

RESEARCH ARTICLE

Temperature effects on the cardiorespiratory control of American bullfrog tadpoles based on a non-invasive methodology

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ABSTRACT

Temperature effects on cardiac autonomic tonus in amphibian larval stages have never been investigated. Therefore, we evaluated the effect of different temperatures (15, 25 and 30°C) on the cardiorespiratory rates and cardiac autonomic tonus of premetamorphic tadpoles of the bullfrog, *Lithobates catesbeianus*. To this end, a non-invasive method was developed to permit measurements of electrocardiogram (ECG) and buccal movements (f_B ; surface electromyography of the buccal floor). For evaluation of autonomic regulation, intraperitoneal injections of Ringer solution (control), atropine (cholinergic muscarinic antagonist) and sotalol (β -adrenergic antagonist) were performed. Ringer solution injections did not affect heart rate (f_H) or f_B across temperatures. Cardiorespiratory parameters were significantly augmented by temperature (f_H : 24.5±1.0, 54.5±2.0 and 75.8±2.8 beats min⁻¹ at 15, 25 and 30°C, respectively; f_B : 30.3±1.1, 73.1±4.0 and 100.6±3.7 movements min⁻¹ at 15, 25 and 30°C, respectively). A predominant vagal tone was observed at 15°C (32.0±3.2%) and 25°C (27.2±6.7%) relative to the adrenergic tone. At 30°C, the adrenergic tone increased relative to the lower temperature. In conclusion, the cholinergic and adrenergic tones seem to be independent of temperature for colder thermal intervals (15–25°C), while exposure to a hotter ambient temperature (30°C) seems to be followed by a significant increase in adrenergic tone and may reflect cardiovascular adjustments made to match oxygen delivery to demand. Furthermore, while excluding the use of implantable electrodes or cannulae, this study provides a suitable non-invasive method for investigating cardiorespiratory function (cardiac and respiratory rates) in water-breathing animals such as the tadpole.

KEY WORDS: Amphibians, Cholinergic tone, Adrenergic tone, Buccal frequency, Heart rate

INTRODUCTION

Amphibian survival depends on an ideal balance of environmental factors, such as temperature and water availability, for completion of the life cycle. Temperature is one of the main factors that affects physiological and metabolic processes in many ectothermic vertebrates (Bicego et al., 2007; da Silva et al., 2013). For instance, increases in heart rate (f_H) owing to elevated body temperature result

mainly from direct influences on the sinoatrial node of the heart (Courtice, 1990; Liu and Li, 2005), in addition to ventilatory adjustments, which probably result from high peripheral chemoreceptor sensitivity (Kruhøffer et al., 1987; Gamperl et al., 1999; Taylor et al., 2010a,b). Anurans are an interesting group to study because of the occurrence of functional changes across their development, passing through water-breathing tadpole stages with gills, to completion of metamorphosis, resulting in the air-breathing adult form with well-developed lungs. Because tadpoles enter early into the external environment, in which they are completely exposed to a highly variable habitat (Burggren and Doyle, 1986; Hill et al., 2012), a short-term oscillation in body temperature is followed by cardiorespiratory adjustments in order to maintain uninterrupted oxygen transport by convective and diffusive steps according to the animals' metabolic demands (Storz et al., 2010).

The autonomic nervous system modulates f_H in all vertebrates, exhibiting inhibitory parasymphathetic and excitatory sympathetic influence on the heart (Taylor et al., 2014). The predominant vagal tone on resting hearts influences instantaneous changes in f_H in a beat-to-beat manner in many vertebrates, while increased adrenergic modulation of the heart is related to activity levels, including fight-or-flight responses (Taylor et al., 1999, 2014). Among vertebrates, autonomic modulation of the heart varies according to the degree of activity, fasting/non-fasting conditions, ontogeny, and environmental factors such as temperature, seasonality and oxygen supply (Burggren and Doyle, 1986; Hoffmann and Romero, 2000; Wang et al., 2001; Taylor et al., 2012; Marks et al., 2013; Sartori et al., 2015; Braga et al., 2016; Zena et al., 2016a; Sandblom, et al., 2016).

During development of the American bullfrog (*Lithobates catesbeianus* Shaw 1802) tadpole, a mild inhibitory vagal tone on the heart has been reported (Burggren and Doyle, 1986), which decreases after metamorphosis (Burggren and Doyle, 1986; Taylor et al., 2012). Additionally, the adrenergic tone seems to be predominant in full-grown specimens of *L. catesbeianus*, in contrast to other anuran species, such as the toad *Rhinella schneideri* (Hoffmann and Romero, 2000) and the frog *Xenopus laevis* (Taylor and Ihmied, 1995). According to Taylor et al. (2012), the sustained adrenergic tone in adult *L. catesbeianus* is virtually independent of temperature, and the changes in f_H evoked by temperature might reflect a thermal compensation of the intrinsic heart pacemaker cells. However, no study has evaluated the effect of temperature on autonomic regulation in premetamorphic tadpoles.

In addition to temperature, resting autonomic tone is also highly dependent on full recovery from surgical procedures (Campbell et al., 2004, 2006). Both minimally instrumented animals with longer recovery periods as well as the use of non-invasive techniques may provide suitable data when studying autonomic modulation in ectothermic vertebrates that have lower resting heart rates, such as amphibians and reptiles (Campbell et al., 2006; Zena et al., 2015, 2016a,b). Cardiorespiratory studies in tadpoles are

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scarce (Burggren and Doyle, 1986; Burggren and West, 1982), which likely reflects the challenging methodology of implantation and maintenance of electrodes, cannulae, etc., especially in the case of long-term measurements.

