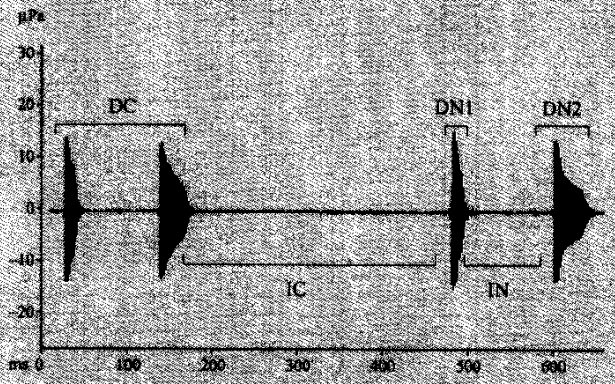
Revista Española de Herpetología



Asociación Herpetológica Española
Volumen 21 (2007)
VALENCIA

ISSN-0213-6686
Rev. Esp. Herp. 21 (2007)
Valencia

The *Revista Española de Herpetología* is the pe... the Asociación Herpetológica Española (AHE). It ... following services: BiologyBrowser, BIOSIS, CINDO... Contents, Revicien, and Zoological Record.

Rev. Esp. Herp. (2007) 21:95-106

Systemic and lymphatic heart performance during forced submersion in the toad *Bufo arenarum* (Anura, Bufonidae)

CLAUDIO O. CERVINO,[1] LILIAN F. CASTILLO [1] & ENRIQUE M. RODRÍGUEZ [2]

[1] *Laboratory of Electrophysiology and Neurophysiology, School of Medicine, University of Morón, Cabildo 134, (1708) Morón, Buenos Aires, Argentina (e-mail: cervino@unimoron.edu.ar)*

[2] *Laboratory of Comparative Animal Physiology, Dept. of Biodiversity and Experimental Biology, University of Buenos Aires, Ciudad Universitaria, Pab. II, 1428, Buenos Aires, Argentina*

Abstract: The activity of systemic (SH) and lymphatic hearts (LH) was characterized in the anuran *Bufo arenarum* during an entire, forced submersion. Prior to submersion, a SH frequency of 28.9 beats / min was recorded, while both posterior LH showed a similar but asynchronic rate of 50.5 beats / min. At the beginning of forced submersion, a reflex apnea (characterized by cessation of buccal pumping) was evident immediately after the nares were covered by water. At the same time, a slow decrease of SH rate was observed, stabilizing at 10 beats / min after 15 min. LH activity completely stopped after 10 min of submersion. Emersion comprised an initial brief phase with no buccal pumping, followed by activation of the buccal pump together with a sudden increase of SH rate and reactivation of LH. Bilaterally vagotomized animals consistently showed a higher SH rate than intact controls and also showed bradycardia during the entire submersion period, following a pattern parallel to that of control toads. Several mechanisms, possibly both nervous and non-nervous (such as blood chemical stimuli), could be triggering and maintaining the systemic bradycardia, as well as the inactivity of LH during submersion. The results reported in this work do not enable to draw definite conclusions about the mechanisms responsible for SH bradycardia and cessation of LH activity during submersion. In view of the correlation of buccal pump activity with the establishment of deep changes in the physiology of both kinds of hearts, said activity becomes a necessary factor for the occurrence of heart rate modifications.
Key words: bradycardia, *Bufo arenarum*, heart rate, lymphatic hearts, submersion, vagotomy.

Resumen: Actividad de los corazones sistémico y linfático en el sapo *Bufo arenarum* (Anura, Bufonidae) durante la sumersión forzada. – La actividad bioeléctrica del corazón sistémico (SH) y de los corazones linfáticos (LH) posteriores fue estudiada durante la sumersión forzada en el sapo *Bufo arenarum*. Previo a la sumersión, la frecuencia cardiaca fue de 28.9 latidos por minuto y la de los LH fue de 50.5 latidos por minuto, y asincrónica. Al comienzo de la sumersión forzada, inmediatamente después que las narinas están bajo la superficie del agua, se registra una detención refleja de la bomba bucal y una lenta y gradual disminución de la frecuencia cardiaca, estabilizándose en 10 latidos por minuto después de 15 min. A los 10 min de sumersión, los LH se detuvieron completamente. La emersión implica una breve fase sin bombeo bucal, seguida por una activación de dicha bomba junto con el repentino incremento de la frecuencia del SH y la reactivación de los LH. Animales vagotomizados bilateralmente mostraron una alta frecuencia cardiaca, comparada con los controles, y bradicardia durante la sumersión. Varios mecanismos, nerviosos y no nerviosos, tales como estímulos químicos sanguíneos, permitirían mantener la bradicardia sistémica y la inactividad de los LH durante el proceso de sumersión. Los resultados no permiten establecer una conclusión definitiva acerca de los mecanismos responsables de la bradicardia y en la detención de la actividad de los LH durante la sumersión, pero la correlación entre la actividad de la bomba bucal y el establecimiento de profundos cambios en la fisiología de ambos corazones, sugiere que la primera es un factor necesario para que se produzcan las modificaciones.
Palabras clave: bradicardia, *Bufo arenarum*, corazones linfáticos, frecuencia cardiaca, sumersión, vagotomía.

