Does the ventricle limit cardiac contraction rate in the anoxic turtle (*Trachemys scripta*)? II. *In vivo* and *in vitro* assessment of the prevalence of cardiac arrythmia and atrioventricular block

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ABSTRACT

Previous studies have reported evidence of atrio-ventricular (AV) block in the oxygen-limited *Trachemys scripta* heart. However, if cardiac arrhythmia occurs in live turtles during prolonged anoxia exposure remains unknown. Here, we compare the effects of prolonged anoxic submergence and subsequent reoxygenation on cardiac electrical activity through *in vivo* electrocardiogram (ECG) recordings of 21 °C- and 5 °C-acclimated turtles to assess the prevalence of cardiac arrhythmia. Additionally, to elucidate the influence of extracellular conditions on the prominence of cardiac arrhythmia, we exposed spontaneously contracting *T. scripta* right atrium and electrically coupled ventricle strip preparations to extracellular conditions that sequentially and additively approximated the shift from the normoxic to anoxic extracellular condition of warm- and cold-acclimated turtles. Cardiac arrhythmia was prominent in 21 °C anoxic turtles. Arrhythmia was qualitatively evidenced by groupings of contractions in pairs and trios and quantified by an increased coefficient of variation of the RR interval. Similarly, exposure to combined anoxia, acidosis, and hyperkalemia induced arrhythmia *in vitro* that was not counteracted by hypercalcemia or combined hypercalcemia and heightened adrenergic stimulation. By comparison, cold acclimation primed the turtle heart to be resilient to cardiac arrhythmia. Although cardiac irregularities were present intermittently, no change in the variation of the RR interval occurred *in vivo* with prolonged anoxia exposure at 5 °C. Moreover, the *in vitro* studies at 5 °C highlighted the importance of adrenergic stimulation in counteracting AV block. Finally, at both acclimation temperatures, cardiac arrhythmia and irregularities ceased upon reoxygenation, indicating that the *T. scripta* heart recovers from anoxia-induced disruptions to cardiac excitation.

1. Introduction

The red-eared slider freshwater turtle (*Trachemys scripta*) can survive ~24 h of anoxic submergence when acclimated to warm temperatures (20–25 °C) and 6–7 weeks of anoxic submergence when acclimated to the cold temperatures (3–5 °C) at which it overwinters in ice-covered ponds (Ultsch, 2006; Warren et al., 2006). During anoxia exposure at both warm and cold temperatures, the heart of *T. scripta* continues to beat (Stecyk et al., 2008). However, in line with the marked suppression of whole animal metabolic rate that occurs under anoxic conditions (Herbert and Jackson, 1985b; Jackson, 1968), cardiovascular status is drastically reduced (Stecyk et al., 2008). The suppression of cardiac activity, which is driven by a large bradycardia, serves to substantially

decrease cardiac work so that cardiac ATP demand falls below the maximum cardiac glycolytic potential (Farrell and Stecyk, 2007).

Modifications intrinsic to the heart have been implicated to contribute to the bradycardia displayed by anoxic turtles. At the level of the cardiac pacemaker, intrinsic heart rate (f_H) is slowed by ~25–30% within 6 h of anoxia exposure at 21 °C, and ~50% within 2 weeks of anoxia exposure at 5 °C (Stecyk et al., 2007, 2009). The response appears to be mediated in part via reduced sarcolemmal Ca^{2+} flux at warm, but not at cold acclimation temperature (Stecyk et al., 2021). Additionally, multiple lines of evidence suggest that the inability of the ventricle to contract in coordination with the pacemaker during anoxia exposure (i.e., ventricular bradycardia) may also contribute to the suppression of cardiac pumping rate in anoxic turtles. Primarily, *T. scripta* forced to exercise while breathing hypoxic air exhibited a

List of abbreviations

AA	combined anoxia and acidosis extracellular conditions (saline solution)
AAK	combined anoxia, acidosis, and hyperkalemia extracellular conditions (saline solution)
AAKCa	combined anoxia, acidosis, hyperkalemia, and hypercalcemia extracellular conditions (saline solution)
AAKCaADR	combined anoxia, acidosis, hyperkalemia, hypercalcemia, and heightened adrenaline concentration extracellular conditions (saline solution)
Control Norm	control normoxic extracellular conditions (saline solution)
ECG	electrocardiogram
f_H	heart rate
I_{Na}	voltage-gated Na$^+$ current density
RA-RA interval	consecutive right atrium contraction interval *in vitro*
RR interval	consecutive ECG R wave peak interval *in vivo*

pronounced atrioventricular (AV) block (Farmer and Hicks, 2002)). Similarly, *in vitro* electrically coupled atrium and ventricular preparations from the anoxia-tolerant Western painted turtle (*Chrysemys picta belli*) exhibited AV block when exposed to anoxia at warm temperatures (Jackson, 1987). Moreover, the intrinsic f_H of *T. scripta* spontaneously contracting right atrium preparations (27 beats min^{-1} at 21 °C; 2.1 beats min^{-1} at 5 °C) (Stecyk and Farrell, 2007) is faster than the *in vivo* f_H (measured from ventricular contraction frequency) of live anoxic turtles treated with atropine to block vagal cholinergic cardiac inhibition (16.7–19.7 beats min^{-1} at 22–25 °C; 1.2 beats min^{-1} at 5 °C) (Hicks and Farrell, 2000; Hicks and Wang, 1998). Further, turtle atria are more resilient to the changes in the extracellular milieu that occur with prolonged anoxia exposure and that induce negative contractile effects (i.e., anoxia *per se*, acidosis, and/or hyperkalemia) under normoxic (Butcher et al., 1952), as well as anoxic conditions (Garner and Stecyk, 2022; Stecyk and Farrell, 2007). Finally, as highlighted in the accompanying study (Garner and Stecyk, 2022), ventricular contraction could limit cardiac pumping rate *in vivo* during prolonged anoxic submergence at cold acclimation temperature if combined hypercalcemia and heightened adrenergic stimulation are insufficient to counteract the negative effects of combined extracellular anoxia, acidosis, and hyperkalemia.

