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Effects of extracellular changes on spontaneous heart rate of normoxia- and anoxia-acclimated turtles (*Trachemys scripta*)

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Summary

Heart rate (f_H) of the anoxia-tolerant freshwater turtle (*Trachemys scripta*) during prolonged anoxia exposure is 2.5- to 5-times lower than the normoxic rate, but whether alterations in blood composition that accompany prolonged anoxia contribute to this bradycardia is unknown. We examined how temperature acclimation, oxygen deprivation, acidosis, hyperkalemia, hypercalcemia and adrenaline affect chronotropy in the turtle myocardium. We monitored spontaneous contraction rates of right-atrial preparations obtained from 21°C- and 5°C-acclimated turtles that had been exposed to either normoxia or anoxia (6 h at 21°C; 2 weeks at 5°C). Sequential exposures to saline solutions were designed to mimic, in a step-wise manner, the shift from a normoxic to anoxic extracellular condition (for normoxia-acclimated preparations) or the reverse (for anoxia-acclimated preparations). Our results clearly show that prolonged anoxia exposure re-sets the intrinsic f_H of turtles at both temperatures, with reductions in intrinsic f_H in the range of 25%–53% compared with normoxia. This intrinsic change would contribute to the bradycardia observed with prolonged anoxia. Further, we found negative chronotropic effects of extracellular anoxia, acidosis and hyperkalemia, and positive chronotropic effects of hypercalcemia and adrenaline. The exact nature of these extracellular effects depended, however, on the acclimation temperature and the prior exposure of the animal to anoxia. With normoxia-acclimated preparations at 21°C, combined anoxia and acidosis (pH reduced from ~7.8 to ~7.2) significantly reduced spontaneous f_H by 22% and subsequent exposure to hyperkalemia (3.5 mmol l^{-1} K^+) further decreased f_H. These negative chronotropic effects were ameliorated by increasing the adrenaline concentration from the tonic level of 1 nmol l^{-1} to 60 nmol l^{-1}. However, in anoxia-acclimated preparations at 21°C, anoxia alone inhibited f_H (by ~30%). This negative chronotropic effect was counteracted by both hypercalcemia (6 mmol l^{-1} Ca^{2+}) and adrenaline (60 nmol l^{-1}). At 5°C, only the combination of anoxia, acidosis (pH reduced from ~8.0 to ~7.5) and hyperkalemia (3.5 mmol l^{-1} K^+) significantly reduced spontaneous f_H (by 23%) with preparations from normoxia-acclimated turtles. This negative chronotropic effect was fully reversed by hypercalcemia (10 mmol l^{-1} Ca^{2+}). By contrast, spontaneous f_H of anoxia-acclimated preparations at 5°C was not affected by any of the extracellular changes. We conclude that prior temperature and anoxia experiences are central to determining f_H during prolonged anoxia in *Trachemys scripta* both as a result of the re-setting of pacemaker rhythm and through the potential influence of extracellular changes.

Key words: acidosis, adrenaline, anoxia, calcium, cardiovascular, intrinsic heart rate, potassium, red-eared slider, temperature, *Trachemys scripta*, turtle.

Introduction

Freshwater turtles of the genera *Chrysemys* and *Trachemys* exhibit a remarkable ability to endure prolonged periods of anoxia. At warm acclimation temperatures (20°C–25°C), these animals can survive 12–24 h of anoxic submergence, whereas at cold-acclimation temperatures (3°C–5°C) *Chrysemys* can recover physiological functions following 5 months of anoxia (Johlin and Moreland, 1933; Jackson and Ultsch, 1982; Ultsch and Jackson, 1982; Herbert and Jackson, 1985a; Herbert and Jackson, 1985b) and *Trachemys* can survive for up to 44 days (Ultsch, 1985; Warren et al., 2006). This exceptional ability to live without oxygen is primarily achieved through a profound ~90% reduction in whole-animal metabolic rate that greatly slows metabolic fuel use and waste accumulation (Jackson, 1968), as well as the utilization of the bone and shell as buffers to ameliorate a catastrophic reduction in pH that would otherwise accompany sustained anaerobic metabolism (Jackson, 2000; Jackson, 2002; Warren et al., 2006).

The heart of the turtle continues to function during prolonged anoxia in order to transport metabolites and waste products among tissues, but cardiac performance is greatly reduced in concert with the reduction in whole-animal metabolic rate and the subsequent decreased demand for blood flow (Herbert and Jackson, 1985b; Hicks and Wang, 1998; Hicks and Farrell, 2000a; Stecyk et al., 2004). For instance, systemic cardiac power output (PO_{sys}) is reduced by 78–85% and by ~95% following 6 h and 14–21 day anoxic exposures in warm- and cold-acclimated turtles, respectively (Hicks and Farrell, 2000a; Stecyk et al., 2004). The large reduction in PO_{sys} with anoxia results from a minor arterial hypotension (~30% decrease in arterial blood pressure) and a large decrease in systemic cardiac output ($\dot{Q}_{sys}$; up to 78% and 92% reductions in warm- and cold-acclimated turtles, respectively). These decreases in $\dot{Q}_{sys}$ are affected by marked bradycardia; systemic stroke volume remains unchanged (Hicks and Wang, 1998; Hicks and Farrell, 2000a; Stecyk et al., 2004). Specifically, heart rate (f_H) decreases by 60% (2.5-fold) from ~25 min^{-1} to ~10 min^{-1} within 1 h during anoxia at 21°C–25°C and by 80% (5-fold) from a normoxic rate of ~5 min^{-1} to less than 1 min^{-1} within 24 h in anoxic turtles at 5°C.

Although the bradycardia and associated depression of cardiac activity exhibited by anoxic turtles is well documented and quantified *in vivo*, its determinants are not fully elucidated. In warm-acclimated turtles, a simultaneous cholinergic, vagal cardiac inhibition and β-adrenergic cardiac stimulation contribute to the setting of anoxic f_H (Hicks and Wang, 1998; Hicks and Farrell, 2000b), but α-adrenergic (Stecyk et al., 2004) and adenosinergic cardiac inhibition do not (J.A.W.S., K.-O. Stenslokken, G. E. Nilsson and A.P.F., unpublished data). However, cholinergic cardiac inhibition only accounts for ~30% of the reduction in f_H that occurs during warm anoxia (Hicks and Wang, 1998; Hicks and Farrell, 2000b). In cold-acclimated, anoxic turtles, autonomic cardiovascular control is blunted and does not account for the large bradycardia (Hicks and Farrell, 2000b; Stecyk et al., 2004). Similarly, there is no adenosinergic cardiac inhibition during prolonged, cold anoxia (J.A.W.S., K.-O. Stenslokken, G. E. Nilsson and A.P.F., unpublished data). Thus, other determinants must contribute to the depression of f_H in both warm- and cold-acclimated turtles during anoxia, i.e. in addition to autonomic cardiovascular control in warm-acclimated turtles and instead of the autonomic control that is turned off in cold-acclimated turtles. The purpose of the present study was to examine the contribution to this anoxic bradycardia made by the significant changes in the extracellular milieu that accompanies prolonged anoxia.

