Trabajo

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Effects of vagotomy and pharmacological blocking on heart rate of the toad *Rhinella arenarum* (Anura: Bufonidae) during forced submersion

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ABSTRACT

Toads of *Rhinella* (=Bufo) *arenarum* were subjected to a condition of forced submersion during a 40-minute period, to deepen the understanding of the mechanisms involved in the control of both submersion and emersion. Animals previously submitted to a first control measurement series, were then injected with atropine or propranolol, or were vagotomized. In animals either injected with atropine or vagotomized, a higher heart rate (HR) than in the control group was observed, before, during and after the forced submersion. These animals were able to decrease HR, although in a lesser extent than controls, suggesting that other mechanisms in addition to vagal activity are also producing and maintaining bradycardia during the submersion period. In those animals injected with propranolol (a β-adrenergic blocker), a HR lower than that of controls were observed only at the beginning of the submersion and during the emersion phase. The low HR caused by propranolol during emersion with buccal pumping shows the importance of the sympathetic system in increasing HR during emersion.

Key words: Amphibians; Submersion; Atropine; Propranolol; Autonomic System; Cardiac Physiology.

Introduction

The heart of amphibians is innervated by vagal fibers that reach the *sinus venosus* via Remak's ganglion and continue along the atrial septum into Bidder´s ganglion at the atrioventricular junction. Some branches also innervate the ventricles (Campbell *et al*., 1982; Preston and Courtice, 1995). Although these vagal fibers are cholinergic, slowing down the heart by means of mechanisms sensitive to atropine, in some amphibians these fiber also contain neuropeptide transmitters co-localized in the those cholinergic fibers, such as somatostatin and galanin (Campbell *et al*., 1982; Morris *et al*., 1989), which can exert both direct and indirect effects on heart rate (HR) (Campbell *et al*., 1982; Preston and Courtice, 1992, 1993; Courtice and Delaney, 1994). Besides, as in most other vertebrates, the heart of amphibians is also innervated by sympathetic fibers (Taylor *et al*., 2014). Moreover, it is assumed that diving bradycardia is caused by both an increased parasympathetic activity and a decreased sympathetic activity (Signore and Jones, 1996; Randall *et al*., 2002; Elliott *et al*., 2002). In amphibians, the causes of bradycardia during submersion are not completely understood. In *Rhinella marina*, the decrease of HR during submersion has been enhanced by electrical stimulation of the vagus nerve (Courtice and Delaney, 1994; Preston and Courtice, 1995). However, the bilateral section of the vagus nerve, thus isolating the heart from the parasympathetic influence, did not impede the development of a moderate bradycardia in *R. pipiens*; at the same time, the vagotomized animals showed a delay to reach a maximum bradycardia, as well as to recover after submersion (Lund and Dingle, 1968). The distinction between the emersion with or without a buccal pump working is also important, since it has been found that the activities of the systemic heart and of lymph hearts in anurans after a prolonged submersion differ, depending on the

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presence or absence of buccal pumping (Affanni *et al*., 1999; Cervino *et al*., 2007).

A first approach to understanding the neural regulation of heart rate would be to block both the sympathetic and parasympathetic activities by means of pharmacological tools. Atropine behaves as an antagonist to acetylcholine; its application in frogs -*Rana pipiens* and *Lithobates catesbeianus*- increases HR during aerial respiration and suppresses bradycardia during submersion (Lillo, 1979). Propranolol has a similar affinity for β_1 and β_2 receptors, therefore being a non-selective β-adrenergic antagonist that causes a decrease in both HR and myocardial contractility. In the specific case of the frog *L. catesbeianus,* propanolol produces a sharp decrease in HR during submersion (Lillo, 1979).

This work was aimed at providing more evidence on the autonomic control of heart rate in the toad *Rhinella (=Bufo) arenarum* during both submersion and emersion, by administering different pharmacological antagonists of either adrenergic or cholinergic receptors, or by means of bilateral vagotomy. We hypothesize that there is more than one autonomic mechanism involved in maintaining the deep bradycardia of systemic heart during submersion.

