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## CARDIO-RESPIRATORY INTEGRATION IN DIVING BULLFROG *Lithobates catesbeianus* (Shaw, 1802)

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**SUMMARY:** Cardiac frequency and blood pressure were measured during voluntary diving in the bullfrog, *Lithobates catesbeianus*, exposed to 15°C or, alternatively, 25°C. Diving caused bradycardia and reduction of blood pressure. Cardiac frequency was  $27.1 \pm 2.2$  for breath periods and  $19.3 \pm 2.7$  for diving periods at 15°C, and  $47.7 \pm 4.4$  and  $35.3 \pm 3.6$  for 25°C. Systolic blood pressure was  $39.6 \pm 0.9$  for breath periods and  $36.2 \pm 0.4$  for diving periods at 15°C, and  $42.4 \pm 1.0$  and  $39.8 \pm 0.5$  at 25°C. Diastolic blood pressure was  $32.3 \pm 0.6$  for breath periods and  $29.5 \pm 0.5$  for diving periods at 15°C, and  $35.3 \pm 0.9$  and  $33.2 \pm 0.6$  at 25°C. The animal was artificially ventilated during diving with normoxic, hypoxic (5% O<sub>2</sub>) and hypercarbic gas mixtures (6% CO<sub>2</sub>). None of these procedures produced any change of blood pressure or cardiac frequency. Cardio-vascular effects have, however, been reported in some other studies on amphibians. As a new aspect, we applied artificial ventilation during a voluntary dive in the frog. Surfacing, elicited tachycardia and elevated blood pressure. These cardiovascular reflexes depend on a stimulus that still needs to be identified.

**Keywords:** Bullfrog. Diving. Bradycardia. Arterial blood pressure. Artificial ventilation. Gas mixtures.

**RESUMO:** A frequência cardíaca e a pressão arterial foram medidas durante o mergulho voluntário na rã *Lithobates catesbeianus*, exposto a 15 ° C ou, alternativamente, a 25 ° C. O mergulho causou a bradicardia e redução da pressão arterial. A frequência cardíaca foi  $27.1 \pm 2.2$  para períodos respiratórios e  $19.3 \pm 2.7$  para os períodos de mergulho à 15°C, and  $47.7 \pm 4.4$  e  $35.3 \pm 3.6$  à 25°C. A pressão arterial sistólica foi  $39.6 \pm 0.9$  para os períodos respiratórios e  $36.2 \pm 0.4$  para períodos de mergulho à 15°C, e  $42.4 \pm 1.0$  e  $39.8 \pm 0.5$  à 25°C. A pressão diastólica foi  $32.3 \pm 0.6$  para períodos respiratórios e  $29.5 \pm 0.5$  para períodos de mergulho à 15°C, e  $35.3 \pm 0.9$  e  $33.2 \pm 0.6$  à 25°C. O animal foi utilizado para ventilação artificial durante o mergulho com normóxia, hipóxia (5% de O<sub>2</sub>) e misturas de gases hipercárbica (6% de CO<sub>2</sub>). Nenhum desses procedimentos produziu qualquer alteração da pressão arterial ou frequência cardíaca. Os efeitos cardio-vasculares, no entanto, têm sido relatado em alguns outros estudos sobre os anfíbios. Como um novo aspecto, foi aplicado a ventilação artificial durante um mergulho voluntário no sapo. O ato de toca a superfície resultou em taquicardia e aumento da pressão arterial. Estes reflexos cardiovasculares dependem de um estímulo que ainda precisa ser identificado.

**Palavras-chave:** Rã-touro. Mergulho. Bradicardia. Pressão arterial. Ventilação artificial. Mistura de gás.

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### INTRODUCTION

Amphibians develop bradycardia when submersed, and the return to the surface is accompanied by tachycardia (ANDERSEN 1966; JOHANSEN, 1966). This coupling of cardiac frequency to ventilation has been reported for forced and voluntaries dives ( JONES

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and SHELTON, 1964; SHELTON and JONES, 1965; JONES, 1966; JONES, 1967; LILLO, 1978). Heath (1980) analyzed larval stages as well as adult specimens of the tiger salamander *Ambystoma tigrinum*, and all stages developed tachycardia during emersion. As a more complicated pattern, adult *Lithobates catesbeianus* developed tachycardia when surfacing, whereas this response was absent in the larval form (BURGGREN; MWALUCANA, 1983; WEST; BURGGREN, 1983). Burggren and Doyle (1986 b) also reported that early stages of *Lithobates catesbeianus* only developed ventilation-heart rate coupling after metamorphosis.