Indeed, in the anoxia-sensitive mammalian heart, oxygen deprivation leads to AV block, in which cardiac electrical conduction is disrupted, causing either a delay or total disruption of ventricular excitation (Harris and Matlock, 1947). However, in contrast, the anoxia-tolerant crucian carp (*Carassius carassius*) showed no evidence of arrhythmia during prolonged exposure to anoxia at cold acclimation temperature, suggesting that the cold-acclimatized heart of anoxia-tolerant vertebrates has some protective mechanisms that protect it from irregularities (Tikkanen et al., 2017). Thus, while there is some evidence of AV block under certain conditions in anoxia-tolerant turtles, if cardiac arrhythmia, including AV block, occurs during prolonged anoxic submergence, if its prevalence is dependent on the acclimation temperature of the turtle, and if its prevalence is determined by extracellular conditions remains unknown.

Here, to fill these information gaps, we assessed *in vivo*, via electrocardiogram (ECG) recordings from 21 °C- and 5 °C-acclimated *T. scripta* exposed to normoxia and prolonged anoxia exposure, as well as *in vitro*, using spontaneously contracting *T. scripta* right atrium and electrically coupled ventricle strip preparations exposed to altered extracellular conditions, the prevalence of cardiac arrhythmia and atrioventricular block in the turtle heart. Given that prior temperature and anoxia experiences are central to determining the intrinsic contractile response of

the turtle myocardium to altered extracellular conditions (Garner and Stecyk, 2022; Overgaard et al., 2005; Stecyk and Farrell, 2007), in part due to alterations to cardiac electrophysiology with anoxia exposure and cold acclimation (Stecyk et al., 2007), we hypothesized that the prevalence of and extracellular contributors to cardiac arrhythmia would be acclimation temperature-specific.

2. Material and methods

2.1. Experimental animals

All animal husbandry and experimental procedures were in accordance with protocols (1362273, 1362274, and 1025890) approved by the University of Alaska Anchorage (UAA) Institutional Animal Care and Use Committee. Twenty-four red-eared slider turtles (*Trachemys scripta*) of both sexes and with a mass of 299 ± 80.4 g (mean ± SD) were utilized. Turtles were obtained from a commercial supplier (Niles Biological, Sacramento, CA, USA) and air freighted to UAA. All animals were initially acclimated to and maintained at 21 °C as detailed in the accompanying study (Garner and Stecyk, 2022).

2.2. Recording and analysis of ECG from unrestrained and unanesthetized turtles

Fourteen of the 21 °C-acclimated turtles were intubated with soft rubber tubing and ventilated with 4% isoflurane in room air prepared by an Isoflurane vaporizer (Dräger, Lubeck, Germany). Ventilation rate was 2–3 breaths per minute and a tidal volume was 25–30 ml kg^{-1} (Harvard Apparatus Inspira Advanced Volume Control Ventilator, Harvard Apparatus, Holliston, MA, USA). Once anesthesia was deemed effective via the absence of a pedal withdraw reflex, the isoflurane level was reduced to and maintained at 1%. Three 1 mm holes were drilled into the plastron such that small stainless-steel screws (thread 1 mm wide x 5 mm long) that were disinfected with 70% ethanol could be implanted approximately 3 mm without penetrating the body cavity. The screws served as the ECG electrodes and their placement was consistent with prior literature for optimum ECG signals (Farmer and Hicks, 2002). Shielded electrical wire, identifiable by different colors, was wrapped around each screw and anchored in place with epoxy resin. Post-surgery, animals were placed individually into experimental containers and allowed at least 48 h to recover.

Half of the instrumented turtles were exposed to anoxia and reoxygenation at 21 °C. The other seven turtles were acclimated to 5 °C in normoxia prior to being exposed to anoxia and reoxygenation. Anoxia exposures and acclimation to 5 °C in normoxia followed protocols described in the accompanying study (Garner and Stecyk, 2022). At 21 °C, ECG signals were continuously recorded for 6 h in normoxia, 16 h of anoxia exposure, and 24 h of reoxygenation. At 5 °C, normoxic ECG recordings were acquired daily over three days. Recordings were 3 h in duration and occurred at a consistent time each day. ECG signals were then recorded continuously throughout the first 24 h of anoxic submergence, for 3 h on alternating days of a 12-day anoxia exposure period, and then continuously for 24 h commencing at 24 h of reoxygenation. A FE136 Animal Bio Amp (AD Instruments; Colorado Springs, CO, USA) and PowerLab 8/35 data acquisition system (AD Instruments) were used to record ECG signals at a sampling rate of 1000 Hz.