Materials and methods

Animals

Adult male and female *R. arenarum* toads, weighing between 115 and 120 g, were used. The specimens were captured on the outskirts of Luján city (Province of Buenos Aires), throughout March and April. They were transported to the laboratory and kept in plastic cages provided with wet wooden chips. The animals were acclimated for fifteen days prior to the beginning of the experiments, and were not fed either during this period or during the experiments. Temperature ranged from 18 to 22°C, while photoperiod was maintained at 12:12 (L:D).

Recording of heart rate (HR)

The animals were chronically implanted with metallic electrodes connected to a pre-fitted cable by means of Michel suture clips. The electrodes were fixed dorsally to the skin, forming a triangle: one electrode positioned on each scapula, and a third electrode on the posterior area, on the left side of the body. Bipolar derivations between an anterior and a posterior electrode were recorded, whereas the third electrode served as ground reference. To record the actual HR, the electrodes were connected to an AC-amplifier of 8 channels (Exxer, Bernal, Argentina). The optimum values of the signals (band pass 10-40 Hz) were set. Moreover, it was checked that the electrode impedance was low enough (typically <5 KΩ). The signals were acquired and digitized at 64 Hz. For programming the acquisition routine and the data visualization and analysis, the Rhythms 10.0d software (Stellate Systems, Canada) was used. HR was determined by counting the electrocardiographic "spikes" in one minute.

Recording of buccal pumping activity

This activity was recorded by means of two nicrome electrodes (0.3 mm wide) inserted in the submaxillary muscles as fish hooks. The insulation coat from the tips was removed and the electrodes were connected to the recording system. Thus, an electric signal related to the activity of the buccal pumping was recorded (Affanni *et al*., 1999).

Submersion

Toads were placed in a glass aquarium (50 cm x 35 cm x 10 cm) with a perforated acrylic cover secured at 6 cm under the upper border, to avoid toads escaping. Another recipient (supply recipient) was filled with tap water, aerated and dechlorinated by means of a vigorous bubbling. Water temperature ranged from 20 to 22º C during all experiments. A plastic pipe connected to the supply recipient provided a continuous water flow (30 L/h) to the glass aquarium (having a capacity of 15 L), where each animal was successively placed. A second pipe allowed the water outflow, at a rate similar to the inflow. The whole device was placed inside a Faraday cage, so as to minimize the electrical noise during the recordings. The animal behavior was monitored throughout the experiments by means of a close-circuit TV system.

Submersion protocol

The toads were placed in an aquarium containing wet sand. An initial electrocardiogram (ECG) was measured while toads ventilated their lungs through buccal pumping (air temperature: 21º ± 2ºC). Animals were then left to rest for 40 min. The moment at which the nares were covered by water (detention of the buccal pumping) was considered as time zero of submersion. All the recordings began between 10 AM and 2 PM.

Pharmacological blocking
Both atropine sulphate (3 mg/kg, John Martin SRL) and propranolol (6 mg/kg, Gador®) to were injected, at concentrations similar to those used in previous studies (Herman and Sandoval, 1983; Gamperl *et al*., 1999); 1 mL of amphibian Ringer´s solution was used as vehicle. Both drugs were specifically applied to block, respectively, cholinergic-muscarinic and β-adrenergic receptors. Both drugs were injected into the dorsal lymph sac.

Bilateral vagotomy
Eight toads, whose HR had been previously recorded during the entire submersion protocol, were submitted to a surgical vagotomy (VGX). After being anesthetized with benzocaine solution (2 $g.L^{-1}$) for 15 min or until the corneal reflex disappeared, an incision on each dorso-lateral zone immediately caudal to the head was made. Once both vagus nerves were localized under stereoscopic Lupe (Zeiss), a section of 2-3 mm was cut from each one. Incisions were then carefully sutured. Animals recovered from this surgery with no apparent change in their behavior. A few animals (n=3) were *sham* operated, following all the steps above mentioned except the vagus cutting.