Based on the effects of temperature on cardiorespiratory parameters in full-grown amphibians (Zena et al., 2015, 2016a,b; Glass et al., 1997; Bicego-Nahas and Branco, 1999; Seebacher and Franklin, 2011) and the fact that no data exist in the literature showing whether autonomic regulation in premetamorphic tadpoles is temperature dependent, the aim of the present study was to determine how temperature (15, 25 and 30°C) affects cardiorespiratory responses, including the sympathetic and parasympathetic balance in the heart, in premetamorphic bullfrog tadpoles (IV–VII; Taylor and Kollros, 1946). Owing to the limitations imposed by the invasive methods available for measuring cardiorespiratory parameters, we developed a non-invasive methodology to measure f_H and buccal movement rates (f_B) in tadpoles based on their resting behavior at the bottom of the water column. To evaluate the extent of the parasympathetic and sympathetic tonus, we performed selective autonomic blockade with an antagonist of β -adrenergic receptors (sotalol hydrochloride) and an antagonist of muscarinic receptors (atropine sulfate).

MATERIALS AND METHODS

Animals

For this study, we used premetamorphic bullfrog (*L. catesbeianus*) tadpoles weighing 3.5–10 g in developmental stages IV–VII (hind limbs are still small buds, the lungs are present but not functional) according to Taylor and Kollros (1946). Animals were obtained from the bullfrog farm (Centro de Aquicultura da Unesp – CAUNESP) at the College of Agricultural and Veterinarian Sciences, São Paulo State University (FCAV-UNESP) in Jaboticabal, São Paulo State, Brazil. All tadpoles were maintained at the animal facility at the Department of Animal Morphology and Physiology. Animals were kept in a water tank (50×40×50 cm) in a room with a light:dark cycle of 12 h:12 h and temperature of 25°C, and were fed daily with a commercial fish food (Purina, Brazil). Water quality was maintained by an external filtration system (mechanical, chemical and biological filtration; model HF-0600, Atman, Santo André, São Paulo, Brazil), and with water replacement of 20 to 30% of the tank volume twice a week. The study was conducted with the approval of the São Paulo State University Animal Care and Use Committee (CEUA-FCAV-UNESP; Protocol 020496).

Cardiorespiratory parameter measurements

f_B and f_H were measured by a non-invasive electrocardiogram (ECG) and surface electromyogram (EMG) method. Buccal movement in the American bullfrog tadpole consists of an opening of the mouth, where water is forced into the buccal cavity by depression of the hyoid, and then a subsequent closing of the mouth, where elevation of the hyoid expels water through the branchial apparatus (Ryerson and Deban, 2010) out of a single spiracle.

The experimental apparatus (Fig. 1) consisted of a homemade chamber (26.5×5×6 cm) containing two parallel, stainless steel filaments 1.5 cm apart from each other and positioned perpendicular to the chamber length. These filaments, which served as recording electrodes, were positioned on the tank floor and extended up into the lateral wall of the chamber (from the bottom to the top of the chamber) from which two wires were attached to them. Both electrodes were connected to a differential AC amplifier (A-M Systems, model 1700, Sequim, WA, USA), allowing for measurements of the animals' buccal floor EMG signals (f_B), in

addition to the ECG for f_H measurements that appeared throughout the buccal movements signal. These biological signals were recorded (1 kHz sample rate) by an acquisition system (PowerLab System, ADInstruments, Sydney, Australia) and later analyzed offline (LabChart Software, version 7.3, ADInstruments). Online signals were amplified (10,000× gain) and band-pass filtered (0.1–5 kHz) using a differential AC amplifier. The water system (water box underneath the experimental tank) was grounded to attenuate noise, using a ground wire connected to the ground input of the amplifier. For analysis, raw signals were further filtered using the software's digital built-in filters (low-pass: 150 Hz).

The experimental tank was covered with black plastic throughout the entire experiment in order to prevent visual disturbances of the animals. In addition, a mini aquarium pump positioned in a box underneath the experimental tank maintained a continuous flow to ensure water renewal; two air pumps were used to guarantee adequate oxygenation. Given the configuration and characteristics of this experimental apparatus, the system was only operational when tadpoles were resting motionless over the electrodes in the experimental chamber (Fig. 1).

In order to validate EMG measurements of buccal muscle contractions, we measured the buccal pressure of one premetamorphic bullfrog tadpole. For this, the tadpole was anesthetized by immersion in benzocaine (Acros Organics, NJ, USA) solution (20 mg l⁻¹) buffered to pH 7.7 with sodium bicarbonate for 10 min or until postural reflexes disappeared. A flared polyethylene cannula (PE-10; Clay Adams, Parsippany, NJ, USA), filled with water, was placed inside the buccal cavity through a hole made by a needle (26 gauge) in the soft dorsal palate. The tadpole was allowed to recover from anesthesia and placed in the experimental apparatus for measurements of EMG (see details above) and buccal pressure, both at the same time, by connecting the buccal cannula to a physiological pressure transducer (MLT844, ADInstruments). The transducer was connected to a data acquisition system (PowerLab System, ADInstruments/Chart Software, version 7.3) via a bridge amplifier (FE221, ADInstruments). Owing to the tadpole's buccal soft tissue, the buccal cannula was easily removed upon any abrupt movement performed by the animal. Thus, the validation protocol was made possible for only a few minutes, right after the animal's recovery from anesthesia.