ECG waveforms were quantified using the ECG Analysis Module or the Scope View of LabChart 8 software (ADInstruments). ECG features were averaged from a minimum of 8 ECG traces per animal during each analysis period. The ECG parameters quantified included the PR interval, which represents conduction time from the onset of atrial depolarization to onset of ventricular depolarization, the QRS complex duration, which represents the time required for AP depolarization to propagate through the ventricle, and the QT interval, which represents the average duration of the ventricular action potential (Fig. 1). Biphasic
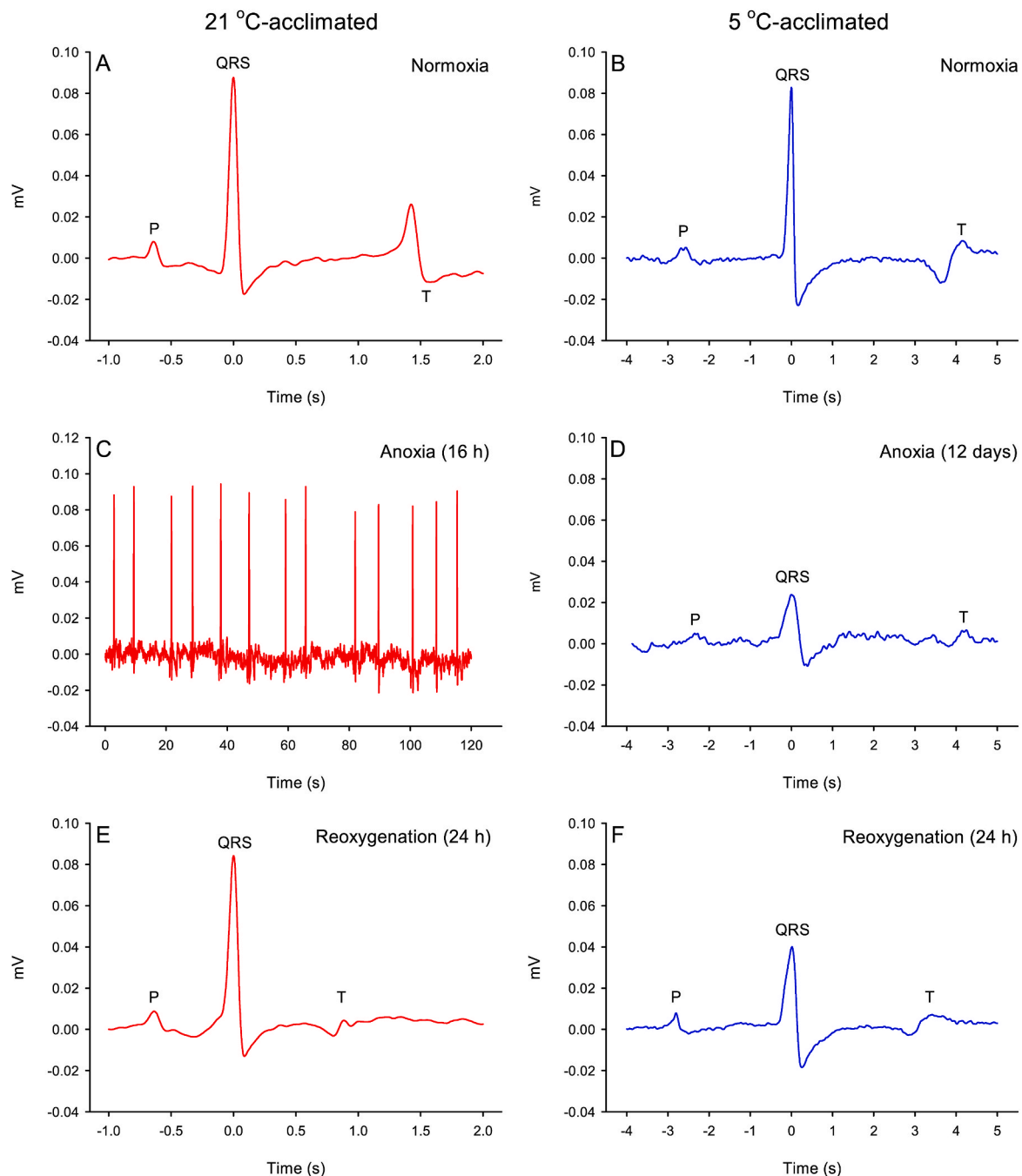
Fig. 1. Representative electrocardiogram (ECG) waveforms from (A, C, and E) a 21 °C-acclimated and (B, D, and F) a 5 °C-acclimated turtle in (A and B) normoxia, (C and E) anoxia (16 h at 21 °C; 12 days at 5 °C), and (E and F) at 24 h of reoxygenation. P, QRS, and T waves are indicated. Note the biphasic T waves, the reduced ECG amplitude in 5 °C anoxia and reoxygenation, and the widening of the QRS complex in 5 °C anoxia.

T waves were quantified at the terminal portion of the wave regardless of orientation. For the control normoxia and reoxygenation recording periods, ECG parameters were calculated separately for putative periods of breathing and post-breathing. Periods of breathing were characterized by tachycardia, and post-breathing data was taken within the first 2 min of the following period of apnea, represented by bradycardia.

Additionally, the RR intervals from a ~25–55 min period from each recording period were utilized to calculate f_H, quantify arrhythmia, and to assess heart rate variability (HRV). Arrhythmia was quantified by calculating the coefficient of variation of the RR interval. The standard deviation of the RR interval was divided by the mean RR interval and is presented as a percentage. HRV was assessed using HRVanalysis version 1.2 (Pichot et al., 2016; Stecyk et al., 2020). RR intervals were plotted

against their successive RR interval to produce Poincaré plots. From the plots, mean short-term (SD1) and long-term (SD2) variabilities were derived, and the SD1/SD2 ratio calculated.

2.3. In vitro spontaneously contracting right atrium with electrically coupled ventricle strip preparation experimental protocol and data analysis

Turtles acclimated to 21 °C or 5 °C in normoxia were weighed, decapitated, the plastron removed with a bone-saw, and the heart dissected and washed with ice-cold saline solution containing (in mmol l^{-1}): 100 NaCl, 25 NaHCO$_3$, 2.5 KCl, 2 CaCl$_2$, 1 NaH$_2$PO$_4$, 1 MgCl$_2$, 5 glucose and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); pH 7.75–7.78. The left atrium was separated from the right

atrium and ventricle, except for a small remnant, and the apex of the right atrium severed to ensure that both sides of the right atrial wall would be exposed to the physiological saline solutions during experimentation. Most of the ventricle was also removed, save for the right atrial-ventricular junction, to ensure electrical integrity between the two tissue types, and a medial, longitudinal strip of ~4–6 mm in length and ~1–2 mm in thickness that was cut using a razor from the dorsal side of the chamber. This location was selected as most muscle fibers are arranged in the longitudinal direction on the dorsal side of the *T. scripta* ventricle, resulting in more consistent responses to experimental manipulation (Ball and Hicks, 1996). Throughout, care was taken to not damage the pacemaker region (Schlomovitz and Chase, 1916).

The remnant of the left atrium was attached to a tissue support using 3-0 surgical silk and the right atrium and ventricular strip were secured individually to separate force-displacement transducers (FT03, Grass Instruments, Quincy, MA, USA) using a small tissue hook and 3-0 silk suture and a small tissue clamp, respectively. This allowed the contractile force generated by the right atrium and ventricle to be recorded independently. The preparation was then immersed in a 30 ml water-jacketed tissue bath (Radnoti, Covina, CA, USA) containing a Control Normoxia (Control Norm) physiological saline solution that was specific to the acclimation temperature of the animal (Table 1) (Stecyk and Farrell, 2007). The length of the right-atrial tissue 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). No stretch was applied to the ventricular strip for 20 min, after which the length of the strip was gradually adjusted to maximize force production (L_{max}). The preparation was then left to stabilize for a minimum of 30 min prior to commencing the experimental protocol.