Experimental protocols
Experiment 1. The effect of both pharmacological blockers was assayed in air-breathing animals, 24 to 48 h following the electrode implantation. This first experiment took 60 minutes and comprised three experimental groups: 1) injected with atropine (n=9) and 2) injected with propranolol (n=7), at the doses indicated above. HR was calculated at 7.5-minute intervals for one hour. A third group composed of five toads was used as control group, injecting 1 mL of amphibian Ringer´s solution into the dorsal lymph sac.

Experiment 2. After a period of 24 to 48 h following the electrode implantation, all animals were submitted to a first recording under submersion, which served as control for comparing to any further treatments made on the same animals (repeated measured design). After an additional period of 24 to 48 h, they were recorded again under submersion, but receiving a pharmacological treatment, i.e., atropine (n=6) or propranolol (n=6). The submersion began 10 minutes after the drugs had been injected in order to allow their maximum effect, as seen from the results of experiment 1. Animals used were different to the ones used in experiment 1. HR was recorded during the 10 min preceding submersion, during the 40-min forced submersion and finally during the first 15 min of emersion. In all cases, HR was calculated at 5-min intervals, for further statistical analysis.

Experiment 3. To study the effect of surgical bilateral VGX, implanted animals were first submitted to a control submersion, to be surgically operated 24 h after; 24 to 48 h after being operating, they were submitted to forced submersion. HR of the systemic heart was calculated during their air-breathing period (before submersion), at different submersion times and every 5 min during emersion, with or without buccal pumping. The experimental groups were 1) toads with bilateral VGX (n=8) and 2) toads undergoing *sham* surgical procedure (n=3).

Ethical procedures
The animals were treated following the code of ethics outlined by the Canadian Council on Animal Care (Olfert et al. 1993) and also according to the Argentine law.

Data collection and analysis
The systemic heart rate was recorded as beats/min for each consecutive period of 5 min, by averaging the ECG systolic frequency from 10 intervals of 10 sec each, randomly taken within each 5 min-period. The collected HR data were analyzed by means of a two-way (treatment and time) ANOVA with repeated measures, followed by planned contrasts (SigmaStat 3.5, Systat Software Inc., 2006). Statistical differences were considered significant when p-values < 0.05.

Results

Effects of pharmacological blocking on HR during air breathing
The control group (injected with Ringer solution) showed a basal and steady HR during the 60-min recording out of the water (27.9 ± 1.3 $beats.min^{-1}$), and did not differ ($p>0.05$) from the initial values (time zero) of both atropine and propanolol groups (Fig. 1). The animals injected with atropine showed a significant increase ($p<0.001$) in HR during the 60-min duration of the experiment, increasing on average up to 40%. Propranolol resulted in a rapid and significant decrease (25 %, $p<0.001$) of HR until the end of the experiment (Fig. 1).

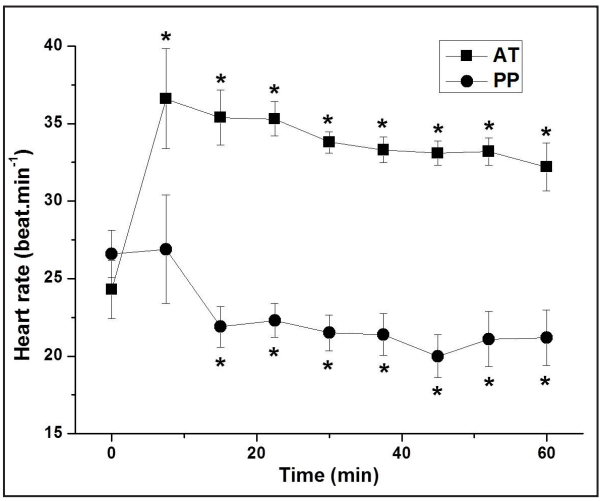

Figure 1. Heart rate (means ± standard errors) during air breathing (Exp. 1). AT: atropine injected (n= 9); PP: propanolol injected (n= 7). Asterisks (*) indicate significant differences ($p<0.05$) with respect to 0 min values.