During prolonged anoxia, turtle blood progressively becomes anoxic, acidic, hypercapnic, hyperkalemic, hypermagnesemic and hypochloremic (Ultsch and Jackson, 1982; Jackson and Ultsch, 1982; Herbert and Jackson, 1985a). Further, blood lactate (Ultsch and Jackson, 1982; Jackson and Ultsch, 1982; Herbert and Jackson, 1985a) and circulating catecholamine levels are greatly elevated during anoxia (Keiver and Hochachka, 1991; Wasser and Jackson, 1991; Keiver et al., 1992). Oxygen deprivation, acidosis and hyperkalemia, either individually or collectively, have negative inotropic effects on turtle hearts (Yee and Jackson, 1984; Wasser et al., 1990a; Wasser et al., 1990b; Farrell et al., 1994; Jackson et al., 1995; Shi and Jackson, 1997; Shi et al., 1999; Kalinin and Gesser, 2002; Overgaard and Gesser, 2004; Overgaard et al., 2005) that can be partially alleviated by increased levels of calcium and/or adrenaline (Jackson, 1987; Nielsen and Gesser, 2001; Overgaard et al., 2005). Similarly, for warm-acclimated, normoxic turtles, anoxia, acidosis and anoxia combined with acidosis have negative chronotropic effects of varying degree on spontaneously contracting cardiac tissue (Reeves, 1963; Yee and Jackson, 1984; Jackson, 1987; Wasser et al., 1990a; Wasser et al., 1990b; Wasser et al., 1992; Farrell et al., 1994; Wasser et al., 1997). However, what is not known are the chronotropic effects of these extracellular changes on spontaneous f_H after cold-acclimation (i.e. at an acclimation temperature similar to the ones turtles experience during prolonged anoxia in their natural environment). Moreover, no one, to our knowledge, has examined chronotropic responses on cardiac tissue taken from turtles that had first been exposed to prolonged anoxia.

In view of this information gap, we conducted a comprehensive study with spontaneously contracting right-atrial preparations from both 21°C- and 5°C-acclimated turtles that had been held under either normoxic or prolonged anoxic conditions. Specifically, we exposed atria preparations to a series of saline solutions that, in a step-wise manner, either mimicked in normoxia-acclimated preparations or reversed in anoxia-acclimated preparations the expected changes in turtle blood composition during prolonged anoxia at these temperatures. Given the effects noted above, we predicted that for normoxia-acclimated preparations, extracellular anoxia, acidosis and hyperkalemia would decrease spontaneous f_H and that this negative chronotropy would be offset by increased concentrations of Ca^{2+} and adrenaline. For anoxia-acclimated preparations, we predicted that the chronotropic responses to the reversed sequence of extracellular changes would restore f_H to that of normoxia-acclimated preparations. Furthermore, we reasoned that if spontaneous f_H of normoxia-acclimated and anoxia-acclimated hearts were the same under comparable simulated conditions, this would be indicative that prolonged anoxia does not affect pacemaker rate.

Materials and methods

Experimental animals

Sixty-four red-eared sliders *Trachemys scripta* Gray, with body masses ranging between 83 and 506 g (228±88 g, mean ± s.d.), were used in this study. Turtles were obtained from Lemberger Inc. (Oshkosh, WI, USA) and airfreighted to Simon Fraser University, Burnaby, BC, Canada. We used a 2×2 exposure design (normoxia and anoxia exposure × 21°C and 5°C acclimation). Turtles studied at 21°C were held indoors in glass aquaria under a 12 h:12 h L:D photoperiod, had free access to basking platforms and diving water and were fed

Table 1. *Composition of saline solutions used for the 5°C and 21°C experiments*

Exposure order	Saline solution	Concentration (mmol l^{-1}) NaCl	$NaHCO_3$	NaH_2PO_4	$MgSO_4$	KCl	$CaCl_2$	Lactic acid	Glucose	Adrenaline (nmol l^{-1})	Gas composition (%CO_2/%N_2)[a]	pH[b]
Normoxia-acclimated right-atrial preparations	Simulated normoxic saline	85 (100)	40 (25)	1 (1)	1 (1)	2.5 (2.5)	2 (2)	0 (0)	5 (5)	1 (1 or 10)	1/99%O_2 (2/98%O_2)	8.00±0.03 (17) [7.75±0.02 (36)]
	Anoxia	85 (100)	40 (25)	1 (1)	1 (1)	2.5 (2.5)	2 (2)	0 (0)	5 (5)	1 (1 or 10)	**1/99** **(2/98)**	7.98±0.03 (12) [7.74±0.02 (24)]
	Anoxia + acidosis	85 (100)	40 (25)	1 (1)	**3** **(3)**	2.5 (2.5)	2 (2)	**14** **(14)**	5 (5)	1 (1 or 10)	**2/98** **(3/97)**	**7.59±0.09 (12)** **[7.23±0.02 (24)]**
	Anoxia + acidosis +hyperkalemia	85 (100)	40 (25)	1 (1)	3 (3)	**3.5** **(3.5)**	2 (2)	14 (14)	5 (5)	1 (1 or 10)	2/98 (3/97)	7.53±0.02 (12) [7.22±0.02 (24)]
	Anoxia + acidosis + hyperkalemia + hypercalcemia	85 (100)	40 (25)	1 (1)	3 (3)	3.5 (3.5)	**10** **(6.0)**	14 (14)	5 (5)	1 (1 or 10)	2/98 (3/97)	7.54±0.02 (12) [7.22±0.02 (24)]
Anoxia-acclimated right-atrial preparations	Simulated anoxic saline	85 (100)	40 (25)	1 (1)	3 (3)	3.5 (3.5)	10 (6.0)	14 (14)	5 (5)	**25** **(60)**	2/98 (3/97)	7.55±0.02 (18) [7.22±0.02 (30)]

Data for the 5°C experiments are presented without parentheses; data for the 21°C experiments are presented in parentheses.

Bold text highlights the saline solution composition difference from the preceding solution for the normoxia-acclimated experimental protocol.

[a]Appropriate gas mixtures were obtained by a gas-mixing pump (Wösthoff, Bochum, Germany) and all solutions were pre-equilibrated with the appropriate gas mixture prior to use.

