hypoxia on ventilation were evaluated by a two-way ANOVA followed by a point-by-point one-way ANOVA and a paired *t*-test, respectively, to assess the differences among groups. A Tukey–Kramer multiple comparisons test was applied as a post hoc test. One way ANOVA was used to compare the effects of aerial hypoxia/hypercarbia before or after aquatic hypoxia/hypercarbia, as well as aquatic hypoxia/hypercarbia before or after aerial hypoxia/hypercarbia. A $P < 0.05$ significance level was used to identify significant differences among the means.

VO_2 was calculated as detailed above. The data from the 2 h time interval were used to fit the regression line to the values of the decrease in O_2 ($\Delta O_2/\Delta t$) in the water and the gas phases. The aerial and aquatic VO_2 mean values were compared using an unpaired *t*-test, with a *P*-value of 0.05.

3. Results

3.1. Breathing pattern in P. carvalhoi

Pipa carvalhoi exhibited an intermittent breathing pattern, *i.e.*, it emerged sporadically to ventilate its lungs and would then dive and spend long time intervals in apnea underwater. Each episode of single breaths was characterized by an expiration followed by an inspiration (Fig. 1A), which is the most common breathing pattern in this species. Two other types of patterns were observed, i.e., one expiration followed by two inspirations (Fig. 1B) and two expirations followed by an inspiration (Fig. 1C). Furthermore, we visually observed that the species exhibits oropharyngeal cavity respiratory behavior. These events are called buccal oscillations and are also present in other anurans, including *Xenopus laevis* (family Pipidae).

The inspiratory volume was equal (1.51 ± 0.47 mL, N = 79) to expiratory volume.

3.2. Aquatic and aerial gas exchange

Fig. 2 summarizes the partitioning of the O_2 exchange between the air (*via* the lung) and the water (*via* the skin). At 25 °C during normoxia normocarbia, aerial gas exchange provided most of the required O_2 (an aerial VO_2 of 63%) in *P. carvalhoi*, whereas the remaining 37% of the VO_2 was cutaneous.
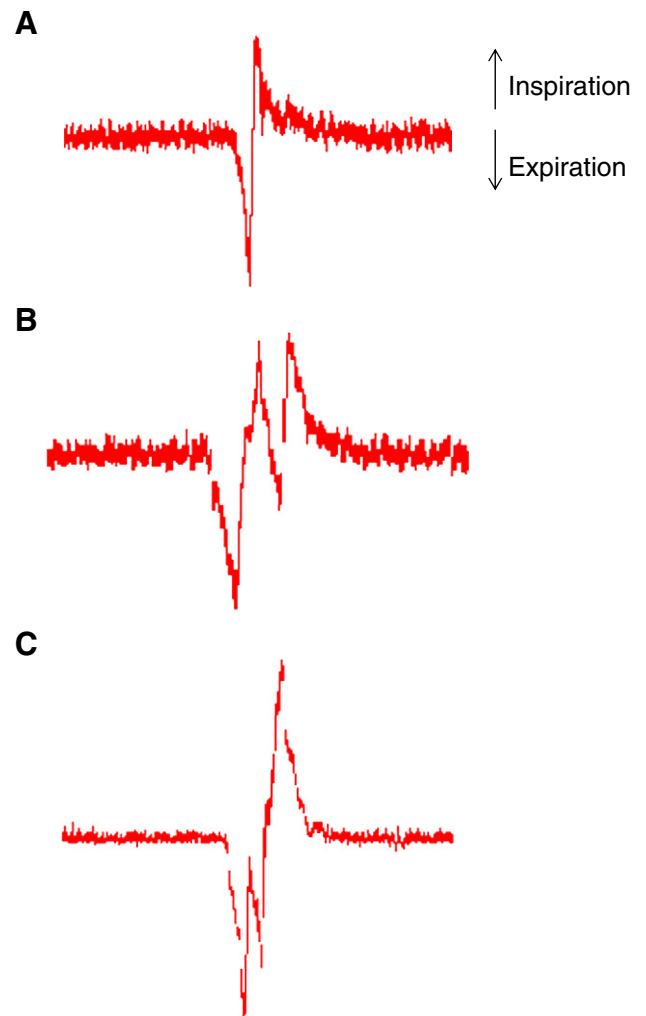