Drugs

Atropine (cholinergic muscarinic antagonist; 3.0 mg kg⁻¹) and sotalol (β -adrenergic antagonist; 3.0 mg kg⁻¹) were purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in amphibian Ringer solution (composition in mmol l⁻¹: 46.9 NaCl; 21.0 KCl; 2.40 CaCl; 1.29 MgCl; 3.14 NaHCO₃; according to Zena et al., 2016a,b). The effectiveness of the blockade was previously confirmed by injecting acetylcholine (cholinergic muscarinic agonist; 100 μ g kg⁻¹) and epinephrine (β -adrenergic agonist; 100 μ g kg⁻¹) before and after cholinergic and adrenergic blockade, respectively.

Experimental protocol

All experiments were performed on unanesthetized premetamorphic tadpoles, which were previously exposed to the experimental apparatus at different temperatures (15, 25 or 30°C) for at least 24 h prior to the start of the experiments. A temperature change and maintenance system was adapted to the existing setup. For this, an external circulation bath (PolyScience 9112A11B Programmable Model 9112 Refrigerated Circulator) and a copper coil were used to change the temperature of the experimental box (Fig. 1). Internal

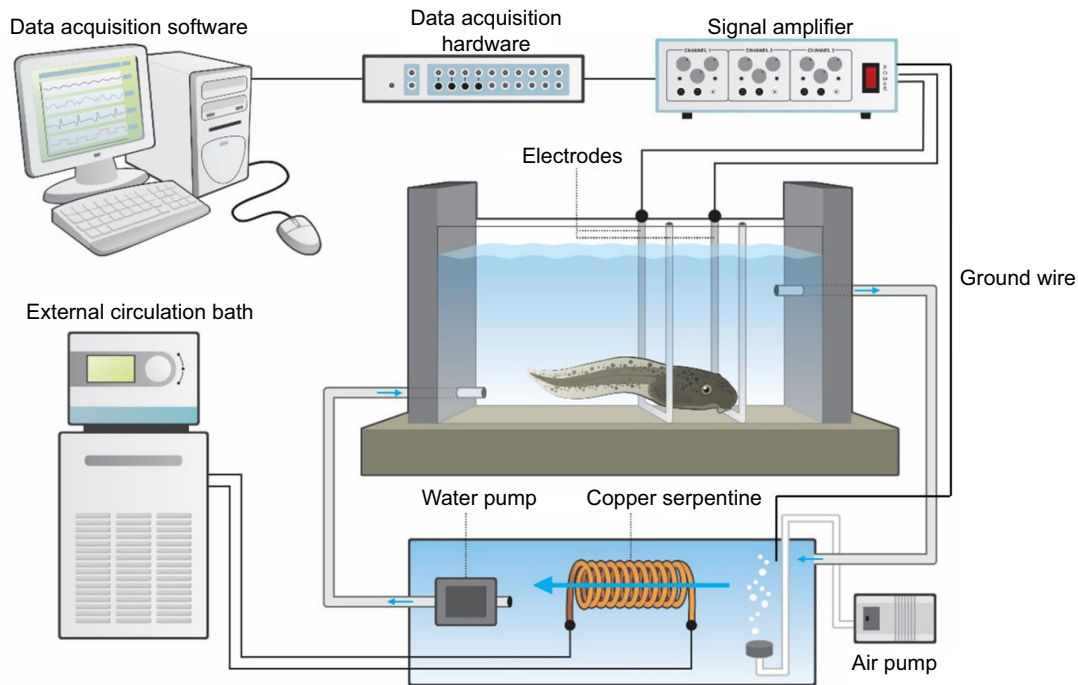


Fig. 1. Representative scheme of the non-invasive experimental setup for electrocardiogram (ECG) and surface electromyogram (EMG) measurements. The experimental apparatus consists of a water chamber containing two parallel stainless steel filaments positioned perpendicular to each other, soldered to two wires and connected to a differential AC amplifier. The electrode configuration allowed measurements of the premetamorphic bullfrog tadpole's EMG and ECG signals (see Materials and methods for details).

temperature settings of the bath were made so as to maintain the desired temperature (15 and 30°C) in the experimental box.

Real-time baseline recordings of buccal movements and ECG were performed for approximately 1 h. After this period, animals were handled in order to administer an intraperitoneal injection of Ringer solution (vehicle) to verify the influence of the injections on the basal cardiorespiratory parameters, which were recorded for an additional hour. Intraperitoneal injections were performed using a thin dental needle (30 gauge) connected by a polyethylene tube (PE-10, Clay Adams) to a Hamilton syringe (5 μ l). The injections were standardized so that the volume injected into the peritoneal cavity was equal to 0.6 μ l g^{-1} . The movement of an air bubble inside the PE-10 polyethylene tubing connecting the microsyringe to the dental needle confirmed drug flow. One day after the baseline measurements were made in tadpoles, the drugs were injected to block sympathetic and parasympathetic influences on the heart. First, the baseline buccal movements and ECG basal were recorded for 40 min, followed by intraperitoneal injection of atropine (antagonist of muscarinic receptors, 3.0 mg kg^{-1}) with collection of buccal movement and ECG data for an additional hour. In sequence, sotalol (β -adrenergic antagonist; 3.0 mg kg^{-1}) was injected, completing the double autonomic blockade, and one more hour of recording was performed. Another group of animals was subjected to the same experimental protocol with administration of the drugs in the opposite sequence (injection of sotalol followed by injection of atropine).

The grouping model previously described by Altimiras et al. (1997) was used in the present study to refer to the different treatments: Group I ($N=23$) for those animals that first received the β -adrenergic antagonist (sotalol), and Group II ($N=24$) for those that received the cholinergic antagonist (atropine) first. The same experimental protocol was used in the experiments conducted at 15 and 30°C with different animal groups to test the same parameters

mentioned above (buccal movements and ECG) at reduced and elevated temperatures.