Following the stabilization period, a tonic level of adrenaline (1 nmol l^{-1}) was added to the bath and baseline (i.e., control normoxic) recordings obtained. The preparations were then sequentially and additively exposed to anoxia (A), combined anoxia + acidosis (AA), combined anoxia + acidosis + hyperkalemia (AAK), combined anoxia + acidosis + hyperkalemia + hypercalcemia (AAKCa) and finally combined anoxia + acidosis + hyperkalemia + hypercalcemia + increased adrenaline concentration (AAKCaADR; Table 1) (Stecyk and Farrell, 2007). The levels of acidosis, hyperkalemia, hypercalcemia, and adrenaline in the AA, AAK, AAKCa, and AAKCaADR saline solutions were selected to strike a balance between the extracellular changes that occur *in vivo* in anoxia-tolerant turtles with 6 h of anoxia exposure at warm acclimation temperature and 12 days of anoxia exposure at cold acclimation temperature (Herbert and Jackson, 1985a, b; Jackson and Ultsch, 1982; Keiver and Hochachka, 1991; Keiver et al., 1992; Warren and Jackson, 2007; Warren et al., 2006), and those employed by past studies investigating the effects of altered extracellular conditions on turtle contractile parameters (Nielsen and Gesser, 2001; Overgaard et al., 2005; Stecyk and Farrell, 2007; Stecyk et al., 2021; Yee and Jackson, 1984). Anoxic solutions were pre-bubbled with the appropriate gas mixtures prior to use and anoxic bath conditions were confirmed with a TROXROB3 robust trace oxygen miniprobe and FireSting fiber-optic oxygen meter (PyroScience GmbH, Aachen, Germany). The tonic adrenergic stimulation of 1 nmol l^{-1} was maintained with each saline change, except when superseded by the high adrenaline concentration. The 20 min exposure time to each saline solution was based on prior study (Stecyk and Farrell, 2007) showing that the duration allows an effective balance between preparations reaching a new steady state with a saline change and maintaining tissue integrity for the duration of the experiment.

Signals from the force transducers were amplified with CP122 AC/DC strain gage amplifiers (Grass Instruments) and digitized at 100 Hz with a PowerLab 8/35 data acquisition system (AD Instruments). Intrinsic f_H was calculated from consecutive right atrium contraction intervals (RA-RA) and the right atrium - ventricle contraction interval was calculated off-line from the duration between the peaks of

Table 1
Composition of saline solutions utilized for the spontaneously contracting right atrium with electrically coupled ventricle strip *in vitro* experiments.

Saline Solution	Acclimation Temperature (°C)	NaCl (mmol⁻¹)	KCl (mmol⁻¹)	NaHCO₃ (mmol⁻¹)	CaCl₂ (mmol⁻¹)	MgSO₄ (mmol⁻¹)	NaH₂PO₄ (mmol⁻¹)	Lactic Acid (mmol⁻¹)	Glucose (mmol⁻¹)	ADR (nmol⁻¹)	Gas Composition (%CO₂/%N₂)	pH
Control Normoxia	21	100	2.5	25	2	1	1	–	5	1	2/98O₂	~7.75
(Control Norm)	5	85	2.5	40	2	1	1	–	5	1	1/99O₂	~7.95
Anoxia (A)	21	100	2.5	25	2	1	1	–	5	1	**2/98**	~7.75
	5	85	2.5	40	2	1	1	–	5	1	**1/99**	~7.95
Combined Anoxia + Acidosis (AA)	21	100	2.5	25	2	**3**	1	**14**	5	1	**3/97**	**~7.25**
	5	85	2.5	40	2	**3**	1	**14**	5	1	**2/98**	**~7.50**
Combined Anoxia + Acidosis + Hyperkalemia (AAK)	21	100	**3.5**	25	2	3	1	14	5	1	3/97	~7.25
	5	85	**3.5**	40	2	3	1	14	5	1	2/98	~7.50
Combined Anoxia + Acidosis + Hyperkalemia + Hypercalcemia (AAKCa)	21	100	3.5	25	**6**	3	1	14	5	1	3/97	~7.25
	5	85	3.5	40	**10**	3	1	14	5	1	2/98	~7.50
Combined Anoxia + Acidosis + Hyperkalemia + Hypercalcemia + ↑ Adrenaline (AAKCaADR)	21	100	3.5	25	6	3	1	14	5	**60**	3/97	~7.25
	5	85	3.5	40	10	3	1	14	5	**25**	2/98	~7.50

Bold text highlights differences in saline solution composition from the preceding solution.
Saline pH was confirmed prior to use using an Orion Star A211 pH meter with Orion ROSS Ultra Glass Triode pH/ATC combination electrode (Thermo Fisher Scientific, Waltham, MA, USA).

consecutive right atrial and ventricular contractions using the cyclic measurements and Peak Analysis functions, respectively, of LabChart 8 software (AD Instruments). 2–3 min of recordings at the conclusion of each saline exposure were analyzed. The coefficient of variation, expressed as a percentage and calculated as the standard deviation of the RA-RA interval divided by the mean RA-RA interval, was calculated to quantify arrhythmia.

2.4. Statistical analysis

One-way repeated measures (RM) analysis of variance (ANOVA) with Holmes-Sidak multiple comparison *post hoc* tests was employed to determine statistically significant differences in ECG parameters, the coefficient of variability, HRV parameters, and contractile properties of the *in vitro* preparations among exposure conditions within an acclimation temperature. Coefficient of variation data was log transformed prior to statistical analysis. Statistically significant differences in ECG parameters between 21 °C- and 5 °C-acclimated normoxic turtles were assessed with *t*-tests. Statistical analysis was conducted with Sigmaplot 12.5 (Systat Software Inc, San Jose, CA, USA) and in all instances $P < 0.05$ was adopted as the level of statistical significance. Results are presented as means ± 95% confidence interval (CI).

3. Results

3.1. Effect of oxygenation state and acclimation temperature on ECG parameters

At 21 °C, 16 h of anoxic submergence caused a 2.3-fold reduction ($P < 0.05$) in f_H, a 1.3-fold prolongation ($P < 0.05$) of QRS duration, and a 1.4-fold lengthening ($P < 0.05$) of QT interval (Table 2). With reoxygenation, f_H was 1.5- to 2.4-times faster ($P < 0.05$) and QT interval up to 0.75 s shorter ($P < 0.05$) during breathing periods, as compared to during anoxia exposure and post-breathing periods pre-anoxia exposure (Table 2; Figs. 1E and 2A).