Fig. 1. Types of breathing patterns observed in *Pipa carvalhoi*. A — a single breath characterized by an expiration followed by an inspiration; B — one expiration followed by two inspirations; C — two expirations followed by an inspiration.
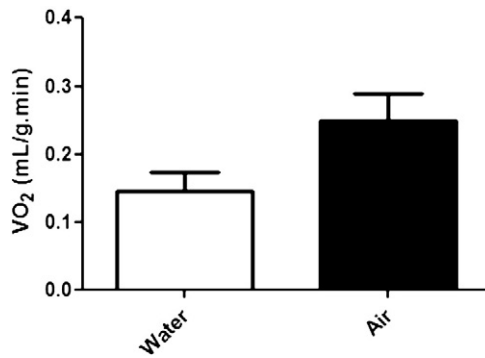
Fig. 2. Aerial and aquatic oxygen consumption (VO_2) in *Pipa carvalhoi* during normoxia normocarbia.

3.3. Ventilatory response to aquatic or aerial hypoxia

Fig. 3 shows representative recordings of pulmonary ventilation during normoxia and aerial or aquatic hypoxia. The values of fR, V_T and V_I for the animals exposed to aquatic or aerial hypoxia are shown in Fig. 4. None of the ventilatory variables were significantly affected by aquatic hypoxia. Aerial hypoxia caused an increase in V_I compared to normoxia ($P<0.0001$) and aquatic hypoxia ($P<0.0001$). This difference was due to an increase in fR, whereas V_T remained unchanged.

Statistical analysis of the data obtained under aerial hypoxia/hypercarbia before or after aquatic hypoxia/hypercarbia, as well as aquatic hypoxia/hypercarbia before or after aerial hypoxia/hypercarbia revealed no carryover effect (data not shown).
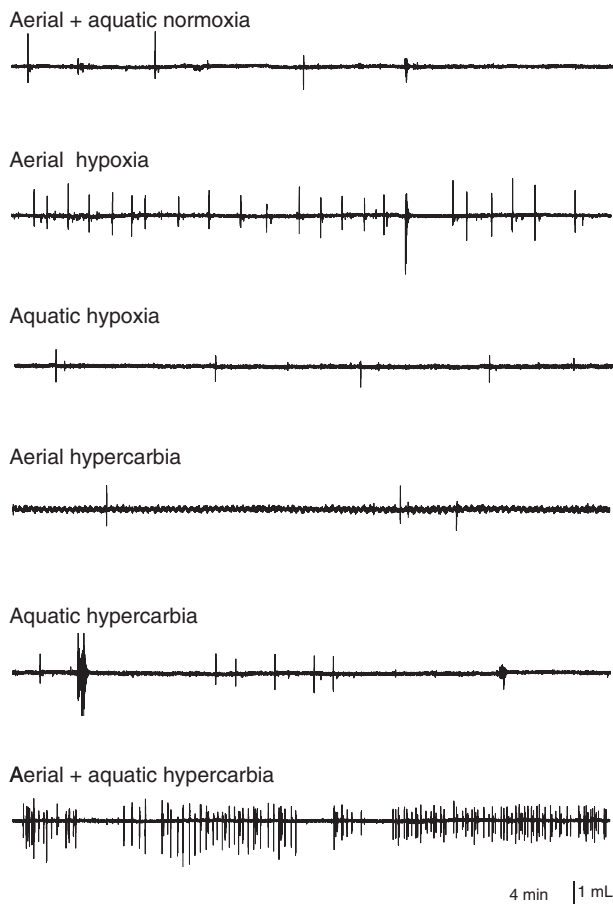