At the end of each experiment, the tadpoles were euthanized by submergence in an aqueous solution of benzocaine hydrochloride (250 mg l^{-1}) buffered to pH 7.7 with sodium bicarbonate.

Analysis of cholinergic and adrenergic tones

Cholinergic and adrenergic tones of the heart were calculated based on the animals' cardiac intervals. For this, the changes induced in the cardiac interval by atropine (3.0 mg kg^{-1}) or sotalol (3.0 mg kg^{-1}) were expressed in relation to the intrinsic cardiac interval obtained after the total autonomic blockade (atropine +sotalol). To quantify the cholinergic and adrenergic tones, we used the equations proposed by Altimiras et al. (1997):

$$T_{\text{Chol}} = \frac{RR_{\text{cont}} - RR_{\text{musc}}}{RR_0} \times 100, \quad (1)$$

$$T_{\text{Adre}} = \frac{RR_0 - RR_{\text{musc}}}{RR_0} \times 100, \quad (2)$$

where T_{Chol} is the cholinergic tone (%), T_{Adre} is the adrenergic tone (%), RR_{cont} is the wave interval control, RR_{musc} is the wave interval after muscarinic receptor blockade and RR_0 is the wave interval after total autonomic block.

Data analysis and statistics

The values obtained are expressed as means \pm s.e.m. Mean f_H and f_B were obtained using the last 5 min of stable recordings made for baseline measurements and drug effects. Heart rate was calculated using the 'cyclic measurement detection' tool from Chart Software, selecting 'ECG mode' and adjusting 'QRS width' as needed. Buccal movement rate was calculated using the same tool as for f_H , except choosing 'general mode' in the 'cyclic measurement

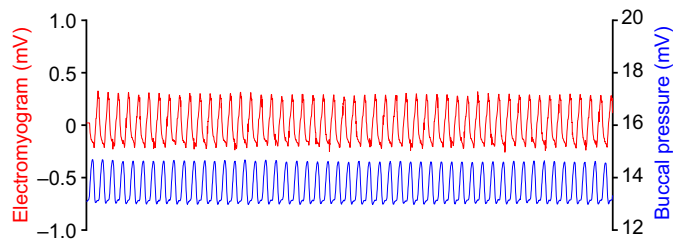


Fig. 2. Representative recordings of buccal movements in premetamorphic tadpoles. Comparison between buccal floor electromyography (red) and buccal pressure signals (blue) from one representative premetamorphic bullfrog (*Lithobates catesbeianus*) tadpole at 25°C. Note that the raw signal (red line) is composed by the electromyogram and the electrocardiogram waveforms.

detection' settings. The effects of animal manipulation and injection of Ringer solution at the three temperatures were evaluated by means of a two-way ANOVA for repeated measures (factors: Ringer solution and temperature). The effects of injections of atropine and sotalol (or sotalol and atropine) at each of the three temperatures were also evaluated by a two-way repeated-measures ANOVA (factors: drugs and temperature). Differences in cholinergic and adrenergic tones were evaluated using a two-way repeated-measures ANOVA. When performing an ANOVA, a Tukey's test was used to access differences between means that were considered significant at $P < 0.05$.

RESULTS

Heart rate and buccal movement recordings

In order to validate data obtained from muscle contractions of the tadpoles' buccal floor, we measured buccal pressure simultaneously with buccal floor EMG signals in a single tadpole. As depicted for the representative recordings in Fig. 2, there is an equivalence for

buccal movement rate between both methodologies tested. Fig. 3A, B shows representative recordings of f_B and f_H during the basal measurements of three premetamorphic bullfrog tadpoles at 15, 25 and 30°C. It can be observed that the frequencies of the cardiorespiratory parameters increase as the temperature rises.

Temperature effects on cardiorespiratory parameters in bullfrog tadpoles

Fig. 3C,D depicts temperature effects (15, 25 and 30°C) on cardiorespiratory parameters before and after injection of Ringer solution (vehicle). At each temperature, f_H ($P=0.24$) and f_B ($P=0.435$) were not affected by Ringer solution injection. In addition, f_H and f_B was significantly augmented across temperatures for either baseline [f_H (beats min^{-1}): 15°C: 24.5 ± 1.0 ; 25°C: 54.5 ± 2.0 ; 30°C: 75.8 ± 2.8 ($F_{2,21}=260.2$, $P < 0.001$) and f_B (movements min^{-1}): 15°C: 30.3 ± 1.1 ; 25°C: 73.1 ± 4.0 ; 30°C: 100.6 ± 3.7 ($F_{2,20}=130.3$, $P < 0.001$)] or Ringer solution values [f_H (beats min^{-1}): 15°C: 26.1 ± 1.8 ; 25°C: 54.4 ± 1.6 ; 30°C: 78.3 ± 2.2 and f_B (movements min^{-1}): 15°C: 31.5 ± 1.4 ; 25°C: 72.1 ± 4.2 ; 30°C: 107.2 ± 6.1]. As expected for a typical thermal effect for physiological and biochemical processes (i.e. $Q_{10}=2.0-3.0$), f_H increased from 15 to 30°C with a Q_{10} of 2.08. Regarding f_B , the same pattern was observed, with a Q_{10} of 2.16 from 15 to 30°C.