[b]Saline pH was measured (Model 220 pH meter, Corning Science Products, NY, USA) prior to every experiment and values presented are mean ± s.d. (*N*).

several times a week with a mixture of commercial trout feed pellets, cat food and fresh vegetables. The turtles studied at 5°C were kept in glass aquaria with shallow water (3–4 cm) under a 12 h:12 h L:D photoperiod in a temperature-controlled room set to 5°C for 5 weeks prior to experimentation to allow adequate time for cold-acclimation (Hicks and Farrell, 2000a). The 5°C turtles were fasted during this period. Normoxic turtles were sampled from these conditions. For prolonged anoxia, turtles were exposed to anoxia for 6 h at 21°C or 14 days at 5°C. The required anoxic conditions were achieved by first individually placing turtles into an enclosed, water-containing plastic chamber that still allowed access to air for 24 h. Then, the plastic chamber was completely filled with water, continuously bubbled with N_2 (water P_{O_2}<0.3 kPa) and the turtle denied air access by means of a metal mesh that was suspended below the surface of the water. All procedures were in accordance with Simon Fraser University Animal Care Guidelines.

Tissue preparation

A spontaneously contracting right-atrial preparation was used to investigate the extracellular effects of anoxia, acidosis, hyperkalemia, hypercalcemia and adrenaline on the spontaneous f_H of normoxia- and anoxia-acclimated turtles at 21°C and 5°C. The turtle was killed by decapitation (which for anoxia-acclimated turtles occurred underwater in the plastic containers) and the heart was accessed by removal of a 3 cm×3 cm piece of the plastron using a bone saw. The vena cava was ligated with 3-0 braided surgical silk, and the entire right atrium separated from left atrium, and from the ventricle at the atrial-ventricular junction, taking care to preserve the pacemaker region of the sinus venosus. The entire procedure lasted approximately 3 to 5 min, after which the surgical silk was immediately fastened to a force-displacement transducer (Grass, FT 10, Quincy, MA, USA). The apex of the atrium was hooked to a fixed arm such that both sides of the atrial wall would be in direct contact with saline solutions. The preparation was then suspended in a water-jacketed organ bath containing the starting saline solution that approximated the *in vivo* extracellular conditions (i.e. atria from normoxia-acclimated turtles were placed in simulated normoxic saline whereas atria from anoxia-acclimated turtles were placed in simulated anoxic saline; Table 1). The length of the mounted atrial preparation was adjusted with a micrometer screw to produce ~90% of maximal contraction force to limit inter-preparation variation due to the chronotropic effects of cardiac stretch (Cooper and Kohl, 2005). The preparation was allowed 20–25 min to stabilize to allow for washout of any inherent adrenergic agents. No further adjustments were made to the length of the atrial preparation during the experiment.

Experimental protocol

Normoxia-acclimated preparations

Following the stabilization period, atrial preparations from normoxia-acclimated turtles were subjected to either a control

or treatment protocol. The control preparations remained in the simulated normoxic saline (Control Normoxic protocol), although the saline solution was refreshed at the same time interval as saline changes in the treatment protocol. The treatment protocol (Normoxic Treatment protocol) involved a series of saline solutions that progressively simulated *in vivo* anoxic extracellular conditions (i.e. atria were sequentially and additively exposed to anoxia, acidosis, hyperkalemia, hypercalcemia and increased adrenaline concentration; see Table 1 for details).

Anoxia-acclimated preparations

Similar to the normoxia-acclimated preparations, atria from anoxia-acclimated turtles were subjected to either a control or treatment protocol (Table 1). The anoxia-acclimated control preparations remained in simulated anoxic saline (Control Anoxic protocol) with refreshment of the saline occurring at the same time interval as saline changes. The purpose of the Anoxia-acclimated Treatment protocol was to return the atria to a simulated *in vivo* normoxic extracellular condition from the simulated *in vivo* anoxic extracellular condition. Therefore, the Anoxia-acclimated Treatment protocol was the exact reverse order of saline solutions to the Normoxic Treatment protocol (see Table 1 for details).

Saline compositions were devised to closely mimic the changes in blood plasma that occur *in vivo* with 6 h (21°C) or 14 days (5°C) of anoxia (the anoxia-acclimation times of our turtles) and not the changes that occur with several months of anoxia (see Ultsch and Jackson, 1982; Jackson and Heisler, 1982; Jackson and Ultsch, 1982; Herbert and Jackson, 1985a; Keiver and Hochachka, 1991; Wasser and Jackson, 1991; Keiver et al., 1992; Warren et al., 2006) (Table 1). Therefore, some of our changes in saline ionic composition differ from those utilized in previous studies that have examined the effects of extracellular factors on turtle cardiac inotropy and chronotropy. Further, it should be noted that we utilized both hypercapnic and lactic acidosis to depress pH. Consequently, the concentration of ionized calcium (Ca^{2+}) in the saline solutions would be slightly less than indicated in Table 1 due to the binding of calcium and lactate to form a calcium–lactate complex (Jackson and Heisler, 1982). Moreover, the anoxic plus acidotic saline solution was simultaneously made hypermagnesemic in order to accurately simulate the changes in blood plasma that accompany anoxia (see Table 1), but no attempt was made to distinguish unique effects of hypermagnesemia from those of acidosis.

21°C acclimation experiments

For experiments with warm-acclimated preparations, exposure time to each saline solution in the Normoxic Treatment and Anoxia-acclimated Treatment protocols was 15 min. Likewise, simulated normoxic and simulated anoxic saline solutions were refreshed every 15 min during the 1.5 h Control Normoxic and Control Anoxic protocols, respectively. Additionally, at 21°C, the Control Normoxic, Normoxic Treatment, and Anoxia-acclimated Treatment protocols were conducted with two levels of tonic adrenergic stimulation (1 nmol l^{-1} and 10 nmol l^{-1}; Table 1).

5°C acclimation experiments

For experiments with cold-acclimated preparations, exposure time to each saline solution in the Normoxic Treatment and Anoxia-acclimated Treatment protocols was 20 min. Likewise, simulated normoxic and simulated anoxic saline solutions were refreshed every 20 min during the 2 h Control Normoxic and Control Anoxic protocols, respectively.