INTRODUCTION

Systemic heart rate (SHR) is the main physiological variable related to cardiac output. Changes in SHR are directly and strongly associated with changes in the activity level of animals, in order to maintain a normal blood pressure as well as an adequate perfusion of active tissues. In this sense, a precise nervous control of SHR is required for the compensatory adjustments needed to face several environmental and internal changes.

The systemic heart (SH) of anurans is myogenic, but externally regulated by the autonomic nervous system (RANDAL *et al.*, 2002). Some sympathetic postganglionic fibers innervate the cardiac pacemakers, releasing norepinephrine as the neurotransmitter that produces an increase in SHR. Other sympathetic branches extensively innervate the myocites (mainly in the ventricles), increasing muscle strength and, therefore, ventricular ejection. Parasympathetic innervation to the SH is provided by the vagus nerve. This nerve provides parasympathetic branches to the venous sinus by means of the Remak ganglion, while the atrialventricular joint is innervated by the Bidder ganglion. A few collateral branches project to the ventricle (CAMPBELL *et al.*, 1982; PRESTON & COURTICE, 1995). A decrease of SHR is caused by acetylcholine as the parasympathetic neurotransmitter that is recognized by muscarinic receptors at the pacemaker cardiac cells. Peptide neurotransmitters have also been reported in some amphibians, as co-localized in the cholinergic heart fibers (CAMPBELL *et al.*, 1982; MORRIS *et al.*, 1989); these peptides can directly or indirectly affect the heart rate (CAMPBELL *et al.*, 1982; PRESTON & COURTICE, 1992, 1993; PRESTON *et al.*, 1993; COURTICE & DELANEY, 1994).

Submersion is the usual behavior for feeding and reproduction in amphibians, representing an adaptive defensive strategy against predators. In all vertebrates considered in a broad sense as divers (pinnipeds, cetaceans, some birds and reptiles and amphibians), bradycardia is the most obvious manifestation of a series of both cardiovascular and respiratory changes that take place during submersion. Under normal, resting condition, the vertebrate heart rate is modulated by vagal (parasympathetic) tone. Bradycardia takes place through an increase in vagal activity, and eventually by a decrease of sympathetic activity (SIGNORE & JONES, 1996). In seals, bradycardia is maintained by the vagus nerve during submersion, but tachycardia during emersion is caused by decreasing parasympathetic activity, as well as by increasing the sympathetic stimulation of the heart (ELLIOTT *et al.*, 2002).

Precise control of bradycardia during submersion has not been clearly established in amphibians. Electric stimulation of the vagus nerve in *Bufo marinus* while breathing out of water enhances bradycardia (COURTICE & DELANEY, 1994; PRESTON & COURTICE, 1995), although it is not clear whether parasympathetic fibers are involved, and evidence was given regarding the participation of other factors. Hence, bradycardia was observed during the submersion of atropinized toads (LUND & DINGLE, 1968; LILLO, 1979).

One of the effects of submersion is the progressive decrease of the oxygen blood level. In most divers, low levels of oxygen detected by arterial chemoreceptors contribute to the maintenance of bradycardia (RANDALL *et al.*, 2002). In the case of anurans, a decrease of blood oxygen would be compensated by cutaneous respiration, possibly due to a highly vascularized integument. The magnitude of bradycardia would therefore be related to the capacity of gas

exchange of the skin, determined by its area, thickness and time of diffusion of gases (refs?). Other physiological changes during submersion have been reported in anurans. For instance, brain bioelectric activity significantly decreased (C. Cervino & J.M. Affanni, unpublished data) and lymphatic hearts stopped in *B. arenarum* (AFFANNI *et al.*, 1999).

Anurans have two pairs of lymphatic hearts (LH), placed at both sides of the antero-posterior axis. The anterior pair is localized under the scapulae, while the posterior pair can be seen at both sides of urostile; their asynchronic beating is easily observable. The structure of LH has been reported (SATOH & NITATORI, 1980; RUMYANTSEV & KRYLOVA, 1990; AFFANNI *et al.*, 1997). These structures drain a high quantity of lymph from interstitial spaces and lymphatic sacs, returning it to systemic circulation through the lymphatic system (JONES *et al.*, 1992, 1997). During submersion, epidermal intake of water takes place. Detention of LH during this period could help to avoid the increase of blood volume due to water absorption (AFFANNI *et al.*, 1999).

The neurogenic nature of LH has been demonstrated, and nervous signals reach these hearts through spinal nerves (BRAUN-MENÉNDEZ & FOGLIA, 1940; DAY *et al.*, 1963). The role of olfactory bulbs has also been proposed in regulating the activity of LH (J.M. Affanni & C. Cervino, unpublished data). Pressure changes inside LH have been reported by means of cannulation techniques (JONES *et al.*, 1992, 1997). AFFANNI *et al.* (1997, 1999) developed an original technique for recording the bioelectrical activity of posterior LH, allowing the chronic and continuous recording of their beating in freely-moving animals in or out of the water.

Concerning ventilation, a complete and sudden cessation of buccal pumping takes place in anurans following submersion, as first described by HOBSON (1967). Buccal pumping plays an essential role in ventilation, allowing the air to fill the nasal and buccal cavities and then forcing it to the lungs (GANS *et al.*, 1969). The beating of both systemic and lymphatic hearts of toads is influenced by the activity of the buccal pumping while emerging from a free submersion (AFFANNI *et al.*, 1999).