Following acclimation to 5 °C from 21 °C in normoxia, f_H was 3.3-fold slower ($P < 0.05$; Table 2). Concomitantly, PR interval, QRS duration, and QT interval lengthened ($P < 0.05$) by 1.8 s, 0.3 s, and 3.9 s, respectively (Table 2). Corresponding Q_{10} values were near or greater than 2 (Table 3).

A marked bradycardia accompanied anoxia exposure at 5 °C (Table 2; Fig. 2C), with the suppression of f_H occurring during the initial 24 h of anoxic submergence. Like at 21 °C, QRS duration increased ($P < 0.05$) with anoxia exposure at 5 °C, but in contrast to warm-acclimated,

anoxic turtles, QT interval was unaffected (Table 2; Fig. 1D). With reoxygenation, f_H and QRS duration returned to normoxic levels (Table 2; Figs. 1F and 2C).

3.2. Prevalence of cardiac arrhythmia during anoxia exposure

Qualitatively, evidence of abnormal cardiac excitation and electrical activity was observed in ECG traces of all seven 21 °C-acclimated turtles exposed to prolonged anoxia. Arrhythmia, which was characterized by groups of beats in pairs and trios, commenced within the first hour of anoxia exposure and continued throughout the duration of the exposure period (Fig. 1C). Upon reoxygenation, the arrhythmia ceased. Quantitatively, the coefficient of variation of the RR interval increased ($P < 0.05$) with prolonged anoxia exposure at 21 °C, but it was less ($P < 0.05$) than in normoxia during reoxygenation (Fig. 2B).

At 5 °C, qualitative evidence of cardiac irregularities during anoxia exposure was less compared to at 21 °C. The prevalence ECG waveforms in pairs and trios was inconsistent between animals and measurement times. Quantitatively, the coefficient of variation of the RR interval was unchanged with prolonged anoxia exposure at 5 °C and subsequent reoxygenation (Fig. 2D).

3.3. HRV analysis

Concomitant with the slowing of f_H with cold acclimation in normoxia, SD1 and SD2 were greater ($P < 0.05$) in 5 °C-acclimated turtles than 21 °C-acclimated turtles (Table 4). However, since the increases in SD1 and SD2 were proportional, the SD1/SD2 ratio remained consistent with acclimation temperature (Table 4). By comparison, with anoxia exposure at both acclimation temperatures, the SD1/SD2 ratio increased ($P < 0.05$), but then returned to levels not significantly different from normoxia upon reoxygenation (Table 4).

3.4. Response of in vitro spontaneously contracting right atrium with electrically coupled ventricle strip preparations to altered extracellular conditions

The intrinsic f_H of spontaneously contracting right atrium with electrically coupled ventricle strip preparations slowed ($P < 0.05$) from Control Norm upon exposure to AA and AAK extracellular conditions (Fig. 3A and B). At 21 °C, the negative chronotropic effect was alleviated by the heightened adrenergic stimulation present in the AAKCaADR solution (Fig. 3A). By comparison, at 5 °C, hypercalcemia (i.e., AAKCa) offset the slowed intrinsic f_H induced by AA and AAK (Fig. 3B).

Table 2
ECG parameters of 21 °C- and 5 °C-acclimated *T. scripta*.

Acclimation Temperature	Exposure Condition	PR Interval (s)	QRS Duration (s)	QT Interval (s)	Heart Rate (min^{-1})
21 °C	Normoxia Breathing	0.60 ± 0.0	0.15 ± 0.005 [a]	1.11 ± 0.09 [a,b]	14.8 ± 3.10 [a]
	Normoxia Post-Breathing	0.58 ± 0.0	0.15 ± 0.007 [a]	1.23 ± 011 [b]	9.5 ± 2.33 [a]
	16 h Anoxia	0.62 ± 0.07	0.20 ± 0.020 [b]	1.59 ± 0.25 [c]	6.3 ± 1.64 [b]
	24 h Reoxygenation Breathing	0.67 ± 0.11	0.15 ± 0.007 [a]	0.84 ± 0.08 [a]	22.9 ± 7.20 [c]
	24 h Reoxygenation Post-Breathing	0.55 ± 0.05	0.15 ± 0.004 [a]	1.09 ± 0.26 [a,b]	14.8 ± 3.98 [a]
5 °C	Normoxia Breathing	2.42 ± 0.31 [*]	0.48 ± 0.05 [A *]	5.00 ± 1.04 [A,B *]	4.5 ± 1.12 [A,C *]
	Normoxia Post-Breathing	2.48 ± 0.28 [*]	0.47 ± 0.04 [A *]	5.36 ± 0.87 [B *]	2.9 ± 0.97 [B,C *]
	24 h Anoxia	2.69 ± 0.19	0.63 ± 0.09 [B,C]	4.37 ± 0.37 [A,B]	2.0 ± 1.13 [B]
	12 days Anoxia	2.90 ± 0.61	0.71 ± 0.10 [C]	4.06 ± 0.80 [A,B]	1.5 ± 0.46 [B]
	24 h Reoxygenation Breathing	2.60 ± 0.36	0.53 ± 0.07 [A]	3.71 ± 0.35 [A]	5.8 ± 0.88 [A]
	24 h Reoxygenation Post-Breathing	2.77 ± 0.19	0.56 ± 0.06 [A,B]	3.94 ± 0.47 [A]	4.6 ± 1.50 [A,C]