Effects of selective autonomic blockade in f_H and f_B

Both f_H ($F_{2,66}=14.380$, $P < 0.001$) and f_B ($F_{2,63}=5.088$, $P=0.01$) were significantly affected by selective pharmacological blockade in Group I (sotalol followed by atropine injections) (Fig. 4). The β -adrenergic blockade (sotalol) reduced f_H significantly only at 25°C ($P=0.014$), but not at 15°C ($P=0.97$) or 30°C ($P=0.28$); at the latter temperature, a decreasing trend in f_H was exhibited (Fig. 4A). In addition, muscarinic blockade (sotalol+atropine) injections brought f_H to near-baseline values at 25°C ($P=0.25$) and 30°C

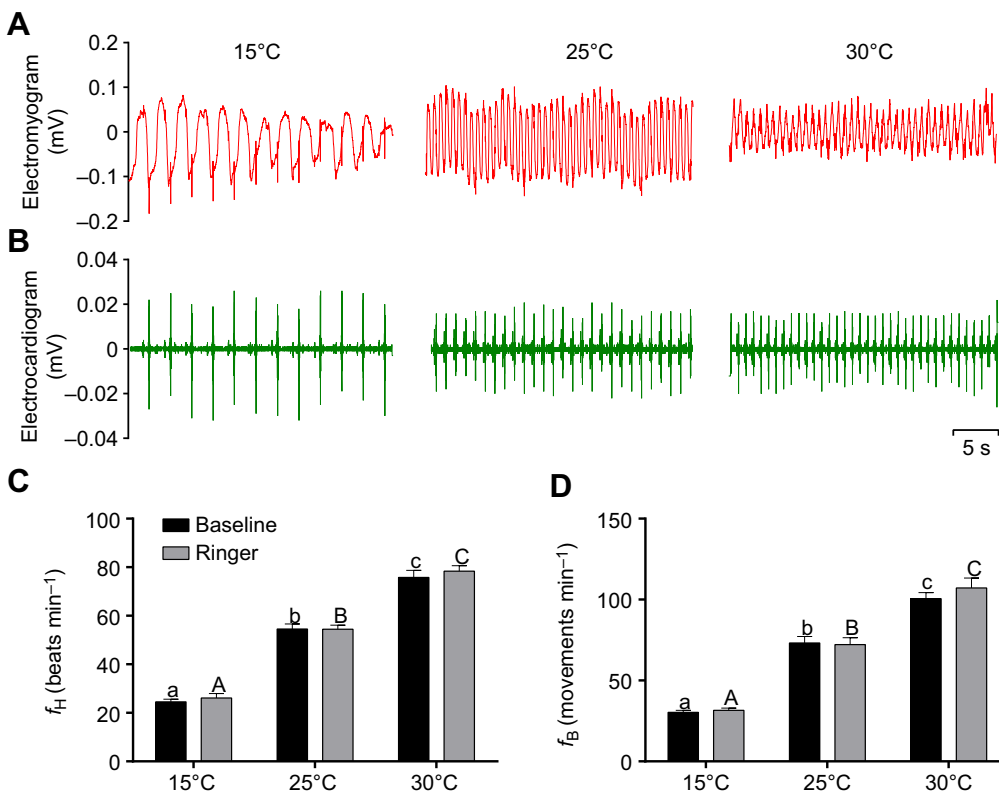


Fig. 3. Temperature effects on cardiorespiratory parameters in premetamorphic tadpoles.

(A,B) Representative records of buccal floor electromyography (EMG; A) and electrocardiogram (ECG; B) signals from three premetamorphic bullfrog (*Lithobates catesbeianus*) tadpoles during resting at 15, 25 and 30°C. (C,D) Effect of Ringer solution injections (vehicle) on heart rate (f_H ; C) and buccal movements (f_B ; D) in premetamorphic tadpoles across temperatures. Different lowercase letters indicate a significant difference between temperatures for baseline values, while different uppercase letters indicate a significant difference between temperatures for Ringer solution injection values. Ringer solution injections did not influence cardiorespiratory parameters within each temperature. Data are presented as means \pm s.e.m. f_H : $N=8$, 8 and 7 for 15, 25 and 30°C, respectively; f_B : $N=7$, 8 and 8 for 15, 25 and 30°C, respectively.

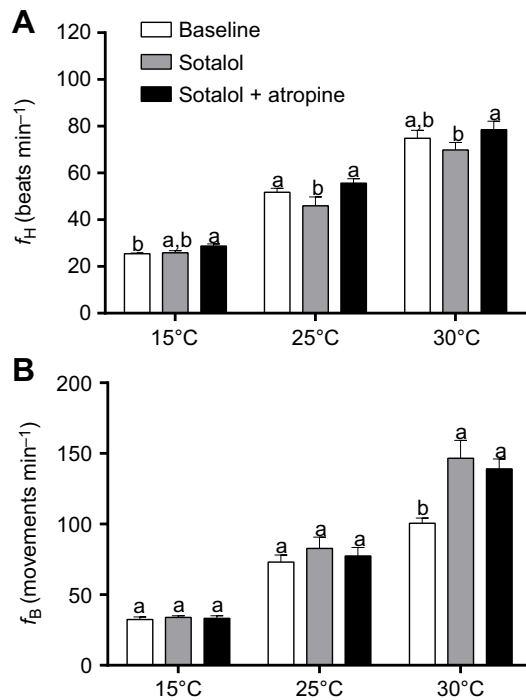


Fig. 4. Effect of Group I pharmacological blockade on cardiorespiratory parameters in premetamorphic tadpoles. Effect of pharmacological blockade (sotalol alone and sotalol+atropine; Group I) on heart rate (f_H ; A) and buccal movements (f_B ; B) of premetamorphic *Lithobates catesbeianus* tadpoles at three different temperatures (15, 25 and 30°C). Different lowercase letters indicate a significant difference within each temperature. Regardless of treatment, f_H and f_B were significantly altered across temperatures. Data are presented as means \pm s.e.m. $N=7-8$.