Behavior and HR during control submersions

During the pre-submersion period, buccal pumping was normal (frequency ~1.5 Hz) with brief apnea episodes. Once submersion began, control animals remained quiet until the water covered the nares. At that time, buccal pumping immediately ceased (post-submersion apnea) and a constant and gradual decrease of HR was observed for approximately 10 min. During the whole forced submersion period (40 min), animals developed a consistent bradycardia (CT: control animals, Figs. 2 and 3). Eventually, abrupt movements of swimming were noted, together with a brief tachycardia. Once emerged, toads remained up to 5 min in tonic immobile state, with no activation of buccal pumping. When buccal pumping was initiated (at a frequency of 2.5-3.0 Hz), the systemic bradycardia eventually reverted to a tachycardia for a few minutes (Figs. 2 and 3). Finally, both HR and buccal pumping reached the values previous to submersion.

Averaging the HR values of the control animals from the three experiments (Figs. 2 and 3), heart rate significantly ($p<0.05$) decreased from 28.9 ± 1.6 beats.min^{-1} to 13.6 ± 1.5 beats.min^{-1} (53% of decrement) within 10 min of submergence, which extended up to the end of the 40-min submersion period. During emersion, HR increased slowly at the first phase (no buccal pumping), averaging 15.9 ± 1.2 beats.min^{-1}, to suddenly increase during the next phase, when buccal pump was initiated. Finally, after 10 min of buccal pumping, HR stabilized to reach the values observed prior to submersion (Figs. 2 and 3).

Effect of cholinergic blockade on HR during submersion

Prior to submersion, there was a marked effect of autonomic blockade on HR, i.e., atropine significantly ($p<0.05$) increased HR from 28.7 ± 2.4 beats.min^{-1} (-10 min) to 43.5 ± 3.93 beats.min^{-1} (0 min) (52% of increment, Fig. 2a). Buccal pumping frequency was only slightly diminished (~1.2 Hz) by the effect of atropine. Ten minutes after submersion the animals developed a bradycardia (22.8 ± 1.89 beats.min^{-1}) which became constant throughout the remaining submersion period. Overall mean HR during submersion (48% of decrement) was significantly ($p<0.05$) different from pre-submerged HR in atropine-injected toad (Fig. 2a). Eventually, HR reverted to the initial values just after emersion, even before activating the buccal pump (Fig. 2a). The