The exposure times of 15 min at 21°C and 20 min at 5°C were chosen to obtain an effective balance between reaching new steady state with a saline change and maintaining tissue integrity for the duration of the experiments. These times were based on a wide range of previous studies reporting that, if present, inotropic and chronotropic responses of turtle myocardium to anoxic, acidotic and/or hyperkalemic changes occur within approximately 5 min to 15 min of exposure in warm-acclimated turtle heart (Poupa et al., 1978; Gesser and Poupa, 1978; Gesser and Jørgensen, 1982; Yee and Jackson, 1984; Wasser et al., 1990a; Wasser et al., 1990b; Wasser et al., 1997; Nielsen and Gesser, 2001), and within 20 min of exposure in 5°C acutely exposed turtle heart (Farrell et al., 1994).

Data analysis and statistics

Atrial contraction force was recorded continuously using an in-house computer-assisted data acquisition program (LabVIEW v5.1; National Instruments, Austin, TX, USA). Intrinsic f_H, determined off-line from the peak-to-peak intervals of the contraction force trace, was recorded from a 1-min interval at the conclusion of each saline exposure. Values presented for each sample time are means ± s.e.m. Two-way repeated measures (RM) analysis of variance (ANOVA) tests were used to compare f_H of comparable Control and Treatment protocols (i.e. 21°C 1 nmol l^{-1} tonic adrenaline normoxia-acclimated preparations; 21°C 10 nmol l^{-1} tonic adrenaline normoxia-acclimated preparations; 21°C 1 nmol l^{-1} tonic adrenaline anoxia-acclimated preparations; 21°C 10 nmol l^{-1} tonic adrenaline anoxia-acclimated preparations; 5°C normoxia-acclimated preparations; 5°C anoxia-acclimated preparations) and to determine statistically significant differences in f_H over time (Control protocols), among saline solutions (Treatment protocols) and between Control and Treatment protocols. Further, two-way RM ANOVAs were used to test for statistically significant effects of tonic adrenaline concentration (i.e. between the 1 nmol l^{-1} and 10 nmol l^{-1} tonic adrenaline groups) on spontaneous f_H of 21°C Control Normoxic, Normoxic Treatment and Anoxia-acclimated Treatment protocols. Statistically significant differences in f_H between comparable normoxic and anoxia-acclimated atria during exposure to simulated *in vivo* normoxic and simulated *in vivo* anoxic saline were determined using *t*-tests. In all instances, $P<0.05$ was used as the level of significance and where appropriate, multiple comparisons were performed using Student–Newman–Keuls tests.

Results

Stability of spontaneously contracting right-atrial preparations

Control experiments conducted to assess the stability of spontaneously contracting right-atrial preparations over time revealed that our preparations were viable throughout the durations of the experiments. At 21°C, Control Normoxic (both 1 nmol l^{-1} and 10 nmol l^{-1} tonic adrenaline groups) and Control Anoxic preparations maintained a stable f_H throughout the control experiments (Fig. 1). Similarly, no statistically significant change in f_H occurred over time in 5°C Control Normoxic preparations. However, spontaneous f_H of 5°C Control Anoxic atrial preparations increased significantly from an initial rate of 2.1±0.2 min^{-1} to a stable rate of ~3 min^{-1} at 40 min (Fig. 1).

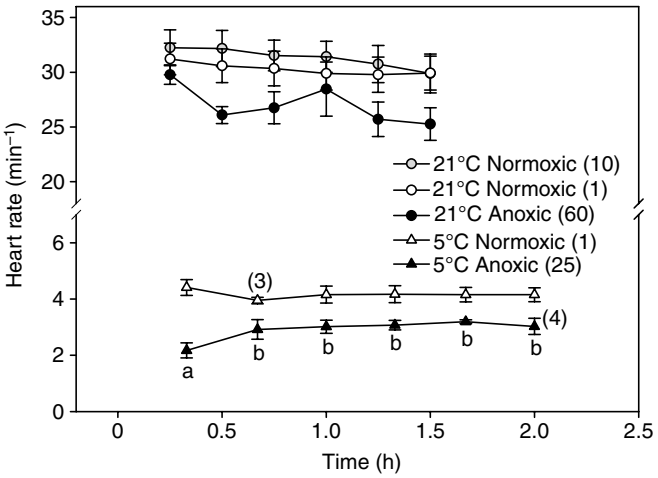

Fig. 1. Spontaneous heart rate (f_H) of 5°C, and 21°C, normoxia- and anoxia-acclimated control preparations during the control experiments. Adrenaline concentration (nmol l^{-1}) is indicated in parentheses. Significant differences (P<0.05) in f_H among time points within an experimental group are indicated by different letters. Values are means ± s.e.m.; N=6 except for the 5°C normoxia-acclimated experimental group where N=5, unless otherwise indicated in parentheses.

Chronotropic effects of extracellular changes on normoxia-acclimated hearts

21°C-acclimated preparations

Exposure to anoxic saline had no effect on spontaneous f_H of normoxic preparations at 21°C (Fig. 2). Combined anoxia and acidosis significantly decreased spontaneous f_H by 22% with 1 nmol l^{-1} tonic adrenaline and by 12% with 10 nmol l^{-1} tonic adrenaline (Fig. 2). Subsequent exposure to combined anoxia, acidosis and hyperkalemia further decreased f_H (to a 33% decrease) with 1 nmol l^{-1} tonic adrenaline, but not with 10 nmol l^{-1} tonic adrenaline (Fig. 2). Hypercalcemia did not reverse these negative chronotropic effects (Fig. 2). However, increasing adrenaline concentration from 1 nmol l^{-1} to 60 nmol l^{-1} completely reversed the negative chronotropic effects and returned f_H to a rate statistically similar to the simulated normoxic saline f_H and the comparable Control Normoxic f_H. Further, this rate was statistically similar to the f_H of the 10 nmol l^{-1} tonic adrenaline Normoxic Treatment group under simulated anoxic conditions. Therefore, increasing adrenaline from 10 nmol l^{-1} to 60 nmol l^{-1} did not have any effect on the already elevated f_H (Fig. 2). Consequently, at least for normoxia-acclimated preparations at 21°C, a tonic adrenaline concentration of 10 nmol l^{-1} in concert with hypercalcemia appears strong enough to produce a maximal adrenergic response and compensate for the negative chronotropic effects of anoxia, acidosis and hyperkalemia.

5°C-acclimated preparations

The 5°C-acclimated preparations revealed an important temperature dependency of extracellular effectors of spontaneous f_H. In contrast to the situation at 21°C, at 5°C f_H was not affected by combined anoxia and acidosis (Fig. 3). But, as at 21°C, combined anoxia, acidosis and hyperkalemia with 1 nmol l^{-1} tonic adrenaline significantly depressed f_H from the simulated normoxic saline rate (by 23%) as well as from the comparable Control Normoxic rate (Fig. 3). Nevertheless, and contrary to the effect at 21°C, this negative chronotropic effect at 5°C was completely offset by increased extracellular Ca^{2+}, whereas subsequent exposure to 25 nmol l^{-1} adrenaline had no further effect on spontaneous f_H (Fig. 3). Therefore, cold acclimation has some form of preconditioning effect for anoxic and acidosis exposure.