Fig. 3. Ventilation (V_I), tidal volume (V_T) and respiratory frequency (fR) of *Pipa carvalhoi* exposed to aquatic and aerial hypoxia (5% O_2, N_2 balance). *Indicates significant difference from normoxia. +Indicates significant difference from aquatic hypoxia.
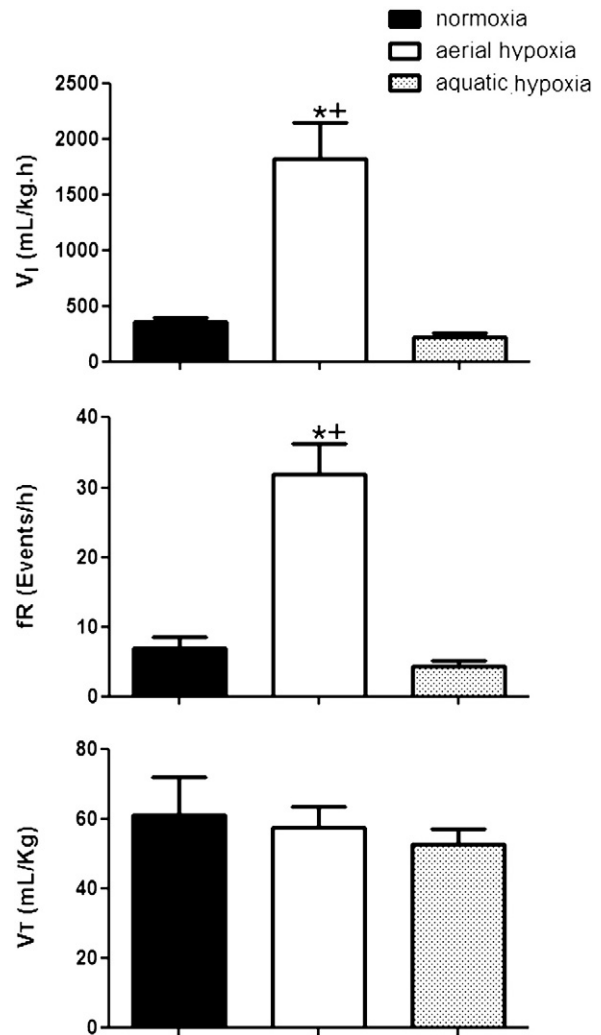

Fig. 4. Ventilation (V_I), tidal volume (V_T) and respiratory frequency (fR) of *Pipa carvalhoi* exposed to aquatic and aerial hipercarbia (5% CO_2, 21% O_2, N_2 balance). *Indicates significant difference from normocapnia. +Indicates significant difference from aquatic hypercarbia. #Indicates difference from aerial hypercarbia.

3.4. Ventilatory response to aquatic or aerial hypercarbia

Fig. 3 shows representative recordings of pulmonary ventilation during aerial and/or aquatic hypercarbia. The responses to aerial and/or aquatic hypercarbia were measured in a separate group of animals (Fig. 5). Aerial or aquatic hypercarbia applied separately did not affect ventilation. Aerial and aquatic hypercarbia applied simultaneously caused a large increase in ventilation ($P<0.0001$) compared with normocarbia, aquatic hypercarbia and aerial hypercarbia. The primary effect of aquatic/aerial hypercarbia on ventilation was a significant increase in fR with no significant change in V_T. The marked increase in fR during aerial/aquatic hypercarbia is noteworthy.