This study was aimed at determining and characterizing different phases of the physiological response of the toad *B. arenarum* during a forced submersion, in terms of changes in the heart rate of both systemic and lymphatic hearts, ventilatory mechanisms and animal behavior. The physiological role of the vagus nerve during submersion was also studied by means of a bilateral vagotomy.

MATERIALS AND METHODS

Animals

Thirty-two adults of *B. arenarum* weighing 150 to 200 g were used. They were captured in the city of Luján (Buenos Aires province) during March and April. In the laboratory, they were maintained in plastic cages with a substrate of wet wooden chips. Before starting the assays, all animals were acclimated to laboratory conditions for 15 days. No food was given during this period or during the assays. Temperature was always maintained within a range of 18 to 22°C, while photoperiod was set up at 12L-12D, the light period beginning at 7:00 h.

Recording of SHR

Animals were chronically implanted with insulated metallic electrodes fixed to the skin. One electrode was set on each of the scapulae, while a third was placed on the posterior, right side of the body, thus forming

a triangle. The best bipolar derivation between one anterior electrode and the posterior one was taken, the third electrode serving as a reference. The electrodes were connected to an Exxer amplifier (bandpass: 5 to 40 Hz, notch: 50 Hz, impedance less than 5 kΩ). Signals were then acquired and digitalized with a sample frequency of 64 Hz, by using a Rhythms 10.0d program (Stellate Systems, 1994, Quebec, Canada).

Recording of bioelectrical activity of lymphatic hearts

The technique of electrode implantation developed by AFFANNI *et al.* (1997) was used. Briefly, this consisted in the insertion and fixation of one electrode on each of the posterior LH. The electrodes were made of stainless steel (1 mm diameter), entirely insulated except in the middle portion (0.5 mm) that was in contact with the LH. Both electrodes were connected to the same amplifier used for recording SHR. Signal processing was the same as described for SHR.

Recording of bucal pumping activity

Two nicrome electrodes were inserted in the submaxillary muscles. Connections and recording were made as mentioned above.

Submersion protocol

Forty-eight hours after the recording electrodes were implanted, toads were placed in an aquarium with only wet sand, where the activity of both kinds of hearts was recorded for 30 min, allowing animals to ventilate through buccal pumping. Air temperature was maintained at 20 ± 2°C. Then animals were gently transferred to the submersion device, i.e. a glass aquarium of 15 l-capacity, initially empty of water and filled with dechlorinated tap at a rate of 30 l/h; this water was previously aerated. Once the desired water level was attained, indicated by a perforated acrylic plaque placed at the top of the aquarium, a continuous water flow (30 l/h) was established, by setting the same outflow as the inflow. The acrylic plaque also avoided any eventual escape of animals. Water temperature was maintained between 19 and 21°C throughout. The behavior of the assayed animals could be monitored at anytime.

The moment at which the nares were covered by water (correlated to a detention of buccal pumping) was taken as time zero of submersion. The duration of submersion was established in 40 min. Additionally, a few animals were subject to short submersions lasting 15 min. Emersion began when decreasing water level left the nares out of the water. Decreasing flow rate during emersion was the same as the increasing flow rate set at the beginning of submersion; in fact, it was achieved by only closing the inflow.

All aquaria used for recording animals were placed inside a Faraday cage, in order to minimize electrical noise. All recordings were made between 10:00 and 15:00 h. SHR was expressed as beats/min, for each consecutive period of 5 min, and was calculated from the mean number of peaks comprised in 10 intervals of 10 s each, randomly taken within each 5 min period. A similar procedure was followed for the estimation of LH rate (LHR).

Bilateral vagotomy

Eight toads whose SHR and LHR had been previously recorded during the submersion protocol were submitted to a surgical vagotomy. After being lightly anesthetized with ether, an incision on each dorso-lateral zone immediately caudal to the head was made. Once both vagus nerves were identified under a stereoscopic microscope, a section of 2-3 mm was cut from each one. Incisions were then 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 mentioned above except for the vagus cutting. After 48 h of being vagotomized, SHR was recorded following the same protocol specified above.

Statistical analyses

Values of both SHR and LHR corresponding to intact toads during submersion were analyzed by means of a one-way repeated measures ANOVA. Student-Newman-Keuls multiple comparisons were then applied in order to establish different phases during the submersion-emersion period. To analyze the results of vagotomized animals, a two-way (time and treatment) repeated measures ANOVA was used, followed by planned comparisons on each considered factor. In all cases, a significant level of 5% was taken.

RESULTS

A typical electrocardiographic (ECG) record obtained from an intact, control toad, chronically implanted and ventilating through the buccal pumping, is shown in Fig. 1. The corresponding mean values for both SHR and LHR can be seen in Table 1. Although asynchronic beating was observed in the posterior LH, their beating frequency was similar (t-test: t = 0.205, d.f. = 62, p = 0.838).

TABLE 1. Cardiac rate (mean ± SD, range in brackets) of both systemic and posterior lymphatic hearts, in *Bufo arenarum* while breathing air. N = 32.

TABLA 1. Frecuencia cardiaca (media ± SD, rango entre paréntesis) del corazón sistémico y de los corazones linfáticos posteriores, en el sapo *Bufo arenarum*, mientras ventila sus pulmones en aire. N = 32.