For each parameter, dissimilar lowercase letters indicate statistically significant differences ($P < 0.05$) between 21 °C exposure conditions, whereas dissimilar uppercase letters indicate statistically significant differences ($P < 0.05$) between 5 °C exposure conditions (one-way RM ANOVA with Holmes-Sidak multiple comparison *post hoc* test). Asterisks demarcate statistical significance difference ($P < 0.05$) between 21 °C- and 5 °C-acclimated animals in normoxia (*t*-test). Values are means ± 95% CI. $N = 7$ per acclimation temperature.
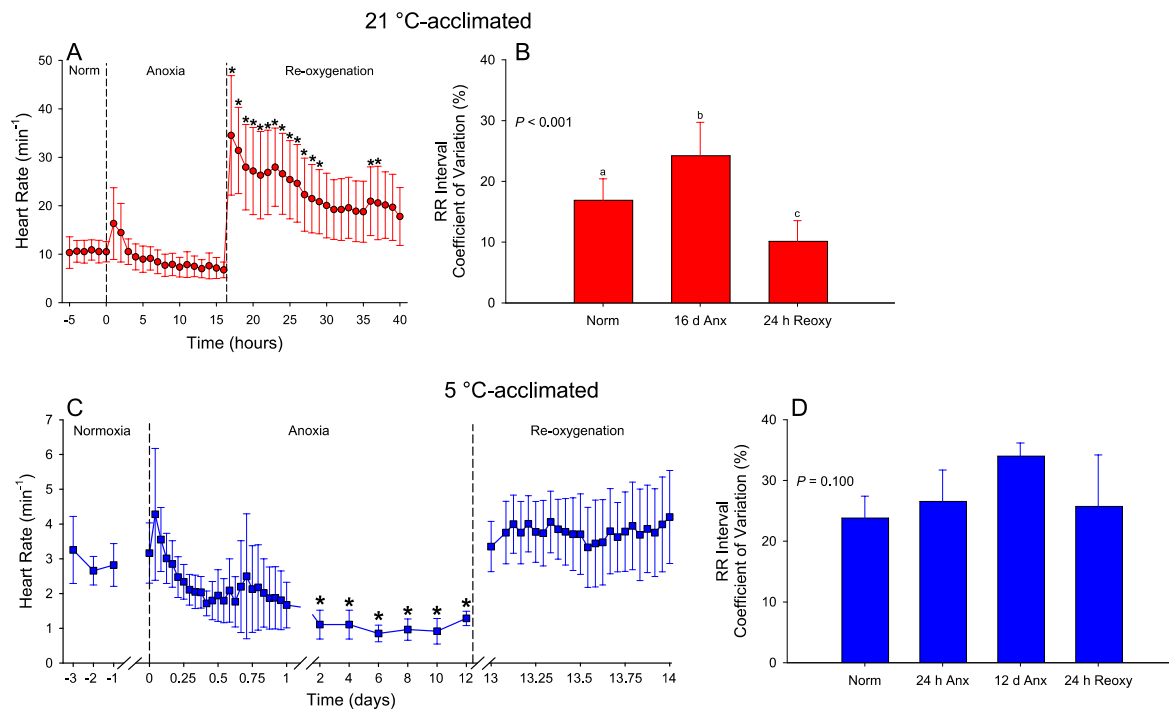
Fig. 2. (A and C) *In vivo* f_H and (B and D) coefficient of variation of the RR interval of (A and B) 21 °C-acclimated and (C and D) 5 °C-acclimated turtles during normoxia (Norm), anoxia exposure, and reoxygenation. In panels A and C, asterisks demarcate statistically significant ($P < 0.05$) differences from control normoxia (i.e., time = 0). In panels B and D, dissimilar lowercase letters demarcate statistically significant ($P < 0.05$) differences between exposure conditions. One-way repeated-measures ANOVA with Holmes-Sidak multiple comparison *post hoc* test. Values are means ± 95% CI. $N = 7$.

Table 3
Q_{10} temperature coefficients for ECG parameters of 21 °C- and 5 °C-acclimated normoxic *T. scripta*.

Exposure Condition	PR Interval*	QRS Duration*	QT Interval*	Heart Rate
Normoxia Breathing	2.42	2.07	2.56	2.10
Normoxia Post-Breathing	2.48	2.13	2.51	2.10

*Q_{10} calculated from reciprocal values.

The response of right atrium - ventricle contraction interval to extracellular changes was a mirror image of the intrinsic f_H response (Fig. 3C and D). AA and AAK prolonged ($P < 0.05$) the right atrium - ventricle contraction interval compared to Control Norm at 21 °C and 5 °C. At 21 °C, the effect was reversed by heightened adrenergic stimulation (i.e., AAKCaADR; Fig. 4C), whereas at 5 °C, the effect was completely reversed by hypercalcemia (i.e., AAKCa; Fig. 4D).

At 21 °C, the coefficient of variation of the RA-RA contraction interval increased ($P < 0.05$) upon exposure to AA and remained elevated ($P < 0.05$) compared to in Control Norm saline solution even in the presence of hypercalcemia (i.e., AAKCa) and combined hypercalcemia with heightened adrenergic stimulation (i.e., AAKCaADR; Fig. 3E). Like observed *in vivo*, the arrhythmia was characterized by groups of beats in pairs and trios (Fig. 4B). Notably, despite the arrhythmia, ventricular contraction always followed right atrium contraction (Fig. 4B).

By comparison, the coefficient of variation of the RA-RA contraction interval remained low and was unchanged by extracellular conditions in 5 °C-acclimated preparations (Fig. 3F). However, one of the five cold-acclimated preparations exhibited AV block (Fig. 4C and D). The AV block was greatest in AA extracellular conditions, but was completely reversed by heightened adrenergic stimulation (i.e., exposure to AAK-CaADR extracellular conditions; Fig. 4C).

Table 4
HRV of 21 °C- and 5 °C-acclimated *T. scripta* exposed to normoxia and prolonged anoxic submergence.

Acclimation Temperature	Exposure Condition	Heart Rate (min⁻¹)	SD1	SD2	SD1/SD2
21 °C	Normoxia	10.7 ± 1.94 [a]	746.5 ± 199.75 [a]	1433.9 ± 355.11 [a]	0.52 ± 0.060 [a]
	16 h Anoxia	7.1 ± 1.85 [a]	1593.0 ± 464.44 [b]	2248.6 ± 982.50 [a]	0.75 ± 0.078 [b]
	24 h Reoxygenation	16.9 ± 5.97 [b]	268.7 ± 63.51 [c]	497.6 ± 136.97 [b]	0.57 ± 0.089 [a]
5 °C	Normoxia	3.1 ± 0.95 [A]*	3077.0 ± 1233.05 [A]*	5120.3 ± 1155.13 [A]*	0.59 ± 0.16 [A]
	24 h Anoxia	1.8 ± 0.79 [B]	7443.6 ± 2912.90 [B]	9560.0 ± 3982.99 [B]	0.79 ± 0.10 [A,B]
	12 days Anoxia	1.6 ± 0.21 [B]	10071.6 ± 1425.19 [C]	12357.7 ± 1965.45 [B]	0.83 ± 0.11 [B]
	24 h Reoxygenation	4.7 ± 0.85 [C]	2582.0 ± 1379.00 [A]	3267.1 ± 1295.01 [A]	0.75 ± 0.13 [A,B]