4. Discussion

The ventilatory cycle of aquatic anurans seems to differ from that of terrestrial anurans. A previous study of the frog *X. laevis*, a species that is almost entirely aquatic, has demonstrated that this animal spends most of its time submerged and emerges episodically to breathe at the surface (Boutilier, 1984). The pulmonary pressure is always maintained above atmospheric levels. As in other anurans, the buccal pump mechanism is used to fill the lungs (Gargaglioni and Milsom, 2007). The respiratory cycle begins with expiration and without any previous buccal movement (Brett and Shelton, 1979).
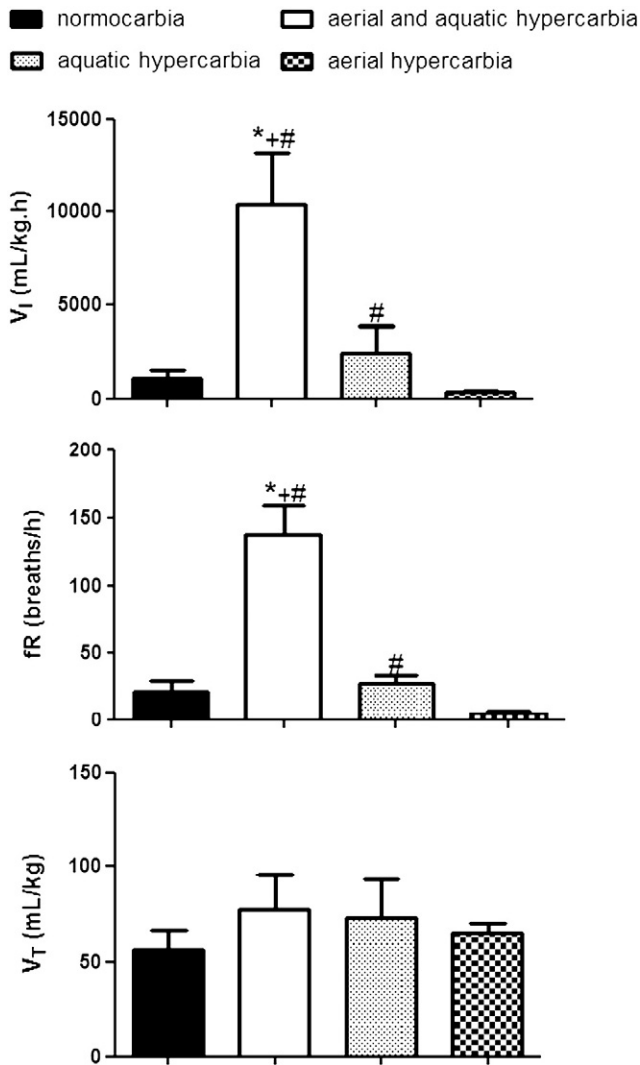
Fig. 5. Pulmonary ventilation recordings obtained of *Pipa carvalhoi* under aerial and aquatic normoxia, aerial hypoxia, aquatic hypoxia, aeria hypercarbia, aquatic hypercarbia or aerial and aquatic hypercarbia.

4.1. Breathing pattern and gas exchange

The breathing pattern observed in *P. carvalhoi* resembles that observed in *X. laevis* (Boutilier, 1984). Normally, the respiratory cycle begins with one expiration followed by one inspiration. According to Boutilier (1984), this pattern decreases the mixture of gases. In the present study, we observed three main types of respiratory events in *P. carvalhoi*: a) an expiration followed by an inspiration, b) an expiration followed by two inspirations, and c) two expirations followed by an inspiration. GeLong dives are periodically interrupted by brief visits to the surface, during which several lung ventilations occur. The animal may also remain submerged in water with its nares at the surface; however this pattern is less frequent. It then ventilates its lungs at intervals over a long time period. In more characteristically terrestrial amphibians, such as the salamanders *Ambystoma maculatum*, *Salamandra salamandra* and *Taricha granulosa* (Whitford and Hutchison, 1965) and the anurans *Rhinella marina* (Macintyre and Toews, 1976) and *Lithobates pipiens* (West and Jones, 1975), lung ventilations are more frequent.

Different from most sarcopterygians, *P. carvalhoi*, *Xenopus* and few other aquatic amphibians are considered four-stroke breathers (Gargaglioni and Milsom, 2007). With this mechanism, the first buccal expansion (first stroke) drives gas from the lungs into the mouth.