Bradycardia may delay depletion of the intrapulmonary O<sub>2</sub> store (JONES and MILSOM, 1982; SHELTON; BOUTILIER, 1982) and, consistently, aquatic hypoxia and hypercarbia reduced diving time in urodeles (*Ambystoma tigrinum*, *Amphiuma tridactylum*) and in anurans (*Rana temporaria*) (JONES, 1966; LUND; DINGLE, 1968; JONES, 1968; JONES, 1972; BURGGREN; DOYLE, 1986 b; LILLO, 1978).

The focus of this study is how diving and surfacing are related to various stimuli. *Rana pipiens* has external mechanoreceptors at the nares, and these prevent water from entering the buccal cavity during dives. Bilateral section of the ophthalmic nerves of *Rana pipiens* had no effect on normal ventilation in air, but submergence was associated with an influx of water to the buccal cavity (WEST; JONES, 1976; WEST; VAN VLIET, 1983).

Little information is available for blood pressures and cardiac frequency during artificial ventilation of the lung of diving anurans. Moreover, the information is scarce as to the mechanisms that cause tachycardia upon surfacing.

To address this question applied artificial ventilation with different gas mixtures in a freely diving *Lithobates catesbeianus*, and cardiovascular reflexes during breathing and diving at were evaluated two temperature (15 and 25°C).

## **MATERIALS AND METHODS**

### **Animals**

Specimens of *Lithobates catesbeianus* (mean body weight  $0.210 \pm 0.25$  kg; mean  $\pm$  SEM; n=7) were collected close to Ribeirão Preto, São Paulo, Brazil. They were maintained at the animal holding facility of the Faculty of Medicine of Ribeirão Preto, USP and kept in 1000 L tanks containing shallow water at 25°C. Earthworms were provided once a day except for the last 48h before experimentation.

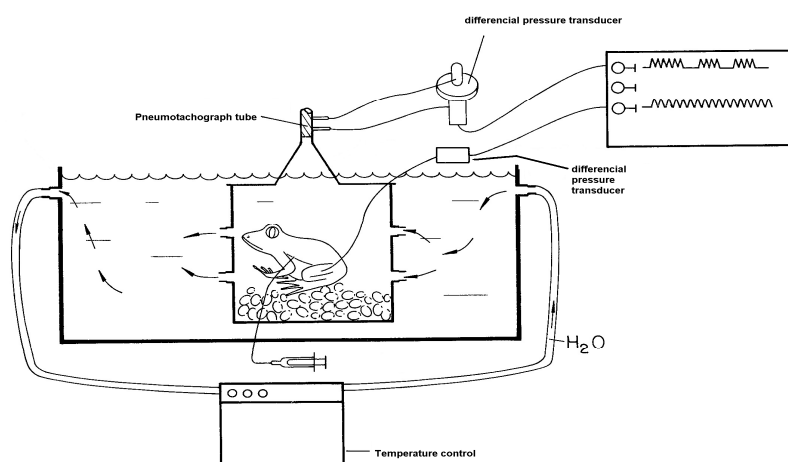
### **Surgical procedure**

The animal was placed into a benzocaine solution (1g·L<sup>-1</sup>) for 10 min, which eliminated

reflexes, which allowed to catheterize the right femoral artery, using a PE50 catheter (BOUTILIER et al., 1979A). The lung was also catheterized, using a PE90 catheter (For details see GLASS et al., 1978). Both catheters were secured to the skin and incisions were closed using interrupted sutures. The frog recovered in less than 15 min, when placed in benzocaine-free water, after which the animal recovered for 24 h.

### Measurements of pulmonary ventilation

Lung ventilation was measured using pneumotachography for diving animals. The animal was placed into a 10 L aquarium, where it could respire within an inverted funnel that sealed the airspace by a slight emersion into the water. A Fleisch tube was attached to the neck of the funnel, which allowed measuring respiratory flows according to the Poiseuille principle (Glass et al., 1978). A differential air pressure transducer (Statham 12123) was attached to a multichannel recorder (Narco Biosystems, Inc., Houston TX, USA), and pulmonary ventilation ( $V_E$ ) was continuously recorded along with blood pressures. The arterial catheter was connected to a pressure transducer (COBE Laboratories, Inc., Lakewood CO; USA) (Figure 1).