($P=0.55$), while increasing f_H at 15°C relative to baseline values ($P=0.048$). Injection of the β -adrenergic receptor antagonist (sotalol) alone or in combination with atropine (double autonomic blockade) did not modify the f_B at lower temperatures (15 and 25°C); however, at 30°C, a significant increase in f_B occurred after sotalol alone ($P=0.001$) and sotalol followed by atropine treatment ($P=0.005$; Fig. 4B).

In relation to Group II (atropine followed by sotalol injections), the selective pharmacological blockade affected both f_H ($F_{2,69}=27.958$, $P<0.001$) and f_B ($F_{2,63}=32.579$, $P<0.001$). Atropine alone caused a significant increase in f_H in two of the three temperatures tested (25 and 30°C, $P<0.001$; Fig. 5A). Nevertheless, even at 15°C, when no significant effect of atropine was observed ($P=0.06$), a slight increase in f_H was observed, which is supported by the higher cholinergic tone in relation to the adrenergic tone ($P<0.001$; Fig. 6). Furthermore, there was an increase in f_B when the double autonomic blockade (atropine +sotalol) was performed at 15°C ($P=0.004$) and 25°C ($P<0.001$; Fig. 5B), while at 30°C, it was possible to observe a significant increase after atropine ($P=0.04$) and double blockade ($P<0.001$).

Autonomic tonus

Fig. 6 shows the relative cholinergic and adrenergic tonus on the heart at different temperatures. In the premetamorphic stages studied (IV–VII), the animals presented a predominant cholinergic tone at 15°C ($32.0\pm 3.2\%$; $P<0.001$) and 25°C ($27.2\pm 6.7\%$; $P=0.025$) relative to the adrenergic tone (15°C: $4.4\pm 4.3\%$ and 25°C: $12.7\pm 2.5\%$). An interaction effect between temperature and autonomic tone was observed ($F_{2,43}=13.766$; $P<0.001$), and cholinergic tone

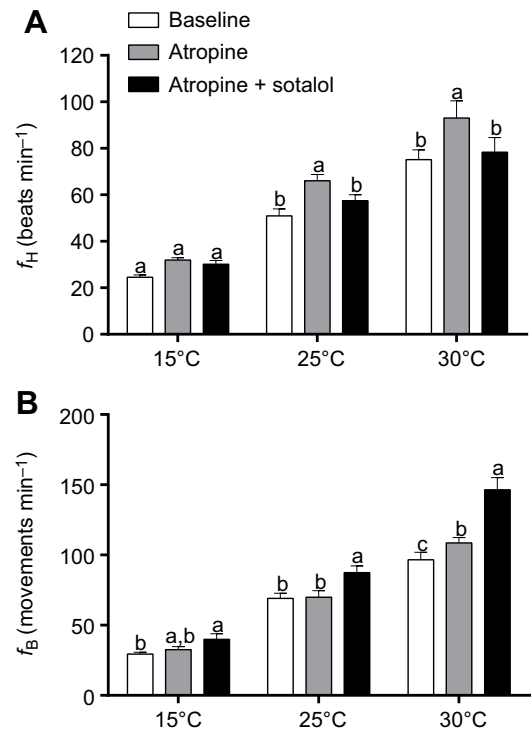


Fig. 5. Effect of Group II pharmacological blockade on cardiorespiratory parameters in premetamorphic tadpoles. Effect of pharmacological blockade (atropine alone, atropine+sotalol; Group II) on heart rate (f_H ; A) and buccal movements (f_B ; B) of premetamorphic *Lithobates catesbeianus* tadpoles at three different temperatures (15, 25 and 30°C). Different lowercase letters indicate a significant difference within each temperature. Regardless of treatment, f_H and f_B were significantly altered across temperatures. Data are presented as means \pm s.e.m. $N=8$.

($13.7\pm 5.3\%$) had a tendency to decrease at 30°C relative to 15 and 25°C, while, conversely, the adrenergic tone increased significantly from 15 to 30°C ($15.7\pm 4.2\%$; $P<0.021$).

DISCUSSION

To our knowledge, this is the first study to demonstrate a non-invasive methodology for measuring cardiorespiratory parameters in tadpoles. Using this methodology, we showed that premetamorphic bullfrog

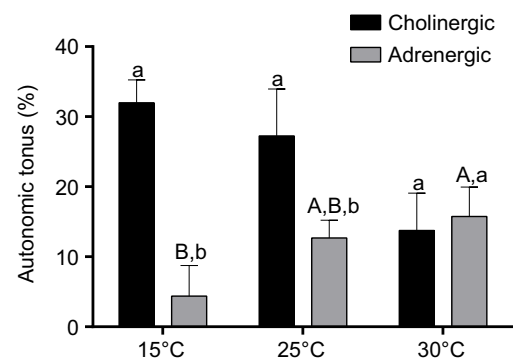


Fig. 6. Calculated autonomic tones on the heart of premetamorphic tadpoles at three different temperatures. Across temperatures (15, 25 and 30°C), cholinergic tone tended to decrease, while adrenergic tone increased (interaction effect, $P<0.001$). Different lowercase letters indicate a significant difference between cholinergic and adrenergic tones within each temperature, while different uppercase letters indicate a significant difference across temperature for adrenergic tone. Data are presented as means \pm s.e.m. $N=8$.

tadpoles (*L. catesbeianus*) exhibit prevailing cholinergic over adrenergic tones at lower temperatures, 15 and 25°C. In contrast, adrenergic tone increases at 30°C, while cholinergic tone trends toward a decrease and may reflect cardiovascular adjustments in order to match oxygen delivery to demand across thermal exposures.