Heart	Cardiac rate (beats / min)
Lymphatic (posterior, right)	50.3 ± 1.36 (34.0 - 68.0)
Lymphatic (posterior, left)	50.8 ± 1.43 (34.0 - 64.0)
Systemic	28.9 ± 1.37 (13.0 - 38.0)

As the forced submersion took place, in both short (15 min) and long (40 min) submersions, animals became immobile and developed a bradycardia of the SH. Eventually, abrupt movements during swimming were noted, with an increase of SHR. LH gradually decreased their beating, and generally stopped around 10 min after submersion, showing an irregular heart rate just before stopping. A typical recording of both kinds of

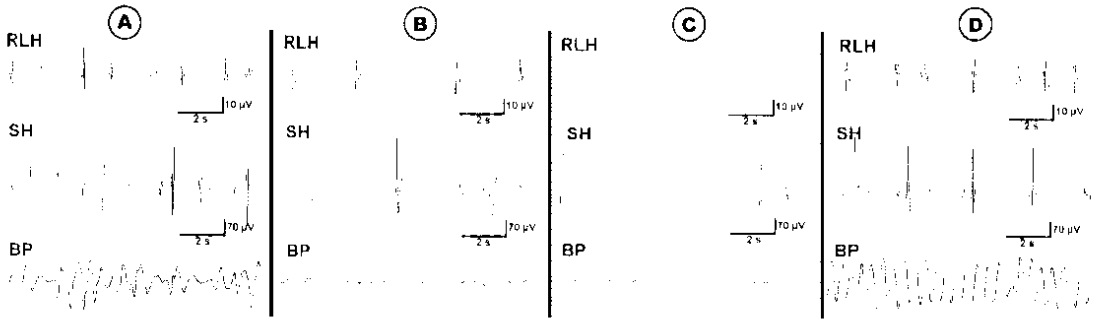


FIGURE 1. Recording of systemic heart (SH) and right lymphatic heart (RLH) bioelectrical activity, in the toad *Bufo arenarum* under different experimental situations. (A) Breathing air, out of water. (B, C) Five and 20 min after the beginning of forced submersion. (D) During emersion, with buccal pumping (BP).

FIGURA 1. Registro de la actividad bioeléctrica del corazón sistémico (SH) y del corazón linfático posterior derecho (RLH), en el sapo *Bufo arenarum* bajo diferentes situaciones experimentales. (A) Respirando aire, fuera del agua. (B, C) Cinco y 20 min después del comienzo de la sumersión forzada. (D) Durante la emersión, con activación de la bomba bucal (BP).

hearts during submersion, as well as the activity of buccal pumping, can be seen in Fig. 1.

Two physiological-behavioral patterns could be recognized during the emersion, according to the time that the animals spent submerged. After long submersions (40 min), toads could remain up to 5 min in tonic immobile state, with no activation of buccal pumping. Also, SHR increased slowly while LH remained inactive. When buccal pumping resumed, systemic bradycardia suddenly reverted to a tachycardia for a few minutes, SHR finally reaching the values previous to submersion. During this phase, LH suddenly increased their activity, reaching values as high as 75 beats / min (Fig. 1), to finally stabilize at the initial, presubmersion values. On the other hand, a second pattern could be recognized after short submersion (15 min or less duration). In this case, the buccal pumping started immediately after emersion, and both SH and LH showed a marked tachycardia.

Mean heart rate values for the submersions assayed (40 min duration) are shown in Fig. 2. A gradual and significant ($p < 0.05$) decrease in SHR can be observed, from a pre-submersion value of 28.9 ± 1.37, to a new steady state around 15 min after the beginning of submersion, that is, a bradycardia involving a mean SHR of 13.6 ± 1.52 that extends to the end of the 40 min submersion period. During emersion, the SHR initially increased , averaging 15.9 ± 1.20 beats / min, suddenly increasing when buccal pump became active. Finally, after 10 min of buccal pumping, SHR stabilized to the values observed prior to submersion.

According to the results of statistical analyses made on heart rates, as well as considering other physiological and behavioral aspects, five different stages could be identified during the submersion process, as shown in Fig. 2. They can be characterized as follows:

Stage I pre-submersion. The animal breathes out of the water. Buccal pumping is normal, alternating some regular pumping periods with more irregular ones, and even with brief apnea episodes. SHR is regular, with an average of 28.9 beats / min. LH beat asynchronically, at a mean frequency of 50.5 beats / min. Eye pupils are dilated and responsive to light changes. Spontaneous movements can be observed, as well as normal responses to environmental stimuli.

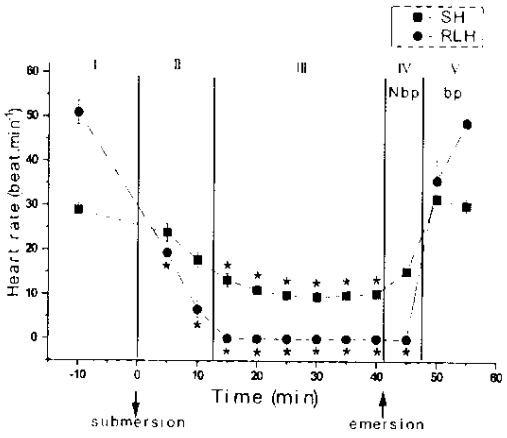


FIGURE 2. Mean values (+ SD) of both systemic heart (SH) and right lymphatic heart (RLH) rate, during forced submersion lasting 40 min. N = 32. Arrows indicate the beginning and end of the submersion period. Asterisks indicate significant differences ($p < 0.05$) with respect to time zero of submersion (breathing air). Vertical lines and roman numbers indicate different stages during the assays (see text), each one significantly ($p < 0.05$) differing from the preceding, according to the multiple comparisons made. bp: buccal pumping; Nbp: no buccal pumping.