For each variable, dissimilar lowercase letters indicate statistically significant differences ($P < 0.05$) between exposure conditions at 21 °C, whereas dissimilar uppercase letters indicate statistically significant differences ($P < 0.05$) between exposure conditions at 5 °C (one-way RM ANOVA with Holmes-Sidak multiple comparison *post hoc* test). Asterisks indicate a statistically significant difference ($P < 0.05$) between acclimation temperatures (*t*-test). SD1: short-term variability; SD2: long-term variability. Values are means ± 95% CI. $N = 7$ per acclimation temperature.
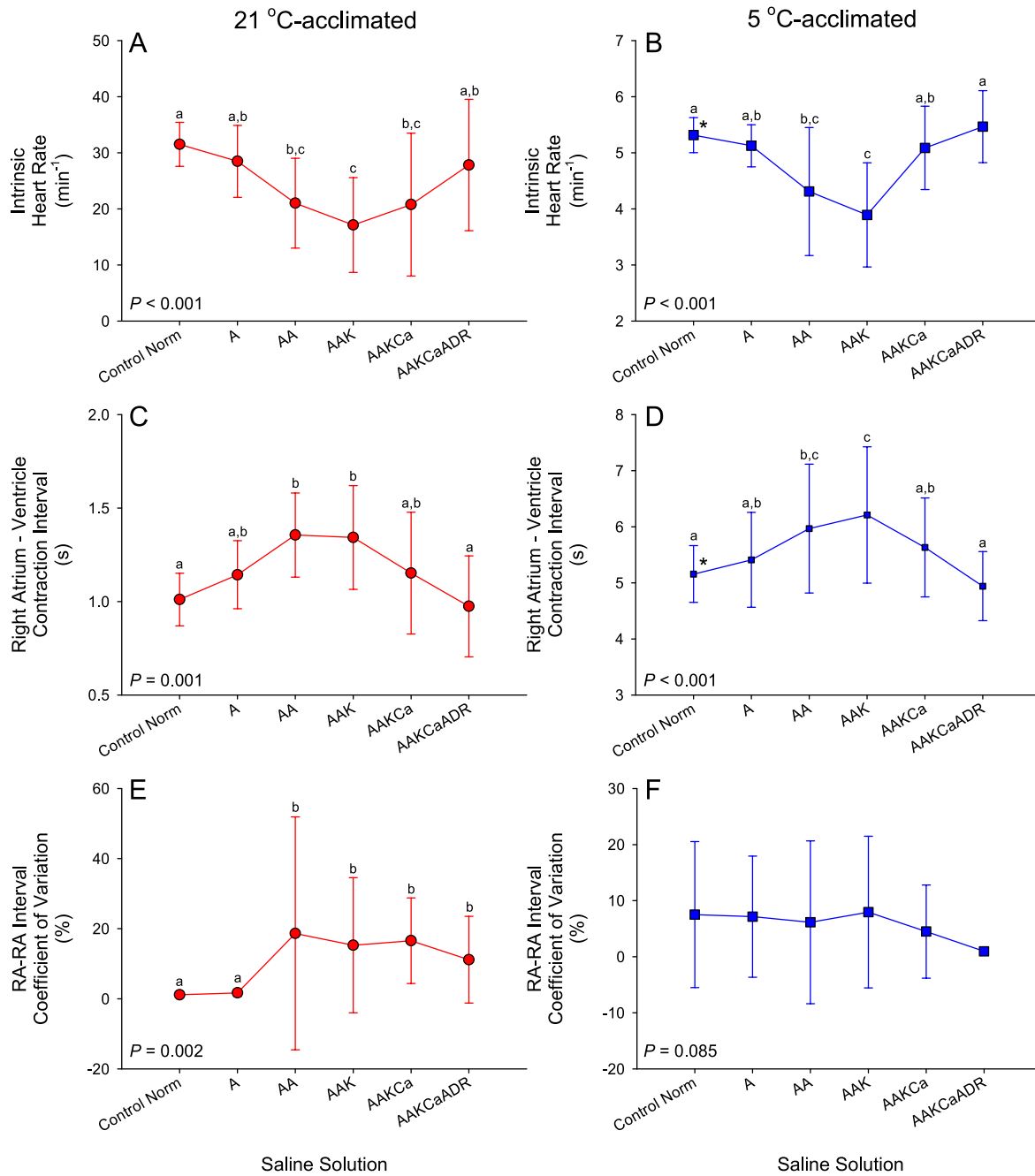
Fig. 3. (A and B) Intrinsic f_H, (C and D) right atrium - ventricle contraction interval, and (E and F) the coefficient of variation of the RA-RA contraction interval of spontaneously contracting *T. scripta* right atrium and electrically coupled ventricle strip preparations during exposure to extracellular conditions that sequentially and additively approximated the shift from the normoxic to anoxic extracellular condition of warm- and cold-acclimated turtles. Panels A, C, and E present data from preparations obtained from 21 °C-acclimated turtles. Panels B, D, and F present data from preparations obtained from 5 °C-acclimated turtles. Dissimilar lowercase letters demarcate statistically significant ($P < 0.05$) differences between saline solutions (Control Norm: Control Normoxia; A: anoxia; A: combined anoxia + acidosis; AAK: combined anoxia + acidosis + hyperkalemia; AAKCa: combined anoxia + acidosis + hyperkalemia + hypercalcemia; AAKCaADR: combined anoxia + acidosis + hyperkalemia + hypercalcemia + heightened adrenergic stimulation; see Table 1). One-way repeated-measures ANOVA with Holmes-Sidak multiple comparison *post hoc* test. Asterisks in panels B and D indicate a difference between acclimation temperatures in Control Norm saline solution (*t*-test). Values are means ± 95% CI. $N = 7$.