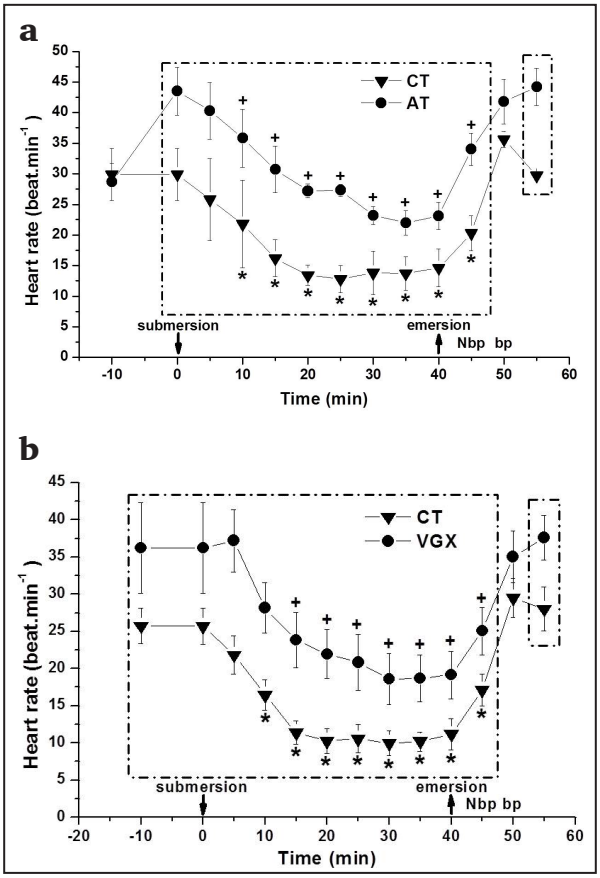

Figure 2. **a**) Heart rate of toads injected with atropine (AT, 3 mg/kg) or controls (CT) (n= 6 in all cases). **b**) Heart rate of intact (CT) or vagotomized (VGX) animals (n=8 in all cases). Means ± standard errors are indicated. Arrows indicate the beginning or ending of submersion. Nbp: no buccal pumping active, bp: buccal pumping active. The (*) and (+) indicate significant differences ($p<0.05$) with respect to 0 min, for any curve. The line-box indicates significant differences ($p<0.05$) between both curves, for any considered time. Data from Fig. 2B has been previously published in Cervino *et al.* (2007).

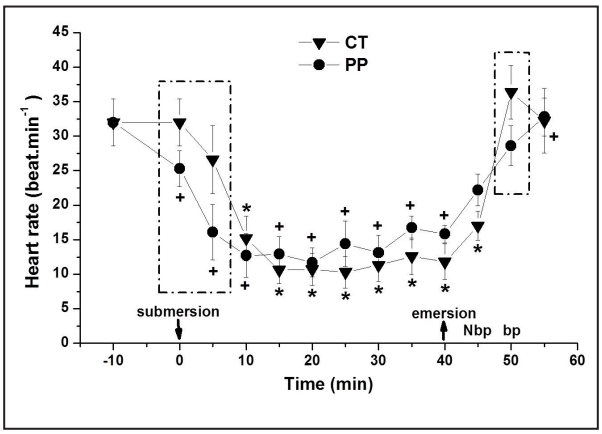

Figure 3. Heart rate in toads injected with propranolol (PP, 6 mg/kg) or controls (CT). Means ± standard errors are indicated. Arrows indicate the beginning or ending of submersion. Nbp: no buccal pumping active, bp: buccal pumping active. The (*) and (+) indicate significant differences ($p<0.05$) with respect to 0 min, for any curve. The line-box indicates significant differences ($p<0.05$) between both curves, for any considered time.

comparison between both experimental series (control and atropinized animals) showed a higher HR in the atropinized group ($p<0.05$), during both the whole submersion period and most of the emersion period analyzed. Consequently, the variation of HR between both groups showed a marked parallelism, as observed in Fig. 2a.

Effect of bilateral VGX on HR during submersion

HR of VGX toads during forced submersion is also shown in Fig. 2b. During air breathing, the eight vagotomized animals showed a significant ($p<0.05$) increase in HR (36.2 ± 6.10 beats.min^{-1}) in comparison with the recording previous to VGX (29.9 ± 4.24 beats.min^{-1}, data not shown in Figures), this is, a 21% of increment. As for atropine, vagotomy procedure only produced a slight decrement of buccal pumping (~1.2 Hz) while *sham* toads did not show any change compared to intact animals.

After 15 min of submerged, VGX animals developed a significant ($p<0.05$) bradycardia (20.5 ± 3.43 beats.min^{-1}; which represented a 43% of decrement), which continued up to 40 minutes (Fig. 2b). The emersion without buccal pumping did not show any significant difference in HR compared to both pre-submersion and the stage of emersion with buccal pumping. During the latter, the HR was re-established at the values recorded previously to submersion. Compared to intact animals, *sham* toads did not evidence any change in systemic HR (26.1 ± 2.1 beats.min^{-1}, data not shown).

Effect of β-adrenergic blockade on HR during submersion

Toads injected with propranolol showed values similar to those of the control series through most of the experiment (Fig. 3). Before submersion, propranolol significantly decreased HR from 32.0 ± 3.40 beats.min^{-1} to 25.3 ± 2.58 beats.min^{-1} (21% of decrement). Propranolol had no effect on buccal pumping frequency, either before or after the submersion period.

During most of the submersion period, HR in propranolol-treated animals (overall mean: 13.9 ± 2.38 beats.min^{-1}) had no differences with respect to control animals (Fig. 3). However, at time 0 and 5 min of submersion, and also at the beginning of the emersion with the buccal pumping, the propanolol-treated group showed HR values significantly ($p<0.05$) lower than those of the control group (Fig. 3).