FIGURA 2. Frecuencia cardíaca media (+ SD) del corazón sistémico (SH) y del corazón linfático posterior derecho (RLH), durante los ensayos de sumersión forzada durante 40 min. N = 32. Las flechas indican el comienzo y el final del período de sumersión. Los asteriscos indican diferencias significativas ($p < 0.05$) con respecto al tiempo cero (ventilando en aire). Las líneas verticales y los números romanos indican diferentes estadios durante los ensayos, cada uno significativamente diferente ($p < 0.05$) del precedente, de acuerdo a pruebas estadísticas de comparaciones múltiples. bp: activación de la bomba bucal; Nbp: sin actividad de la bomba bucal.

Stage II, beginning of submersion. - The animal remains quiet until the water covers its nares. At that time, buccal pumping immediately ceases (post-submersion apnea) and a constant and gradual decrease of SHR takes place for approximately 10 min. This is a transition stage previous to reaching a consistent bradycardia. During this period, toads remain immobile, although sporadic swimming movements can be observed, from a few seconds to about 1 min. LH decrease their frequency, become arrhythmic, and finally stop.

Stage III, deep bradycardia during submersion. – After 15 min of the beginning of submersion, the animal presents a deep bradycardia that extends to the end of the forced submersion period (40 min). During this period, SHR can decrease up to 70%, compared to the pre-submersion values. Behavior of animals is similar to that described in the previous stage, but swimming movements are less frequent and SHR slowly accelerates when they finish. LH are completely stopped, as well as the buccal pumping. Eye pupils are intensely contracted and unresponsive to light stimuli.

Stage IV, emersion with no buccal pumping. – From the vertical position that animals normally take into the submersion device, a change to a horizontal position takes place as the water level decreases, to finally remain immobile on the aquarium bottom, once the water is completely drained. During this first, brief (5 min) phase of emersion, a discrete increase of SHR is observed. Animals remain immobile, without responding to mechanical stimuli. Contracted pupils are still observed. LH remain inactive in most animals.

Stage V, emersion with bucal pumping. – This stage begins with the abrupt activation of buccal pumping. SHR also increases abruptly, reaching values even higher than the pre-submersion ones. During the following minutes, SHR stabilizes in the same way as the buccal pumping. Then, posterior LH resume their normal activity. During this late stage, the animal slowly recovers its normal activity level, including eye movements and spontaneous locomotion. It also becomes responsive to any normal stimuli.

Figure 3 shows the results of the assay with vagotomized animals. Vagotomy pro-

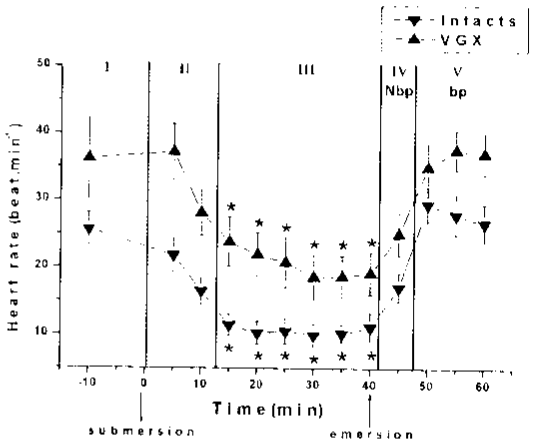


FIGURE 3. Systemic heart rate (SHR) in intact and vagotomized (VGX) animals. N = 8. Arrows indicate the beginning and end of submersion. Asterisks indicate significant differences ($p < 0.05$) with respect to the SHR at time zero of submersion, for any curve. Significant differences ($p < 0.05$) were observed between both curves (intact and vagotomized). Vertical lines and roman numbers indicate different stages during the assays (see text), each one significantly ($p < 0.05$) differing from the preceding, according to the multiple comparisons made. bp: buccal pumping; Nbp: no buccal pumping.

FIGURE 3. Frecuencia cardiaca sistémica (SHR) en animales intactos y vagotomizados (VGX). N = 8. Las flechas indican el comienzo y el final del período de sumersión. Los asteriscos indican diferencias significativas ($p < 0.05$) de la SHR con respecto al tiempo cero de sumersión, en ambas curvas. Diferencias significativas ($p < 0.05$) fueron observadas para ambas curvas (animales intactos y VGX). Las líneas verticales y los números romanos indican diferentes estadios durante los ensayos, cada uno significativamente diferente ($p < 0.05$) del precedente, de acuerdo a pruebas estadísticas de comparaciones múltiples. bp: activación de la bomba bucal; Nbp: sin actividad de la bomba bucal.

duces a significant ($p < 0.05$) increase in SHR, at any time of the entire submersion process. For instance, animals breathing air before submersion shifted their SHR, by effect of vagotomy, from a mean value of 25.7 ± 2.39 beats/min (intact animals) to 36.2 ± 6.10 beats/min. On the contrary, LH frequency was not altered by vagotomy, while sham toads did not show any change compared to intact animals, neither regarding SHR nor LHR (data not shown).