The extent to which adrenergic and cholinergic tones modulate f_H can vary greatly among species and developmental stages within species, and is further compounded by the degree of surgical instrumentation and recovery time (Taylor et al., 2014; Sandblom and Axelsson, 2011; Burggren and Doyle, 1986). Because the autonomic nervous system is very sensitive to stress and anesthesia (Campbell et al., 2004), we used a non-invasive methodology, which eliminated the need for implantation of electrodes and buccal cannula, to evaluate f_H and f_B in bullfrog tadpoles. Some studies have already used non-invasive methodologies to measure fish physiological parameters (Altimiras and Larsen, 2000; Yoshida et al., 2009), but there is no report on amphibians, especially in the larval stage. In addition, the recorded signals for buccal movements made by the same electrodes were compared with pressure oscillations recorded by a polyethylene cannula (P-10) inserted into the buccal cavity and connected to a pressure transducer (Fig. 2), and the recordings were identical. Thus, we believe the method described here is perfectly suitable for studies in intact and undisturbed tadpoles and opens the possibility for investigation of cardiorespiratory physiology in other amphibian species, especially when considering limitations such as size and number of individuals available.

In the present study, the premetamorphic tadpoles increased f_H as temperature increased, as expected. Thermal acclimation effects on resting f_H implicate adjustments in the intrinsic f_H and control systems that modulate f_H , such as the sympathetic and parasympathetic nervous system (Sandblom et al., 2016). Besides the existence of an inverse relationship between vagal and sympathetic tones across temperature exposures in the American bullfrog tadpoles, the major changes in f_H are dictated by thermal acute effects on the intrinsic pacemaker cells, as the f_H thermal effect (Q_{10} ; thermal interval 15–30°C) of full autonomic blockaded tadpoles varies very little from that of resting animals ($Q_{10}=1.92$ versus 2.08, respectively). This suggests that, for short-term acclimation, thermal effects in American bullfrog tadpoles involve mainly intrinsic, rather than extrinsic, cardiac rate adjustments. A long-term acclimation (weeks) might reveal compensatory adjustments in magnitude, such as an increased cholinergic tone, as seen in rainbow trout and perch (Sandblom et al., 2016; Ekström et al., 2016). Nevertheless, chronic acclimation to high temperatures may impose additional challenges for the study of autonomic balance for a specific developmental phase in tadpoles because increases in temperature reduce the duration of amphibian metamorphosis (Orizaola and Laurila, 2009). Our data in resting and double-blockaded tadpoles are in agreement with those from a previous study in adult bullfrogs, demonstrating that temperature-induced rises in f_H are mostly due to intrinsic pacemaker adjustments (Taylor et al., 2012).

Although the drop in the cholinergic tone is not statistically significant, adrenergic tone increases significantly, and this may indicate that 30°C represents a stressful condition for the bullfrog tadpoles. It is worth mentioning that we performed some tests at 35°C, but the animals did not withstand the 2 days of experiments, so we chose to perform the protocols at 30°C. Amphibian larvae may develop in environments in which their adult forms spend part or all of their life cycle, experiencing stressful conditions such as predation, desiccation and temperature changes. The significance of

developmental changes in autonomic tone is directly linked to maturation of their cardiovascular system and reflex mechanisms involved in cardiovascular homeostasis. Thus, adjustments in the metabolic and autonomic modulation would be particularly important for tadpole species that occupy puddles with little water volume that undergo intense heating (Katzenberger et al., 2012). In contrast to premetamorphic tadpoles, full-grown bullfrogs exhibit elevated adrenergic tone that is virtually independent of temperature (10, 20 and 30°C; Taylor et al., 2012), and cholinergic tone that increases slightly from –5% to 10% with increasing temperature (10 to 30°C). Conversely, the cholinergic tone is highly augmented by a 3-day acclimation to a decreasing temperature (from 25 to 15°C, then to 5°C) in the aquatic *Xenopus laevis*, showing that thermal effects on f_H are attenuated by higher cholinergic tone (Taylor and Ihmied 1995).

Having performed experiments at ~21.5°C in bullfrog tadpoles, Burggren and Doyle (1986) described an autonomic nervous system modulation involved in the regulation of f_H . According to the authors, the resting vagal tone appears at stages X–XIV, during which tadpoles already exhibit lung ventilation. Interestingly, our data suggest that resting parasympathetic modulation of f_H in bullfrog tadpoles appears earlier in their development, as intraperitoneal injections of atropine increased f_H in our animals at stages IV–VII (Fig. 5). This difference could be related to the methodology used in the first study, as ECG electrode implantation required surgical procedures performed under anesthesia with MS-222, which may have changed the autonomic balance in their tadpoles. Although Burggren and Doyle (1986) did not report a significant increase in f_H with atropine treatment, it is still possible to observe a slight increase in f_H in their tadpoles. The absence of changes in f_H may be justified by prior use of anesthesia and/or a lower experimental temperature as compared with our study, as no significant increase in f_H was observed at 15°C after treatment with atropine (Fig. 5), aside from the cholinergic tone being higher than adrenergic tone.