Buccal compression (the second stroke) then forces this gas out through the nares. Subsequent buccal expansion (the third stroke) drives in fresh air through the nares and buccal compression (the fourth stroke) then forces this air into the lungs (Gargaglioni and Milsom, 2007). The similarity between the breathing patterns in aquatic amphibians suggests that this breathing pattern is typical of dual medium gas exchangers regardless of ancestry (breathing patterns of primarily terrestrial amphibians).

Our results for O_2 uptake showed that the lungs are responsible for 63% of the VO_2. These findings are similar to previous results for *Xenopus* (Emilio and Shelton, 1974). According to these authors, approximately 70% or more of the total VO_2 is provided by the lungs, whereas the remaining through the skin. Czopek (1955) suggested that the skin capillary network is relatively undeveloped in *Xenopus* compared to other amphibians. In several species of amphibians, especially in terrestrial species, a calcified layer occurs between the *stratum spongiosum* and *stratum compactum* of the dermis (Elkan, 1968; Toledo and Jared, 1993). This layer is absent in *P. carvalhoi*, but it can be detected in *X. laevis* (Greven and Richter, 2009). Therefore, it appears that the skin of *Pipa* is more permeable than that of *Xenopus*.

4.2. Ventilatory response to hypoxia

We observed that the exposure to aquatic hypoxia did not change the breathing ventilation of the animal. PO_2 is an important variable that drives ventilation (Branco and Glass, 1995). The lack of response observed in the present study may result from the absence of changes or the presence of only minimal changes in the blood gases under exposure to aquatic hypoxia. The animal was still able to ventilate its lungs, and this process provided most of the required O_2. Nevertheless, the impact of aquatic hypoxia on the blood gases remains to be tested. However, given the animal's size, this determination is technically very challenging. Our results are in agreement with previous studies on the lungfish *Lepidosiren paradoxa* (Sanchez et al., 2001) and the aquatic caecilian *Typhlonectus natans* (Gardner et al., 2000). In addition, the lack of an effect of aquatic hypoxia on ventilation suggests that these animals may reduce the cutaneous blood flow during aquatic hypoxia, as has been observed in anurans (Malvin and Hlastala, 1986; Gardner et al., 2000).

In contrast to aquatic hypoxia, aerial hypoxia increased the pulmonary ventilation due to an increase in fR without affecting V_T. This pattern of V_I increase was similar to that reported by Boutilier (1984) in *Xenopus*, in which fR produced an increase in V_I. Aerial hypoxia also causes an increase in pulmonary ventilation in the lungfish *L. paradoxa* due to changes in fR (Sanchez et al., 2001). The ventilatory response to aerial hypoxia in *P. carvalhoi* might be attributed to the presence of receptors sensitive to internal oxygen, such as carotid labyrinth as described in the toad *R. marina* (Van Vliet and West, 1992). However, in *X. laevis*, the carotid labyrinth does not play a major role in regulating breathing in hypoxia or hypercapnia (Jones and Chu, 1988). Thus, further studies are needed to identify such receptors in the species *P. carvalhoi*. The large responses to aerial hypoxia compared to aquatic hypoxia suggest that internal PO_2 monitoring is more important than external PO_2.

4.3. Ventilatory response to hypercarbia

Aerial hypercarbia did not affect ventilation in *P. carvalhoi*. A similar lack of response to aerial hypercarbia has been observed for other aquatic amphibians, including *Ambystoma tigrinum* (Boynton and Smatresk, 1993), *Amphiuma sp.* (Toews, 1971) and the aquatic caecilian *T. natans* (Gardner et al., 2000). Interestingly, all of these species depend on aquatic gas exchange for the elimination of CO_2. During aerial hypercarbia, the animal may be eliminating CO_2 through the skin and not altering the pulmonary ventilation.