Despite the differences observed between intact and vagotomized animals, in both experimental groups a marked bradycardia could be seen at any time during submersion. In other words, although vagotomized animals always showed a higher SHR than intact animals, both experimental groups displayed bradycardia during submersion, paralleling their patterns of SHR; even the latency up to the occurrence of bradycardia was 15 min in both groups. Emersion patterns were also similar in vagotomized animals, compared to intact ones, although the transient tachycardia observed in the latter was not detected in the former (Fig. 3).

DISCUSIÓN

Bradycardia is one of the most evident cardiovascular adaptive responses of vertebrates during submersion, together with peripheral and visceral vasoconstriction. In the case of mammals, birds and reptiles, both under voluntary and forced submersion, bradycardia is achieved by a neural reflex mediated by the vagus nerve (SIGNORE & JONES, 1995, 1996; RANDALL *et al.*, 2002). Bradycardia of the anuran SH has been explained by several mechanisms: a) a neural, parasympathetic reflex, as for the other mentioned vertebrates (this mechanism implicates a sudden decrease of heart frequency; LUND & DINGLE, 1968), or b) non-reflex mechanisms, independent from vagal activity, proposed for instance for frogs presenting a delayed bradycardia, i.e. 15 to 30 min after submersion (JONES & SHELTON, 1964).

In the species under study, *B. arenarum*, a reflex bradycardia has been reported after a voluntary submersion of animals (AFFANNI *et al.*, 1999). In that study, it was observed that voluntary submersion of the nares coincided with a cessation of buccal pumping as well as an intense and sudden bradycardia of the SH, while LH completely stopped. In the current study, involving forced submersion, bradycardia slowly developed, reaching values significantly different from those at the beginning after 15 min. In principle, this relatively long latency seems to preclude a reflex mechanism for the observed bradycardia. However, since the submersion was forced, animals were exposed to a stressful situation that could produce, for instance, the secretion of catecholamines from the adrenal medulla, therefore compensating a possible increased and inhibitory vagal influence on heart frequency.

Nevertheless, the results obtained with vagotomized animals allow us to evaluate the relevance of vagal reflex during forced submersion. As expected, vagotomy abolished the inhibitory vagal tone that is normally present in animals resting out of water. Hence, SHR increased 40% in vagotomized animals, compared to intact ones, prior to submersion. These results were similar to those obtained in *Rana pipiens* and *R. temporaria* after bilateral section of the vagus nerve (LUND & DINGLE, 1968). However, in the current study a significant bradycardia was observed in vagotomized *B. arenarum* during submersion. Moreover, the temporal pattern of SHR in vagotomized animals was parallel to that of intact animals during the entire submersion period, the former having higher values than the latter at

any time of submersion, and the difference between both groups was consistent throughout. In *R. pipiens*, bilateral vagotomy did not completely abolish bradycardia during submersion, but the relative decrease of SHR was low (LUND & DINGLE, 1968).

Therefore, a bradycardia independent from vagal activity was evident during the forced submersion of *B. arenarum*, i.e. no acetylcholine, somatostatin or any inhibitory neuropeptide secreted through vagus nerve (COURTICE & DELANEY, 1994; PRESTON & COURTICE, 1995) made the SHR decrease. Even a similar latency to develop bradycardia, once submerged, was observed in vagotomized and intact animals, together with a parallel time course of SHR in both groups of animals during the entire period of submersion. These results suggest that the parasympathetic system could maintain a constant inhibitory tone in intact animals before and during forced submersion, being the cause of bradycardia other than the increase of vagal activity, i.e, the same as for vagotomized animals. However, an eventual increase of sympathetic activity as an indirect consequence of vagotomy or by other reasons could have contributed to increasing the SHR in vagotomized toads.

Concerning the non-reflex factors that could be producing bradycardia during submersion, previous evidence about bradycardia mediated by parasympathetic independent factors were reported for several anuran species, such as *R. temporaria, R. pipiens, R. esculenta, Xenopus muelleri, X. laevis*, and *B. bufo* (JONES & SHELTON, 1964; JONES, 1966, 1967; LUND & DINGLE, 1968). Such factors, not mutually exclusive, can be summarized as follows: a) activity of nervous pathways different from parasympathetic vagal ones; b) changes in the circulatory pattern (blood volume, arterial pressure, venous return, etc.) that could imply a compensatory adjustment of SHR by intrinsic mechanisms, and c) changes in hemolymphatic parameters, such as O_2 content and several metabolite levels, that could decrease the metabolic rate and the global physiological performance of submerged animals.

According to the latter possibility, a report from EMILIO & SHELTON (1974) shows that short voluntary dives (10 min) cause blood O_2 partial pressures to fall from approximately 80 to 40 mmHg in *Xenopus*.We cannot disregard other metabolic changes or modification in the blood respiratory properties. BOUTILIER & SHELTON (1986) found in *X. laevis* the existence of differences in certain hemolymphatic parameters, by comparing the response obtained during a voluntary submersion with that corresponding to a forced one. Hence, after 30 min of forced submersion, but not after the relatively short voluntary submersions, animals showed a marked hemoconcentration, together with an increase of the hemolymphatic levels of lactate and other metabolic acids. Further research on this point is needed in *B. arenarum*.