**Figura 1:** a experimental hydraulic system apparatus permitting voluntaries dives, in normoxic conditions, at 25°C. The animal was kept in a 7,3 L receptacle inside of a 30 L aquarium. The receptacle was equipped with holes in the walls, that permitted the free movement of water, and a funnel-shaped chamber in a surface, open to the air. To monitor pulmonary ventilation, a pneumotachograph tube, was placed onto the outlet of the chamber. the duration of submersions and cardio-respiratory responses (cardiac frequency and blood pressure) in voluntaries dives of *Lithobates catesbeianus* during artificial lung ventilation, at 15°C. The artificial lung ventilation was performed during animal dives, using a 20 ml syringe connected with a lung catheter, with a tidal volume of 10 ml.mim<sup>-1</sup>. Tree gas conditions was used in the artificial lung ventilation, normoxic (atmospheric air), hipoxic (5% O<sub>2</sub> in N<sub>2</sub>) and hyperoxic (6% CO<sub>2</sub> in air).

## Experimental protocols

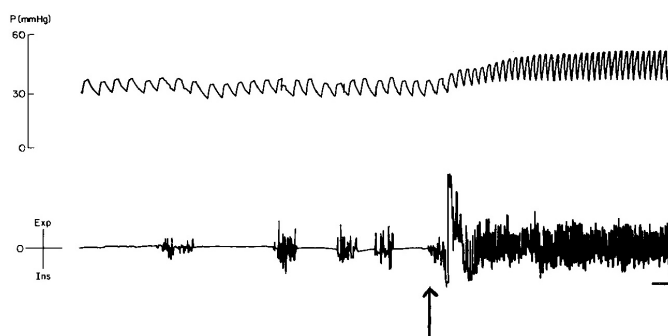
We recorded the duration of dives and lung ventilation, while cardiac frequency ( $f_H$ ) and blood pressures were recorded at 15°C or, alternatively, 25°C. We calculated systolic arterial pressure (Ps) and diastolic arterial pressure (Pd). At 15°C, the measurements were repeated, but artificial lung ventilation was applied during the dives, using a 20 ml syringe connected to the intrapulmonary catheter. Three gas mixtures were used for artificial lung ventilation at 10 ml min<sup>-1</sup>: atmospheric air, hypoxia (5% O<sub>2</sub> in N<sub>2</sub>) and hypercarbia (6% CO<sub>2</sub> in air).

## Statistics

Statistics was performed using one-way analysis of variance, followed by Bartlett's test for equal variances. With equal variances ( $P < 0.05$ ) we applied Bonferroni's multiple comparison test for differences between individual means. Logarithmic transformations were applied in case of unequal variance. If this failed, Friedman's test was applied followed by Dunn's multiple comparison test for differences between means. Values are expressed as mean  $\pm$  SEM with significance level  $P < 0.05$ ;  $n = 7$ .

## Results

The cardiac frequency was significantly different for diving and respiration at the surface ( $P < 0.05$ ), with  $27.1 \pm 2.2$  for breath periods and  $19.3 \pm 2.7$  for diving periods at 15°C, and  $47.7 \pm 4.4$  and  $35.3 \pm 3.6$  for 25°C. The cardiac frequency in water at 15°C was significantly lower than at 25°C. The figure 2 illustrates an example of transition from bradycardia to tachycardia when surfacing.



**Figure 2:** Cardiograph register showing the moment that the animal touch the water surface to ventilate his lungs, with a notable tachycardia. We can note bradycardia in the registers during diving. The arrow show the animal emersion and the right small trace below the register represent the time of 10 seconds.

Diving significantly reduced Ps and Pd (Pressures during systole and diastole) relative to values during surface ventilation (Table 1).

**Table 1.** Cardiac frequency during ventilatory periods (VP) and non ventilatory periods (NVP), at 15°C and 25°C.

$f_H$	Condition	Temperature	Mean $\pm$ SEM
	VP	15°C	27.1 $\pm$ 2.2 <sup>+</sup>
(beat/min)	NVP	15°C	19.3 $\pm$ 2.7* <sup>+</sup>
	VP	25°C	47.7 $\pm$ 4.4
	NVP	25°C	35.3 $\pm$ 3.6*

Mean  $\pm$  SEM,  $n=7$ . \* indicate significant difference from PV and + significant difference from 25°C.

An increase of temperature from 15 to 25°C significantly influenced Pa (Tables 2 and 3) and cardiac frequency in general. Artificial lung ventilation had no significant effect ( $P < 0.05$ ) on any of the cardiac variables ( $f_H$ , Pa, Pd), and this was independent of the gas mixture applied. (Table 4).

**Table 2.** Systolic blood pressure during ventilatory periods (VP) and non ventilatory periods (NVP), at 15°C and 25°C.