Discussion

The most evident cardiovascular response observed in vertebrates during submersion is a pronounced bradycardia, together with peripheral vasoconstriction and changes in both gases and metabolites blood levels. In the case of mammals, birds and reptiles, bradycardia takes place immediately (from a few seconds to minutes) under both voluntary and forced submersions; this implies a reflex regulation mechanism mediated by the vagus nerve activity (Lund and Dingle, 1968; Kooyman *et al.*, 1981; Kanwisher *et al.*, 1981; Signore and Jones, 1995, 1996; Panneton *et al.*, 2000; Randall *et al.*, 2002).

In anurans, the bradycardia during submersion has been explained by several different factors. Early studies only referred vagal activity, as the efferent branch of a nervous reflex (Lund and Dingle, 1968) without discriminating whether this reflex response was stimulated by submersion or not (Lillo, 1979). Vagal activity, stimulated as a consequence of cardiocirculatory adjustments induced by submersion, is another possibility to be considered. However, it has been reported in frogs that bradycardia stabilization comprised from 5 to 30 min, therefore suggesting that it would be mediated by non-reflex mechanisms, i.e., independent from vagal activity (Jones and Shelton, 1964). To this respect, it must be considered that a relatively extended submersion could change some internal variables such as the partial pressure of gases in the blood, lactate plasmatic concentration and others (Portner *et al.* 1991; West *et al.* 2006),

which might act as either direct or indirect stimuli on the heart activity.

According to the obtained results, all the control animals submitted to forced submersion developed slow bradycardia, with a significant fall (53%) in HR after 10 to15 min after the beginning of the submersion. In principle, this relatively long latency argues against a reflex mechanism triggered by water receptors in the nares or by arterial baroreceptors (Bianchi da Silva *et al*., 2000). However, it must be considered that the possible stress of forced submersion might interfere with reflex mechanisms. Indeed, *R. arenarum* exhibits a sudden and vigorous bradycardia when they submerged voluntarily (Affanni *et al*., 1999). However, even when the possible stress of animals could avoid a quick bradycardia at the beginning of the forced submersion conditions, no stress that might interfere with the observed deep bradycardia is expected after that initial period.

Atropine caused HR to increase in air-breathing toads kept out of the water (Fig. 1), compared to the control group, which might indicate a suppression of the cardiac cholinergic vagal inhibitory tone. The atropined toads also exhibited a tachycardia when they were forcefully submerged (Fig. 2a), but were nevertheless able to develop a marked relative bradycardia, i.e., a significant decrease (48%) of HR before submersion, although of a lower magnitude than that observed in the control group. These results contrast with those reported by Lillo (1979) on bullfrogs, where atropine fully abolished the bradycardia response during submersion, but resemble the results obtained by Lund and Dingle (1968), who did observe bradycardia in atropinized *R. pipiens,* although with a longer latency than in *R. arenarum.*

On the other hand, the possibility that the bradycardia observed in atropinized animals may be due to the extinction of the atropine effect can be discarded, since the results obtained from the previous experiment performed out of the water (first experiment) showed that the atropine effect persisted for more than one hour (Fig, 1). Likewise, by comparing the results on atropine administration with those upon a bilateral VGX, a clear parallelism in HR variation during submersion is evident (48% and 43% of decrement, respectively). This comparison also suggests that, at least for cardiac regulation during both submersion and emersion, the active vagal fibers are parasympathetic and cholinergic in nature, without any peptidic co-transmission. These fibers would maintain a relatively steady discharge tone, both during the stage prior to submersion and throughout the whole periods of submersion and emersion. In short, it could be asserted that, under the experimental conditions used, the bradycardia observed during submersion in *R. arenarum* is caused, at least partly, by factors other than the vagal innervation of the heart.