These changes could in turn inhibit the general metabolism and particularly the cardiac activity, in terms of the intense heart bradycardia maintained during the entire submersion period. Even the relatively slow recovery of SHR during the first phase after emersion (no buccal pumping active) could be explained by the time needed for returning those metabolite levels toward normal values. Interestingly, the few animals submitted in the current study to a short forced submersion period (15 min) did not show that first, slow response, but only a rapid increase of SHR was seen after emersion. This suggests that short times of submersion are not enough to produce significant metabolic changes in hemolymph that could act as inhibitory stimuli.

Concerning LH, a sudden cessation of their activity was observed, even in vagotomized animals, just at the beginning of submersion. These results are in accordance with those reported for the same species during voluntary submersions (AFFANNI *et al.*, 1999). On the other hand, cessation of LH activity persists throughout the submersion period. The water detected by the nares would be the stimulus that would tonically acting on higher encephalic centers that in turn would inhibit the spinal pacemaker that controls the activity of LH. This explanation is strongly supported by the fact that animals whose spinal cord had been sectioned at the cervical level, never showed detention of LH during forced submersion (AFFANNI *et al.*, 1999). Additionally, baroreceptors in the pulmocutaneous artery responding to an increase in blood pressure could trigger a central reflex that finally inhibits LH rate. To this respect, the increment of systolic pressure in the pulmocutaneous artery of *B. marinus* breathing air was correlated to a significant decrease of LH frequency (CROSSLEY & HILLMAN, 1999).

The duration of submersion appears as an important factor influencing the prolonged breathing cessation and heart bradycardia and LH arrest that we observed after emergence. In fact, buccal pumping, SH activity and LH beating are immediately reinitiated in the emergence occurring after short submergence. Contrarily, buccal pump cessation, intense bradycardia and LH arrest persist during several minutes after long submersions.

The rapid increase of SHR during the second phase of emersion coincided with the re-initiation of buccal pumping. LILLO (1979) reported a fast increment of SHR in *R. catesbeiana* at emerging, probably due to both an increase of sympathetic and a decrease of parasympathetic activities, but no relationship with activity of the buccal pump was described. Re-initiation of buccal pumping, perhaps activated by baroreceptors or nasal receptors, would trigger a reflex response on SH, possibly mediated by the sympathetic system.

The water surrounding the toads could act as a stimulus to produce more than one reflex mechanism related to diving. In fact, endings of both trigeminal and terminal nerves, which have been previously described in certain anurans, are probably involved in the detection of water in the nares, which may lead to cessation of buccal pumping. The resulting apnea implies changes in nasal, pharynx and / or pulmonary activities, which in turn could produce, by several mechanisms, inhibition of systemic and lymphatic hearts.

JONES & SHELTON (1964) were able to induce bradycardia of the SH by blocking the activity of the buccal pump. Re-initiation of this pump after emersion would act as a signal to increase the activity of both kinds of hearts, i.e. producing tachycardia in the SH and a reactivation of LH. This increased cardiac activity could help to pay the oxygen debt acquired during submersion.

These experiments do not permit us to draw definite conclusions about the mechanisms involved in heart bradycardia and LH arrest during forced submersion. First, neither the SH bradycardia nor the LH arrest depend on the activity of vagus nerve. However, for the maintenance of these physiological conditions some participation of other factors or of interference with oxygen supply cannot be excluded. Second, in view of the correlation of buccal pump activity with the establishment of deep changes in the physiology of both kinds of hearts, said activity becomes a necessary factor for the occurrence of heart rate modifications.

Acknowledgements

This study was partially supported by CONICET and UBACyT programs. We wish to thank Mrs. P. López for revising translation. The animals were treated following criteria established by "Principles of Animal Care" (NIH publication 86-23, revised 1985) and according to Argentine law.

REFERENCES

AFFANNI, J.M., CERVINO, C.O. & BEDNERS, A.S. (1997): A technique for chronically recording the EKG of the lymph hearts en the toad *Bufo arenarum* (Hensel). *Biocell*, 21: 91-94.

AFFANNI, J.M., CERVINO, C.O., ALDANA MARCOS, H. & BOLIS, G. (1999): Permanent cessation of the activity of posterior lymph hearts in the voluntarily and forcibly submerged toad *Bufo arenarum*. *Comparative Biochemistry and Physiology*, 122A: 291-298.

BOUTILIER, R.G. & SHELTON, G. (1986): Respiratory properties of blood from voluntarily end forcibly submerged *Xenopus laevis*. *Journal of Experimental Biology*, 121: 285-300.

BRAUN-MENENDEZ, E. & FOGLIA, V.G. (1940): Biologie et pharmacologie des coeurs lymphatiques des batraciens. *Archives Internationales de Pharmacodynamie et de Thérapie*, 64: 273-307.

CAMPBELL, G., GIBBINS, I.L., MORRIS, J.L., FURNESS, J.B., COSTA, M., OLIVER, J.R., BEARDSLEY, A.M. & MURPHY, R. (1982): Somatostatin is contained in and released from cholinergic nerves in the heart of the toad, *Bufo marinus*. *Neuroscience*, 7: 2013-2023.