The aquatic hypercarbia did not alter the ventilation of the animals (Fig. 5). No response may have occurred because the exposure to aquatic hypercarbia may have caused a vasoconstriction and prevented the animal from becoming hypercarbic. Another hypothesis is that these animals do not have chemoreceptors for CO_2. This response differs from that of *Xenopus*, in which the ventilatory response increased during aquatic hypercarbia (Boutilier, 1984). Most amphibians have CO_2 chemoreceptors located in the central nervous system, nasal epithelium, in the labyrinth and in the pulmocutaneous artery (Gargaglioni and Milsom, 2006). However, no previous studies have investigated the possible occurrence of such chemoreceptors in this species.

In air-breathing fish, aerial hypercarbia has no effect on ventilation in certain species but stimulates ventilation in others (Milsom, 2002). Whether or not central CO_2 receptors are present, the nature of the response may depend on the efficacy of gill ventilation in eliminating CO_2. In species in which branchial CO_2 excretion is rapid, the inhalation of CO_2 may not result in any change in the arterial PCO_2. All of the CO_2 inspired by the air exchanger may be eliminated by the aquatic exchanger (Milsom, 2002). In the present study, we did not observe any change in ventilation during separated exposures to aerial and aquatic hypercarbia (Fig. 5). It is reasonable to hypothesize that similarly to what happens in air-breathing fish, CO_2 taken up by air may be rapidly eliminated via skin in *P. carvalhoi*. In this case, the net result would be no change in blood PCO_2. Since, we had no change in ventilation after aerial hypercarbia, it seems unlikely that olfactory chemoreceptors is involved in this response, because such receptors are described to inhibit the ventilatory response to inhaled CO_2. In fact, there is no evidence of the presence of this type of receptors in *P. carvalhoi*. If aquatic and aerial hypercarbia were applied simultaneously, however, we observed a pronounced increase in pulmonary ventilation caused by a marked increase in the breathing frequency. This finding indicates that CO_2 plays a role as a driver of breathing in *P. carvalhoi*. Accordingly, this result suggests that CO_2 chemoreceptors are present in this species. Central chemosensitivity appears to develop slowly in amphibian tadpoles. This type of chemosensitivity is not present in young animals but develops over time (Milsom, 2002). Central chemoreception occurs in other anurans (Gargaglioni and Milsom, 2007).

We conclude that the breathing pattern of *P. carvalhoi* is episodic with single events (*single breaths*) and buccal ventilation. The results also show that this species is more sensitive to reduced oxygen (hypoxia) in the air. In contrast, aerial and aquatic hypercarbia produce little or no change in ventilation in this animal. The increased ventilatory response to combined aerial and aquatic hypercarbia found by this study suggests the presence of CO_2 chemoreceptors.

4.3.1. Perspectives

In the present study we demonstrated that the breathing pattern of *P. carvalhoi* differs from that of other anurans belonging to different families, including *Rhinella* and *Lithobates* (Gargaglioni and Milsom, 2007), and even to the same family, such as *Xenopus* (Boutilier, 1984). This fact gives support to the idea the breathing pattern as well as other respiratory processes in amphibian, or specifically in anurans, vary widely. The accumulation of data about respiratory function in diverse species, which may habit different environments, will certainly bring new insights regarding phylogenetic relations in this group and the evolution of air breathing.

Furthermore, *P. carvalhoi* is known to occur on fish farms, where it is controlled as a pest (Arzabe et al., 2010). The main threats to this species are habitat loss due to agriculture and grazing and pollution due to livestock and pesticides. Therefore, we hope to assist in the conservation of these habitats by contributing to the information produced by research for use by both biologists and the general public. Amphibians are used extensively in physiological studies aimed at generating new insights in evolutionary biology, especially in the

investigation of the evolution of air breathing and terrestriality. Thus, we consider that the study of *P. carvalhoi* will contribute a very useful model for investigating this issue.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10. 1016/j.cbpa.2012.03.020.

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