Effect of prolonged anoxia exposure on spontaneous f_H

An important finding of this study was that the initial spontaneous f_H of 5°C-acclimated turtles exposed to prolonged anoxia and placed in simulated anoxic saline was not the same as the spontaneous f_H of 5°C normoxia-acclimated preparations exposed to the same saline (Fig. 3). Specifically, spontaneous f_H of 5°C anoxia-acclimated preparations was approximately half the rate of normoxia-acclimated preparations. Moreover, in simulated normoxic saline, spontaneous f_H of 5°C anoxia-acclimated preparations was 47% lower than the initial f_H of normoxia-acclimated preparations in the same saline (Fig. 3), whereas spontaneous f_H of 5°C Control Anoxic preparations at t=2 h was 32% lower than comparable normoxia-acclimated preparations, despite the step-wise increase in f_H that occurred at t=40 min (Fig. 1). Thus, anoxia acclimation at 5°C re-sets the spontaneous f_H of turtle hearts to about half of that found in normoxia-acclimated preparations.

Anoxia acclimation at 21°C produced a similar re-setting of f_H to a reduced level. Spontaneous f_H of 21°C anoxia-acclimated preparations in simulated normoxic saline was 25% (with 1 nmol l^{-1} tonic adrenaline) and 34% (with 10 nmol l^{-1} tonic adrenaline) lower than the initial spontaneous f_H of comparable normoxia-acclimated preparations (Fig. 2). However, in contrast to 5°C, no statistically significant differences in f_H existed between normoxia- and anoxia-acclimated preparations in simulated anoxic saline at 21°C. This indicates that 60 nmol l^{-1} adrenaline compensated for the

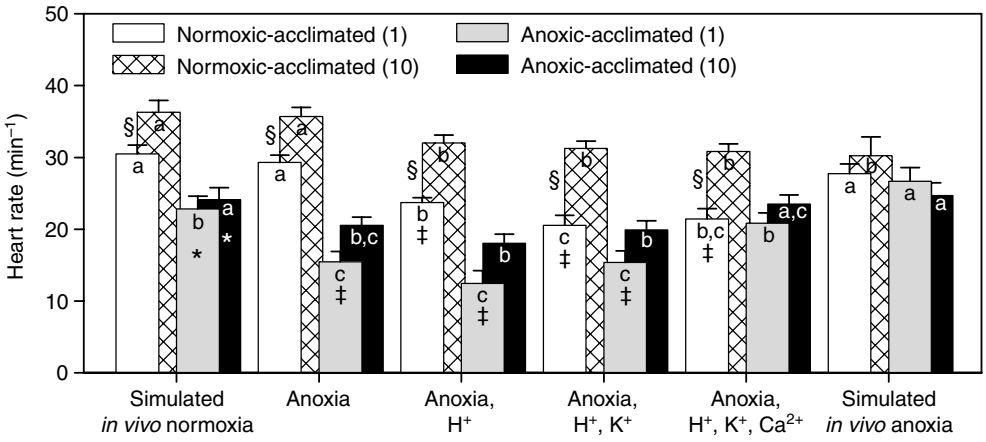

Fig. 2. Spontaneous heart rate f_H of 21°C normoxia- and anoxia-acclimated right-atrial preparations during exposure to various saline solutions devised to simulate or reverse the changes occurring in turtle blood plasma during 6 h of anoxic submergence, in the presence of 1 or 10 nmol l^{-1} tonic adrenaline (indicated in parentheses). Exposure order for normoxia-acclimated preparations was as given in the figure, reading from left-to-right, whereas exposure order for anoxia-acclimated preparations was as reading from right-to-left. Significant differences (P<0.05) in f_H among saline solutions within an experimental group are indicated by different letters. ‡Significant differences (P<0.05) in f_H from a comparable Control protocol f_H. §Significant differences (P<0.05) in f_H between 1 nmol l^{-1} and 10 nmol l^{-1} tonic adrenaline groups within an acclimation condition (i.e. normoxia- or anoxia-acclimated). *Significant difference (P<0.05) in f_H between normoxia- and anoxia-acclimated preparations (of the same level of tonic adrenergic stimulation) under simulated *in vivo* normoxic or simulated *in vivo* anoxic conditions. Values are means ± s.e.m.; N=6 in the 1 nmol l^{-1} tonic adrenaline normoxia- and anoxia-acclimated groups, 5 in the 10 nmol l^{-1} tonic adrenaline normoxia-acclimated group and 6 in the 10 nmol l^{-1} tonic adrenaline anoxia-acclimated group.

re-setting of intrinsic f_H that occurs with prolonged anoxia at 21°C.

Chronotropic effects of extracellular changes on anoxia-acclimated hearts

21°C-acclimated preparations

The reversed exposure of anoxia-acclimated hearts at 21°C to extracellular changes revealed that some important differences existed between anoxia- and normoxia- acclimated preparations in their chronotropic responses. Removal of hypercalcemia significantly reduced f_H of preparations with 1 nmol l^{-1} and 10 nmol l^{-1} tonic adrenaline, which contrasted with the lack of a protective effect of hypercalcemia in normoxia-acclimated preparations (Fig. 2). Thus, prolonged anoxia exposure at 21°C appears to heighten the protective role of extracellular Ca^{2+} on cardiac chronotropy. Further, 21°C anoxia-acclimated preparations were less susceptible to the negative chronotropic effects of hyperkalemia and acidosis than 21°C normoxia-acclimated hearts. Neither decreasing the extracellular K^+ concentration to normoxic levels nor removing extracellular acidosis altered spontaneous f_H of anoxia-acclimated preparations (Fig. 2). Finally, spontaneous f_H did increase significantly with the cessation of extracellular anoxia in 21°C anoxia-acclimated preparations independent of the tonic adrenaline concentration, whereas no decrease in f_H with exposure to extracellular anoxia was observed in normoxia-acclimated preparations (Fig. 2).

Even so, some chronotropic responses of anoxia-acclimated preparations were consistent with the normoxia-acclimated preparations at 21°C. For instance, reducing the adrenaline concentration from 60 nmol l^{-1} to 1 nmol l^{-1}, but not to 10 nmol l^{-1} significantly reduced spontaneous f_H of anoxia-acclimated preparations (Fig. 2).