COURTICE, G. & DELANEY, D. (1994): Effect of frequency and impulse pattern on the non-cholinergic cardiac response to vagal stimulation in the toad, *Bufo marinus*. *Journal of Autonomic Nervous Systems*, 48: 267-272.

CROSSLEY D.A. & HILLMAN, S.S. (1999): The role of pulmocutaneous baroreceptors in the control of lymphatic heart rate in the toad *Bufo marinus*. *Physiological and Biochemical Zoology*, 72: 109-115.

DAY, J.B., RECH, R.H. & ROBB, J.S. (1963): Pharmacological and microelectrode studies on the frog lymph heart. *Journal of Cellular and Comparative Physiology*, 62: 33-41.

ELLIOTT, N.M, ANDREWS, R.D. & JONES, D.R. (2002): Pharmacological blockade of the dive response: effects on heart rate and diving behaviour in the harbour seal (*Phoca vitulina*). *Journal of Experimental Biology*, 205: 3757-3765.

EMILIO, M.G. & SHELTON, G. (1974): Gas exchange and its effects on pH and bicarbonate equilibria in the blood of the amphibian, *Xenopus laevis*. *Journal of Experimental Biology*, 60: 567-579.

GANS, C., DE LONGH, H.J. & FARBER, J. (1969): Bullfrog (*Rana catesbeiana*) ventilation: how does the frog breathe? *Science*, 163: 1223-1225.

HOBSON, J. (1967): Electrographic correlates of behavior in the frog with special reference to sleep. *Electroencephalography and Clinical Neurophysiology*, 22: 113-121.

JONES, D.R. (1966): Factors affecting the recovery from diving bradycardia in the frog. *Journal of Experimental Biology*, 4: 397-411.

JONES, D.R. (1967): Oxygen consumption and heart rate of several species of anuran amphibians during submergence. *Comparative Biochemistry and Physiology*, 20: 691-707.

JONES, D.R. & SHELTON, G. (1964): Factors influencing submergence and the heart

rate in frog. *Journal of Experimental Biology*, 41: 417-431.

JONES, J.M., WENTZELL, L.A. & TOEWS, D.P. (1992): Posterior lymph heart pressure and rate and lymph flow in the toad *Bufo marinus* in response to hydrated and dehydrated conditions. *Journal of Experimental Biology*, 169: 207-220.

JONES, J.M., GAMPERL, A.K., FARRELL, A.P. & TOEWS, D.P. (1997): Direct measurement of flow from the posterior lymph heart of hydrated and dehydrated toads (*Bufo marinus*). *Journal of Experimental Biology*, 200: 1695-1702.

LILLO, R.S. (1979): Autonomic cardiovascular control during submergence and emergence in bullfrogs. *American Journal of Physiology*, 237: R210-216.

LUND, G.F. & DINGLE, H. (1968): Seasonal temperature influence on vagal control of diving bradycardia in the frog (*Rana pipiens*). *Journal of Experimental Biology*, 48: 265-277.

MORRIS, J.L., GIBBINS, I.L. & OSBORNE, P.B. (1989): Galanin-like immunoreactivity in sympathetic and parasympathetic neurons of the toad, *Bufo marinus*. *Neuroscience Letters*, 102: 142-148.

PRESTON, E. & COURTICE, G. (1992): Vagal stimulation and somastotatin potentate cardiac vagal action in the toad, *Bufo marinus*. *Neuroscience Letters*, 134: 285-288.

PRESTON, E. & COURTICE, G. (1993): Cardiac vagal effects in the toad are attenuated by repetitive vagal stimulation. *Neuropeptides*, 25: 193-198.

PRESTON, E. & COURTICE, G. (1995): Physiological correlates of vagal nerve innervations in lower vertebrates. *American Journal of Physiology*, 268: 1249-1256.

PRESTON, E., CARLO-STELLA, R. & COURTICE, G. (1993): Desensitization of the cardiac response to somatostatin in isolated toad atria but not in whole toads. *Journal of Autonomic Nervous Systems*, 43: 151-158.

RANDALL, D., BURGGREN, W. & FRENCH, K. (2002): *Eckert Animal Physiology*. Freeman and Company, New York.

RUMYANTSEV, P.P. & KRYLOVA, M.J. (1990): Ultrastructure of myofibers and cells synthesizing DNA in the developing and regenerating lymph-heart muscles. *International Review of Cytology*, 120: 1-52.

SATOH, Y.I. & NITATORI, T. (1980): On the fine structure of lymph hearts in amphibia and reptiles. Pp. 149-169, *in*: Bourne, G.H. (eds), *Hearts and Heart-like Organs, Vol. I, Comparative Anatomy and Development*. Academic Press, New York.

SIGNORE, P.E. & JONES, D.R. (1995): Effect of pharmacological blockade and cardiovascular responses to voluntary and forced diving in muskrats. *Journal of Experimental Biology*, 198: 2307-2315.

SIGNORE, P.E. & JONES, D.R. (1996): Autonomic nervous control of heart rate in muskrats during exercise in air and under water. *Journal of Experimental Biology*, 199: 1563-1568.

ms # 228
Recibido: 05/09/06
Aceptado: 14/03/08