Ps (mmHg)	Condition	Temperature	Mean $\pm$ SEM
	VP	15°C	39,6 $\pm$ 0,9 <sup>+</sup>
	NVP	15°C	36,2 $\pm$ 0,4* <sup>+</sup>
	VP	25°C	42,4 $\pm$ 1,0
	NVP	25°C	39,8 $\pm$ 0,5*

Mean  $\pm$  SEM,  $n=7$ . \* indicate significant difference from PV and + significant difference from 25°C.

**Table 3.** Diastolic blood pressure during ventilatory periods (VP) and non ventilatory periods (NVP), at 15°C and 25°C.

Pd (mmHg)	Condition	Temperature	Mean $\pm$ SEM
	VP	15°C	32,3 $\pm$ 0,6 <sup>+</sup>
	NVP	15°C	29,5 $\pm$ 0,5* <sup>+</sup>
	VP	25°C	35,3 $\pm$ 0,9
	NVP	25°C	33,2 $\pm$ 0,6*

Mean  $\pm$  SEM,  $n = 7$ . \* indicate significant difference from PV and + significant difference from 25°C.

**Table 4.** Cardiovascular values during artificial ventilatory periods (AVP) and non ventilatory periods (NVP), at 15°C.

	NVP	AVP (AR)	AVP (6% CO <sub>2</sub> )	AVP (5% O <sub>2</sub> )
F <sub>H</sub> (beat/min)	25,3 $\pm$ 0,6	24,9 $\pm$ 0,6	24,6 $\pm$ 0,7	25,2 $\pm$ 0,1
SAP (mmHg)	44,4 $\pm$ 0,9	43,9 $\pm$ 0,8	43,3 $\pm$ 1,1	44,9 $\pm$ 0,5
DAP (mmHg)	36,6 $\pm$ 0,8	36,4 $\pm$ 0,6	35,7 $\pm$ 0,7	37,1 $\pm$ 0,4

Mean  $\pm$  SEM,  $n = 7$ . \* indicate significant difference from PV and + significant difference from 25°C.

## DISCUSSION

*Lithobates catesbeianus* confirmed the developed of bradycardia during diving, like showed in the last studies with different species of amphibians. Therefore, the new information from this study is that artificial ventilation of the submerged animal will not elicit any cardio-vascular responses and this is independent of the composition of the intrapulmonary gas. This invites to try to identify a possible stimulus to tachycardia at the surface.

Numerous studies report on increased pulmonary perfusion during lung ventilation and reduced perfusion during breath-holds (SHELTON, 1970, 1976, with *Xenopus*; Johansen et al.(1970) with *Lithobates catesbeianus*; West and Burggren (1984); White and Ross (1966) and Wang et al. (2004), with *Bufo marinus* ). Moreover, diving involves a left-to-right shunt that reduces perfusion of the lung (SHELTON, 1985). In *Amphiuma* 60% of the cardiac output perfuse systemic tissues during apnea. Conversely, this percentage was reduced 20%

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during ventilation of the lung (TOEWS et al., 1971). Lung inflation of *Rana temporaria* seems to overturn bradycardia (JONES, 1966) and cause pulmonary vasodilatation (EMILIO and SHELTON, 1972).

Our data are, however, consistent with Wang et al. (2004) on decerebrated and unidirectional ventilated *Bufo marinus* that exhibited small but distinct cardiovascular changes associated with apnea.

*Lithobates catesbeianus* had no cardiovascular responses to artificial ventilation with gas mixtures. By contrast, Jones (1966) reported tachycardia during forced diving in *Bufo bufo*, *Rana pipiens* and *Rana temporaria*. Anesthetized *Bufo arenarum* increased fc and Pa in response to artificial ventilation. Segura et al., (1981) reported that autonomic blocking combined with removal of bulbo-mesencephalic centers failed to abolish cardiovascular responses to artificial lung ventilation, and they suggested that these reflexes depend on pulmonary receptors. Amphibians inflate the lungs before diving, which motivated (SHELTON; BOUTILIER, 1982) to exclude the hypothesis that the increases of cardiac frequency results from expansion of the lung. Wang et al., (2004) have shown in spite of no statistically significant cardiovascular changes during lung inflation of *Bufo marinus*, there was a tendency for heart rate, blood pressure and pulmocutaneous flow to be reduced particularly in hypercapnic animal.

Moreover, Johansen et al. (1977) recorded tachycardia during diving of the turtle *Pseudemys scripta*, exposed to artificial ventilation. Composition of the gas mixture had no specific effects.