In the frog *R. temporaria* bradycardia took place with a 15 to 30 min delay and, in this case, the regulation is independent of the action of the vagus nerve (Lund and Dingle, 1968). Other amphibians -*R. pipiens, R. esculenta, Xenopus muelleri, X. laevis*- develop similar patterns of non-reflex bradycardia (Jones and Shelton, 1964; Jones, 1966, 1967). Besides the contact with water, other possible stimuli maintaining bradycardia during the submersion stage would be an increased volemia, a decrease of oxygen levels in the blood, and/or an increase of metabolite blood levels, such as lactic acid. Concerning volemia, it is well known that amphibians absorb water through the skin during submersion (Jones *et al*., 1992, 1997); water enters into the lymph sacs and through the lymphatic system into the blood stream, increasing blood volume and consequently displaying several adjustments at cardiovascular, endocrine and renal levels (Randall *et al*., 2002). However, no mechanisms through which the increase of volemia might cause bradycardia without involving any vagal efferent have been reported in the studied species.

Regarding the decrease of oxygen levels in the blood, it has been reported that short diving periods (10 min) cause in *Xenopus sp* a fall in the partial pressure of O_2 of about 50 % (Emilio and Shelton, 1974). The hypoxia has been, in fact, suggested as a stimulus to reduce the HR in other species of frogs (Jones and Shelton, 1964). Boutilier and Shelton (1986) showed in *X. laevis* the existence of significant differences in some blood parameters during voluntary and forced submersions, by comparing the blood composition of frogs that had finished a voluntary submersion with that of others that had finished a 30-min period of forced submersion; the latter had a higher concentration of plasmatic lactate and other metabolic acids. These chemical stimuli, apart from triggering nervous reflexes via chemo-receptors, could have a direct effect on the heart, maintaining an intense bradycardia as the forced submersion takes place. In such sense, a metabolic depression caused by chemical stimuli (*i.e.* lactic acid), and by low temperatures, was described in diving animals (Hochachka and Somero, 1984).

The current study also studied the possible physiological role of the sympathetic system and the circulating catecholamines, either to develop bradycardia during submersion or to re-establish the HR during following emersion. Firstly, blocking of β-adrenergic receptors with propranolol significantly decreased HR in toad breathing out of water and at the beginning of the submersion period (0 and 5 min), to further produce a bradycardia similar to that of controls throughout the remaining 35 min of submersion. This result shows that a certain sympathetic tone on the heart is maintained by control animals at the beginning of submersion, which disappeared (i.e., becomes similar to that produced by propranolol) 5 min after that. Thus, during most of the submersion period, the sympathetic tone on the heart appears to be abolished, therefore contributing to the observed bradycardia. Since the initial HR decrement caused by the adrenergic blockage was important (45%, a magnitude comparable to the HR increase caused by either cholinergic blockage or VGX) the further physiological inhibition of sympathetic control during submersion seems to be a critical factor for allowing the development of a full bradycardia.

Secondly, at the moment of activating buccal pumping during the emersion stage, it was evident the inability to increase HR up to control levels, in animals treated with propranolol. This result shows that the sympathetic system would be in charge of increasing HR during emersion, and that its activation linked to the re-starting of buccal pumping. The activation of buccal pump once forced submersion is over would trigger a reflex which, by means of the sympathetic system, would stimulate heart activity in order to re-establish the circulatory pattern existing prior to submersion. Although bradycardia has been reported even when buccal pump activity was blocked (Jones and Shelton, 1964), the mechanisms by which heart sympathetic activation would be coupled to the activation of the buccal pump are still unknown in amphibians. Nevertheless, the presence of bulbospinal preganglionic sympathetic neurons in the pFRG/Bötzinger region described for air-breathing aquatic animals (Guyenet *et al.,* 2013; Baghdadwala *et al*., 2016) could be the appropriate substrate to explain such coupling.

We conclude that mechanisms other than vagal activity are also producing and maintaining bradycardia during the submersion period. Among them, the suppression of sympathetic tone seems to play a physiological role at the beginning of the submersion period, while a sympathetic activation of heart rate would become relevant during the emersion phase.

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