5°C-acclimated preparations

Again, the reversed exposure of anoxia-acclimated hearts at 5°C to extracellular changes revealed that some important differences existed between anoxia- and normoxia-acclimated preparations. Specifically, in 5°C anoxia-acclimated atrial preparations a negative chronotropic effect of combined anoxia, acidosis and hyperkalemia was not present, in contrast to 5°C normoxia-acclimated preparations (Fig. 3). In fact, chronotropy of 5°C anoxia-acclimated preparations was not affected by any extracellular change and spontaneous f_H was unchanged throughout the entire procedure.

Discussion

Our objective was to comprehensively investigate how temperature acclimation, oxygen deprivation, acidosis, hyperkalemia, hypercalcemia and adrenaline affect chronotropy in the turtle myocardium. The present study is different from earlier works in two ways. Foremost, this is the first study to investigate the effects of extracellular changes on turtle cardiac chronotropy after anoxia acclimation at any temperature. To do this, we immediately exposed heart preparations from anoxia-acclimated turtles to a simulated *in vivo* anoxic saline and then progressively restored *in vivo* normoxic conditions with saline changes. Second, this is the first study to investigate the chronotropic effects of extracellular changes on cold-acclimated turtle hearts. We discovered that: (1) prolonged anoxia exposure re-sets intrinsic f_H to a reduced level in both warm- and cold-acclimated turtles, and (2) the chronotropic responses to extracellular changes are temperature dependent in both normoxia- and anoxia-acclimated turtle hearts, indicating that cold-acclimation has some form of preconditioning effect for anoxic and acidosis exposure.

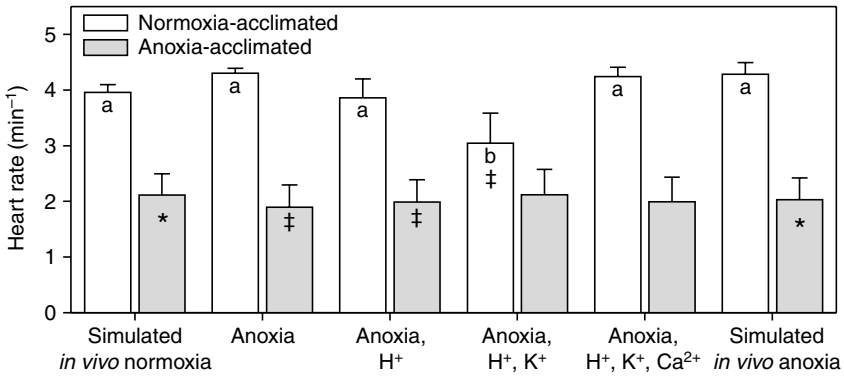

Fig. 3. Spontaneous f_H of 5°C, normoxia- and anoxia-acclimated right-atrial preparations during exposure to various saline solutions devised to simulate or reverse the changes occurring in turtle blood plasma during 14 days of anoxic submergence. Exposure order for normoxia-acclimated preparations was as given in the figure, reading from left-to-right, whereas exposure order for anoxia-acclimated preparations was as reading from right-to-left. Significant differences (P<0.05) in f_H among saline solutions within an experimental group are indicated by different letters. ‡Significant differences (P<0.05) in f_H from a comparable Control protocol f_H. *Significant difference (P<0.05) in f_H between normoxia- and anoxia-acclimated preparations under simulated *in vivo* normoxic or simulated *in vivo* anoxic conditions. Values are means ± s.e.m.; N=6 in both experimental groups.

Critique of methods

To make useful extrapolation to the *in vivo* situation, the spontaneously contracting right-atrial preparations should contract at rates comparable to *in vivo* intrinsic f_H and be stable for the duration of the experimental protocol. Further, compositional changes in saline solutions should be physiologically relevant and the exposure time should be sufficient to reach a new steady state f_H. This was the case. Spontaneous f_H of normoxia-acclimated preparations at 21°C and 5°C closely matched previously reported *in vivo* and *in vitro* intrinsic rates (Yee and Jackson, 1984; Wasser et al., 1990a; Wasser et al., 1990b; Farrell et al., 1994; Wasser et al., 1992; Wasser et al., 1997; Hicks and Farrell, 2000b) and were stable throughout control experiments (Fig. 1). Similarly, spontaneous f_H of 21°C and 5°C anoxia-acclimated right-atrial preparations, which were recorded with tonic adrenergic stimulation, were similar to *in vivo* f_H for anoxic turtles following cholinergic blockade (Hicks and Farrell, 2000b). 5°C anoxia-acclimated control preparations did exhibit a step-wise increase in f_H at 40 min (Fig. 1). However, given that this increase in f_H was an initial step change and not a continuous change, that the increased f_H remained statistically lower than 5°C Control Normoxic f_H, and that f_H at 2 h was not statistically significantly different from the f_H of 5°C Anoxia-acclimated Treatment preparations in simulated normoxic saline, we are confident of our findings for 5°C anoxia-acclimated turtle hearts. Moreover, as described above, our saline compositions were devised to closely mimic the changes in blood plasma that occur *in vivo* with 6 h (21°C) or 14 days (5°C) of anoxia (see Ultsch and Jackson, 1982; Jackson and Heisler, 1982; Jackson and Ultsch, 1982; Herbert and Jackson, 1985a; Keiver and Hochachka, 1991; Wasser and Jackson, 1991; Keiver et al., 1992; Warren, 2006). Finally, visual inspection of traces at both acclimation temperatures revealed that most of the change in f_H occurred within the first 5 min of a saline switch.

Chronotropic effects of extracellular changes

Given the previously reported inotropic and chronotropic effects of extracellular changes on the turtle myocardium (Reeves, 1963; Yee and Jackson, 1984; Jackson, 1987; Wasser et al., 1990a; Wasser et al., 1990b; Wasser et al., 1992; Farrell et al., 1994; Jackson et al., 1995; Shi and Jackson, 1997; Wasser et al., 1997; Shi et al., 1999; Kalinin and Gesser, 2002; Overgaard and Gesser, 2004; Overgaard et al., 2005; Nielsen and Gesser, 2001), for normoxia-acclimated preparations, we predicted negative chronotropic effects of extracellular anoxia, acidosis and hyperkalemia, and positive chronotropic effects of hypercalcemia and adrenaline. For anoxia-acclimated preparations, we predicted that the chronotropic responses to the reversed sequence of extracellular changes would restore f_H to that of normoxia-acclimated preparations.