Tadpoles use the skin, gills and lungs for gas exchange and throughout their development, the relative role of each of these organs changes (Burggren and West, 1982). Tadpoles selected in the present study did not exhibit surfacing behavior for pulmonary gas exchange, suggesting that most of the gas exchange occurred through their skin and gills. Gill ventilation in tadpoles is directly related to buccal movements comprising both buccal and pharyngeal force pumps to produce a continuous unidirectional flow of water from the mouth and across the gills. A high-frequency and low-amplitude activity burst of motor trigeminal (V), facial (VII) and vagus (X) nerves innervates the oropharyngeal muscles, providing the driving force for gill ventilation (reviewed by Gargaglioni and Milsom, 2007). Temperature may also influence the relative contribution of the gills and skin to gas exchange, and the obvious reduction in f_B (and sometimes the occurrence of apneas) at 15°C likely occurs because of a combination of decreased oxygen demand and increased oxygen availability, as oxygen solubility in water increases as temperature decreases (Dejours, 1981). Therefore, we suggest that a reduced metabolic rate at 15°C (inferred by a reduced f_H and f_B) in our tadpoles caused a reduced demand for oxygen, which could be obtained either by the gill or, more commonly, by the cutaneous route, where the skin is highly vascularized and has a large surface-area-to-body-mass ratio (Burggren and West, 1982).

Noteworthy, premetamorphic tadpoles used in the present study exhibited f_B greater than f_H at 25°C ($f_B/f_H>1$). Despite temperature changes and a decrease or increase in both f_B and f_H , the relationship between f_B and f_H was kept relatively constant across temperatures,

where f_B was always higher than f_H (15°C: 1.24±0.04; 25°C: 1.43±0.06; 30°C: 1.33±0.07). Breathing in water requires great energy expenditure to extract sufficient oxygen from an environmental medium in which oxygen is limited by its solubility, especially at high temperature. Therefore, after β -adrenergic blockade, f_H decreased while f_B increased as a compensatory response to maintain oxygen homeostasis at 25 and 30°C. A contrasting result was observed when muscarinic blockade was performed, elevating f_H while f_B remained unchanged at 25°C or increased slightly at 30°C relative to baseline values. It is not clear why f_B was not proportionally reduced while f_H increased after muscarinic blockade; however, a possible explanation may involve temperature effects on oxygen chemosensitivity preventing reductions in f_B when oxygen becomes the limiting factor in a highly metabolically demanding environment.

In summary, our data show that the cholinergic tone originates earlier than previously suggested in the development of American bullfrog tadpoles. The use of a successfully non-invasive methodology for measuring f_H and f_B (and thus avoiding the use of anesthetic agents and surgical procedures) was essential to assess the autonomic tones in the present study. For the thermal interval between 15 and 25°C, cholinergic and adrenergic tones are temperature independent, while at higher temperatures (30°C), the cholinergic tone trends toward a decrease and the adrenergic tone increases. Besides changes in autonomic balance, f_H adjustments in tadpoles are mainly dictated by thermal effects in the intrinsic f_H because the Q_{10} effects on tadpoles' both resting and intrinsic (full autonomic blockade) heart rates are very similar (1.92 versus 2.08). Based on the methodology developed in the present study, we encourage further cardiorespiratory studies in other tadpole species, which may provide valuable data for understanding how larval amphibians may cope with climate changes that likely affect amphibian life cycles.

APPENDIX

To verify the effectiveness of the autonomic blockade, we performed complementary experiments with two groups of animals at 25°C: one group received intraperitoneal injection of acetylcholine (100 $\mu\text{g kg}^{-1}$; $n=6$) before and after muscarinic blockade with atropine, and another group received intraperitoneal injection of epinephrine (100 $\mu\text{g kg}^{-1}$; $n=7$) before and after β -adrenergic blockade with sotalol. Both epinephrine and acetylcholine have instantaneous effects on heart rate, which could also be affected by the manipulation of the animals for intraperitoneal injections. For this reason, we have considered the effects right after the manipulation of the animals, as explained as follows.

We found that epinephrine has an instantaneous tachycardic effect; however, because animal manipulation itself for intraperitoneal injections induces increases in heart rate, we opted to perform an additional injection of Ringer solution to quantify the magnitude of the tachycardia owing to animal manipulation and that owing to epinephrine effects on heart rate (Fig. S1A). In any case, β -adrenergic blockade with sotalol was able to block most of the tachycardic response evoked by epinephrine itself as well as that caused by animal manipulation (Fig. S2A).

Regarding acetylcholine, we found an instantaneous bradycardia right after the intraperitoneal injection of acetylcholine (Figs S1B and S2B), while atropine increased heart rate accordingly and abolished the bradycardic effect, induced by the muscarinic agonist, when a second injection was performed (Figs S1B and S2B). Therefore, because the dosage used in the present study was able to block most or all of the agonist's effects, it was also able to produce a complete blockade of the tadpole's autonomic tone.

Acknowledgements

We thank Euclides Roberto Secato for his excellent technical assistance.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.S.L., L.A.Z., G.S.S., K.C.B., L.H.G.; Methodology: L.S.L., L.A.Z., G.S.S., L.H.G.; Validation: L.A.Z.; Formal analysis: L.S.L., L.A.Z., G.S.S., L.H.G.; Investigation: L.S.L., K.C.B.; Writing - original draft: L.S.L., L.A.Z., G.S.S., K.C.B., L.H.G.; Writing - review & editing: L.S.L., L.A.Z., G.S.S., K.C.B., L.H.G.; Supervision: L.A.Z., L.H.G.; Project administration: L.H.G.; Funding acquisition: L.H.G.

Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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