There are evidences that chemoreceptor's are involved with these reflex. In *Xenopus* the lung insuflation with pure oxygen result in higher vasodilatation than atmospheric air, but the last one responded better than nitrogen mixtures (EMILIO; SHELTON, 1972). Studies with *Lithobates catesbeianus* showed increase in lung perfusion during hypoxic condition in water and the opposite was observed when the animal inspired hypoxic mixture (BOUTILIER et al., 1986). These receptors can be involved within cardiac debt and intracardiac shunt, influence the blood flow resistance in different ways (SHELTON, 1985). Studies with reptiles have shown similar responses, for example in turtle *Pseudemys scripta*, the spontaneous diving result in bradycardia, reduction of the lung blood flow and increase pulmocutaneous blood flow. The opposite was observed when the turtle came to surface (SHELTON; BURGGREN, 1976; SHELTON; BOUTILIER, 1982). Last studies with this turtle showed that Right-to-Left intracardiac shunt was modulated for vagal eferences (HICKS, 1993; WANG; HICKS, 1996).

The amphibian lung mechanoreceptors sensitive to CO<sub>2</sub> in spite to don't be complete understated , probably are involved in the cardio-respiratory regulation during ventilation (SHELTON, 1970; KUHLMANN; FEDDE, 1979; WEST; BURGGREN, 1983, 1984 ) and byonce of aquatic animals (TAGLIETTI; CASELLA, 1966; EVANS; SHELTON, 1984).

White (1966) suggested that superior centers were involved too in these cardio-respiratory reflexes. It has been shown that cardiac vagal motoneurons are located close to the respiratory centers in the medulla of *Xenopus* (IHMIED; TAYLOR, 1992; WANG et al., 1999a ; 1999b).

Jones (1966) observed the elimination of the tachycardia during the animal surface, after bilateral vagotomy. The same wasn't observed after cut sympathetic heart connections. It suggested that tachycardia during animal surfacing is result to the heart reduction parasympathetic activate, had been proposed by Jones (1966) that lung receptor and baroreceptor were involved in this reflex. The pulmonary vagotomy didn't abolish the cardiac frequency increase associated with lung ventilation in *Xenopus laevis* (EVANS; SHELTON, 1984), suggesting that stretch receptors don't are totally responsible in cardio-respiratory modulation.

We observed cardiac frequency alteration before the lung ventilation, when *Lithobates catesbeianus* was moving to surface. Although the artificial lung ventilation during *Lithobates catesbeianus* diving haven't shown any cardiovascular response, the contact of nares in water surface was immediately followed by significative cardiovascular alterations, with tachycardia and arterial blood pressure increase.

Results obtained from *Lithobates catesbeianus* after olfactory receptor denervation indicate that tonic stimulation of olfactory receptors by airways CO<sub>2</sub> inhibited breathing during hypercarbia (KINKEAD; MILSOM, 1996). These authors suggested that although olfactory and pulmonary receptors feed-back shape the breathing pattern, they were not responsible for initiating or terminating the episodes of breathing.

The cardiovascular responses to touch water surface in *Lithobates catesbeianus* is similar to trigemio reflex observed in birds and mammals, characterized by bradycardia when animal toch its noise in water (DYKES, 1974; ELSNER et al., 1977; DALY et al., 1979; ECKBERG, et al., 1984). In this reflex, the central cutaneous receptors aferences of trigemio nerve reduce cardiac frequency by vago eferences reflex (ECKBERG et al., 1984).

Dykes (1974) suggested the hypothesis that in mammals, the diving bradycardia was influence by two sensorial ways: stretch lung receptors associated with cutaneous face receptors. Dykes (1974) noted the bradycardia during apnea in seal, similar condition like observed in amphibians patterns respiration.



Milsom et al. (2004) has been shown in a revised study that there are CO<sub>2</sub>/H<sup>+</sup> sensitive pulmonary receptors (intra pulmonary chemoreceptor's and pulmonary stretch receptors) regulating the breathing patterns in all vertebrates in a manner that reduces dead space ventilation and enhances the efficiencies of CO<sub>2</sub> excretion under conditions of environmental hypercarbia and/or reduces de CO<sub>2</sub> loss from hyperventilation.

Although more studies are necessary, our studies corroborate with the vision of a complex receptors integration responses during the physiology function of amphibians diving, showing that cardiovascular responses and ventilatory movements are intergraded with environment conditions and animal behaviors, with efficient O<sub>2</sub> up take, CO<sub>2</sub> elimination and evicting water to get in the lungs.

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