Normoxia-acclimated hearts

Our results for 21°C normoxia-acclimated preparations were consistent with our predictions. Combined anoxia with acidosis, as well as combined anoxia, acidosis and hyperkalemia were found to depress spontaneous f_H, whereas adrenaline reversed or diminished these negative chronotropic effects (Fig. 2). These present findings are akin to previous studies showing that anoxia and acidosis act synergistically to depress turtle f_H (Wasser et al., 1990a; Wasser et al., 1990b), whereas individually, anoxia and acidosis may (Reeves, 1963; Yee and Jackson, 1984; Jackson, 1987; Wasser et al., 1990b; Farrell et al., 1994; Wasser et al., 1997) or may not induce bradycardia (Wasser et al., 1990b; Wasser et al., 1992). Further, the decreased spontaneous f_H with hyperkalemia reported here for the turtle is similar to the negative

chronotropic effect of increased extracellular K^+ concentration on the anoxia-intolerant rainbow trout heart (*Oncorhynchus mykiss*) (Hanson et al., 2006).

The apparent lack of a beneficial effect of hypercalcemia on f_H of 21°C normoxia-acclimated preparations contrasts with a previous finding that increased extracellular Ca^{2+} concentration partially alleviates the depression in spontaneous f_H of 20°C-acclimated turtle hearts caused by combined anoxic and acidotic insult (Wasser et al., 1990a). However, this difference can be resolved by considering the additive and protective role of adrenaline and extracellular calcium on cardiac contractility and the differences in adrenaline (1 nmol l^{-1} or 10 nmol l^{-1} in the present study *versus* 0 nmol l^{-1}) as well as extracellular Ca^{2+} concentrations (6 mmol l^{-1} in the present study *versus* 10 mmol l^{-1}) between studies. Ca^{2+} entry into vertebrate myocytes occurs through voltage-gated L-type Ca^{2+} channels and the Na^+/Ca^{2+} exchanger (NCX), with the amount of Ca^{2+} entering determined by the electrochemical driving force for Ca^{2+} (L-type Ca^{2+} channels and NCX), the duration of L-type Ca^2 channel opening and the number of activated L-type Ca^2 channels. Adrenaline increases the open probability of L-type Ca^{2+} channels (Reuter, 1983). Therefore, results from the present study indicate that at 21°C, 6 mmol l^{-1} extracellular Ca^{2+} with 1 nmol l^{-1} tonic adrenaline is insufficient to offset the negative chronotropic effects of the combined anoxic, acidotic and hyperkalemic extracellular insult associated with 6 h of anoxia exposure. However, 6 mmol l^{-1} hypercalcemia in conjunction with 10 nmol l^{-1} adrenaline appears adequate to protect f_H.

Unlike at 21°C, the 5°C normoxia-acclimated turtle heart was resistant to combined anoxia and acidosis, but when combined with hyperkalemia, f_H decreased by 23%, a negative effect that was slightly less than at 21°C (Fig. 3). Conversely, the negative inotropic effect of hyperkalemia alone is greater in cold-acclimated turtle hearts than in warm-acclimated hearts (Overgaard et al., 2005). Further, unlike at 21°C, hypercalcemia fully reversed the negative chronotropic effect of combined anoxia, acidosis and hyperkalemia in normoxia-acclimated preparations at 5°C, which precluded the positive chronotropic effect of subsequently increasing the adrenaline concentration (to 25 nmol l^{-1}; Fig. 3). This heightened importance of extracellular Ca^{2+} in protecting chronotropy at 5°C suggests that cold acclimation modifies cellular calcium cycling in the turtle heart, a preconditioning effect that would make sense, given the normal mobilization of calcium from turtle bone and shell during cold anoxia (Jackson, 2002). The lack of positive chronotropy in response to 25 nmol l^{-1} adrenaline is consistent with the attenuation of adrenergic control in cold-acclimated turtle hearts (Hicks and Farrell, 2000b).

Anoxia-acclimated hearts

Our results indicate that intrinsic f_H is re-set to a level 32%–53% (at 5°C) and 25%–34% (at 21°C) lower than with normoxia as a result of prolonged anoxia exposure (Figs 2 and 3). *In vivo* f_H decreases by 5-fold at 5°C and by 2.5-fold at 21°C with prolonged anoxia exposure (Hicks and Farrell, 2000a; Stecyk et al., 2004). Thus, in cold-acclimated anoxic turtles, when autonomic cardiovascular control is blunted (Hicks and Farrell, 2000b; Stecyk et al., 2004), this re-setting of intrinsic f_H could contribute to 40%–66% of the anoxic bradycardia. In warm-acclimated turtles, the re-setting of intrinsic f_H could contribute to 42%–57% of the anoxic bradycardia, but the relative contribution of the re-setting of intrinsic f_H towards the anoxic bradycardia is more difficult to discern. Autonomic cardiovascular control is not blunted at 21°C (Hicks and Farrell, 2000b; Stecyk et al., 2004), and results from this study revealed that with an *in vivo* level (60 nmol l^{-1}) of adrenergic stimulation, spontaneous f_H of normoxia-acclimated and anoxia-acclimated preparations were the same (Fig. 2). Thus, *in vivo* at 21°C, circulating catecholamines may be able to compensate for the re-setting of intrinsic f_H.

Beyond the clear re-setting of intrinsic rate, anoxia acclimation also resulted in different chronotropic responses to extracellular changes, indicating that the mechanisms underlying the effects of extracellular factors on spontaneous f_H are modified with anoxia. Primarily, extracellular factors do not appear to be important controlling factors of cardiac chronotropy during prolonged, cold anoxia (Fig. 3). At 21°C, anoxia-acclimated preparations were less susceptible to the negative chronotropic effects of hyperkalemia and acidosis than 21°C normoxia-acclimated hearts (Fig. 2), indicating that extracellular anoxia is a potent trigger for bradycardia in anoxia-acclimated hearts.

Potential mechanisms underlying the observed chronotropic effects

The mechanisms underlying the differing chronotropic effects of extracellular factors among 21°C and 5°C, normoxia- and anoxia-acclimated turtle heart preparations, as well as the rapid, anoxia-induced re-setting of intrinsic f_H remain to be clarified. However, a number of possibilities exist. Primarily, pacemaker mechanisms could be altered by extracellular factors and/or anoxia exposure with the exact specifics of modification varying with acclimation temperature. Pacemaker cells exhibit a highly regulated diastolic depolarization that results in regular firing of pacemaker action potentials. In mammalian species, this diastolic depolarization results from the coordinated action of various sarcolemmal K^+, Ca^{2+} and Na^+ currents as well as interaction between sarcoplasmic reticulum Ca^{2+} release and the NCX, which, through an elevation of intracellular Ca^{2+} leads to an accelerated diastolic depolarization *via* inward NCX current (Irisawa, 1978; DiFrancesco, 1986; Campbell et al., 1992; Maltsev et al., 2006). Also, adrenaline directly affects pacemaker currents in mammals (Gadsby, 1983; Satoh and Hashimoto, 1983). Therefore, in the turtle, changes in extracellular K^+ and Ca^{2+} concentrations could modify the electrochemical gradients driving ionic sarcolemmal currents and thus alter diastolic depolarization rate and subsequently f_H. Likewise, the positive chronotropic effects of adrenaline on turtle spontaneous f_H could arise from its direct effect on pacemaker currents.

Differences in pacemaker activity and its susceptibility to extracellular factors between warm and cold acclimation temperatures and normoxia and anoxia exposure could potentially arise from variations in density of functional sarcolemmal ion channels involved in pacemaking and/or brought about by changes in channel phosphorylation, transcription, translation, rate of protein degradation, and trafficking of channels to the sarcolemmal membrane.

In addition to effects on pacemaker rate, it is also foreseeable that any occurrence or change that affects the length of the cardiac cycle, either directly or indirectly, could also potentially influence intrinsic f_H. Previous studies in turtles, fish and mammals have revealed that anoxia, acidosis, hyperkalemia, hypercalcemia and adrenaline can all affect cardiac cycle length. For example, anoxia inhibits excitation-contraction coupling (Nielsen and Gesser, 1984) and contractile proteins (Matthews et al., 1986), elevates intracellular inorganic phosphate, which decreases Ca^{2+} sensitivity of the myofilament (Gesser and Jørgensen, 1982), and modifies myocardial action potential shape (Stern et al., 1988). Likewise, acidosis interferes with many steps of excitation-contraction coupling, including reducing the amount of Ca^{2+} entering myocytes and competitively hindering calcium-troponin binding (Williamson et al., 1976; Gesser and Jørgensen, 1982; Orchard and Kentish, 1990). Indeed, in warm-acclimated turtle hearts, extracellular acidosis decreases cardiac myocyte intracellular pH (Wasser et al., 1990a; Wasser et al., 1990b) and slows the maximum rate of force development during cardiac contraction (Shi and Jackson, 1997; Shi et al., 1999). Hyperkalemia reduces resting myocyte membrane potential (Nielsen and Gesser, 2001), which in mammals, negatively affects voltage-gated Ca^{2+} channels and inactivates a proportion of the ventricular Na^+ channels, thereby slowing cardiac conduction (Chapman and Rodrigo, 1987; Bouchard et al., 2004). By contrast, hypercalcemia enhances the inward Ca^{2+} gradient, and has been shown to alleviate the negative inotropic effects of hyperkalemia, acidosis or anoxia in warm-acclimated turtles (Yee and Jackson, 1984; Jackson, 1987; Nielsen and Gesser, 2001). Similarly, adrenaline increases myocardial Ca^{2+} influx through sarcolemmal L-type channels (Frace et al., 1993), which counteracts the acidotic impairment of calcium-troponin binding (Tibbits et al., 1992) and restores the action potential upstroke lost with hyperkalemia (Paterson et al., 1992). However, decreased myofilament Ca^{2+} sensitivity, as a result of adrenergic stimulation, can also lead to a decrease in the systolic interval (Bers, 1991).

In this regard, the reduced chronotropic sensitivity of 5°C normoxia-acclimated turtle hearts to acidosis, and both 21°C and 5°C anoxia-acclimated turtle hearts to extracellular acidosis and hyperkalemia (Figs 2 and 3), could be related to respective changes induced by cold acclimation and anoxia exposure in atrial sarcolemmal ion channel densities or kinetics and/or that contractile protein isoforms that offset the slowed functioning of intracellular pH regulation in turtle myocytes during anoxia compared to normoxia (Shi et al., 1997). However, alteration of pacemaker mechanisms may be more important in facilitating the re-setting of f_H with cold anoxia than a change in cardiac cycle length since time-to-peak twitch force and time to relaxation does not differ between 5°C normoxia- and anoxia-acclimated turtle ventricular strips (Overgaard et al., 2005). Future studies investigating the effects of cold acclimation and anoxia exposure on turtle cardiac electrophysiology are of course needed to clarify these possibilities and these are underway in our laboratory.

Adrenaline and chronotropy

Additionally, this study revealed some important differences in the effect of adrenaline on cardiac chronotropy between warm- and cold-acclimated, as well as normoxia- and anoxia-acclimated turtles. The positive chronotropic effect of adrenaline present at 21°C disappeared with cold acclimation, and at 21°C, anoxia-acclimation modified the interplay between extracellular Ca^{2+} concentration and adrenergic stimulation without affecting the sensitivity of spontaneous f_H to adrenergic stimulation (Figs 2 and 3). These findings open the possibility that cold temperature and anoxia acclimation alter the interplay between adrenaline and excitation-contraction coupling and/or calcium cycling in the turtle heart. Finally, our 21°C experiments with two levels of tonic adrenergic stimulation (1 nmol l^{-1} and 10 nmol l^{-1}) revealed that adrenergic stimulation protects the turtle heart equally well after, as well as concurrently with, the anoxic challenge (Fig. 2). This finding contrasts recent findings in the anoxia-intolerant rainbow trout heart where concurrent adrenergic stimulation better protected cardiac performance during, rather than following a combined hypoxic, hyperkalemic and acidotic insult (Hanson et al., 2006). Given that ventricular β-adrenoreceptor density decreases with prolonged anoxia in the turtle (Hicks and Farrell, 2000b), but not during hypoxia exposure in the rainbow trout (Gamperl et al., 1998), the possibility exists that changes in turtle cardiac β-adrenoceptor density with prolonged anoxia exposure may be tissue specific.

Concluding remarks

This study is the first to report on the temperature-dependent effect of prolonged anoxia exposure on intrinsic f_H of the anoxia-tolerant freshwater turtle and on how the extracellular changes that accompany prolonged anoxia, namely anoxia, acidosis, hyperkalemia, hypercalcemia and increased adrenaline, affect spontaneous f_H. We discovered that a re-setting of intrinsic f_H to a reduced level as a result of prolonged anoxia exposure in both warm- and cold-acclimated turtles plays an important role in generating anoxic bradycardia. Further, our results revealed that the chronotropic responses of the turtle heart to extracellular changes varies with acclimation temperature in both normoxia- and anoxia-acclimated turtle hearts, indicating that cold-acclimation has some form of preconditioning effect for anoxic and acidosis exposure. Future electrophysiological studies on turtle pacemaker currents, working myocyte sarcolemmal currents and excitation–contraction coupling are needed to fully comprehend the

temperature- and anoxia-dependent differences in chronotropic responsiveness of the turtle heart to extracellular changes.

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