4. Discussion

4.1. Cardiac arrhythmia was prominent during anoxia exposure at 21 °C

Our primary research objective was to assess if cardiac arrhythmia, including AV block, occurs during prolonged anoxic submergence in

T. scripta, if its prevalence is dependent on the acclimation temperature of the turtle, and if its pervasiveness is determined by extracellular conditions. In clinical medicine, arrhythmia is defined as abnormal or irregular heart rhythm or rate that is not physiologically justified. Often, it is caused by degenerative processes and certain manifestations are considered pathological. In the present study, because f_H of normoxic
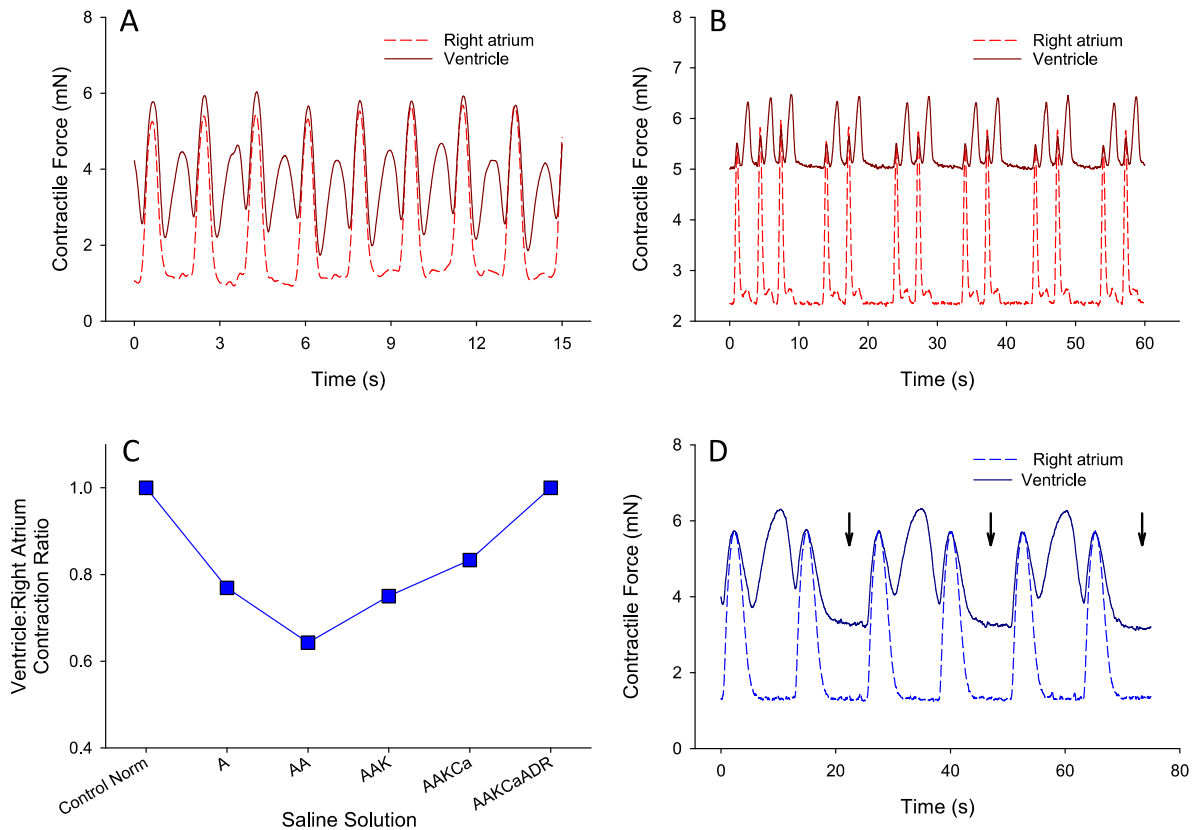
Fig. 4. Representative traces from spontaneously contracting *T. scripta* right atrium and electrically coupled ventricle strip preparations obtained 21 °C-acclimated turtles showing (A) regular contractions in Control Normoxia and (B) arrhythmia in AAK (i.e., combined anoxia + acidosis + hyperkalemia) extracellular conditions. Note that in both panles, ventricular contraction follows each right atrial contraction. (C) Ventricle:right atrium contraction ratio of and (D) representative trace in AA (i.e., combined anoxia + acidosis) extracellular conditions from the sole (out of 5 preparations) spontaneously contracting *T. scripta* right atrium and electrically coupled ventricle strip preparation obtained from a 5 °C-acclimated turtle that exhibited atrioventricular block during exposure to extracellular conditions that sequentially and additively mimicked the shift from the normoxic to anoxic extracellular condition of cold-acclimated turtles (Control Normoxia: Control Norm; A: anoxia; A: combined anoxia + acidosis; AAK: combined anoxia + acidosis + hyperkalemia; AAKCa: combined anoxia + acidosis + hyperkalemia + hypercalcemia; AAKCaADR: combined anoxia + acidosis + hyperkalemia + hypercalcemia + heightened adrenergic stimulation; see Table 1). Arrows denote when ventricular contraction did not follow right atrium contraction.

T. scripta fluctuates (i.e., is irregular) with periods of breathing and apnea, and bradycardia accompanies the metabolic depression necessary for survival anoxic, we defined arrhythmia as coefficient of variation values for RR interval *in vivo* and for RA-RA interval *in vitro* that were statistically significantly different from normoxic control.

In this regard, our most prominent discovery is that cardiac arrhythmia was pronounced in 21 °C-acclimated turtles exposed to prolonged anoxia. Arrhythmia was evidenced qualitatively by ECG waveform groupings in duplets and trios that were not present in normoxia, as well as quantitatively by increased variability of the RR interval. The prevalence of cardiac arrhythmia in anoxic 21 °C-acclimated *T. scripta in vivo* is consistent with the anomalies of cardiac electrical excitation and arrhythmia reported previously for *T. scripta* forced to exercise at room temperature while inspiring hypoxic air (Farmer and Hicks, 2002) and for *in vitro* C. picta belli cardiac strips subjected to anoxia at warm temperature (Jackson, 1987). However, unlike in the past studies, Type III AV block (i.e., third-degree or complete heart block), which is characterized by the absence of conduction from the atria through the AV node to the ventricle, was not apparent *in vivo* in 21 °C-acclimated anoxic turtles. Indeed, the maintained PR interval during anoxia exposure at 21 °C indicates the passage of electrical excitation from the atria to ventricle is not disrupted by oxygen deprivation. Rather, the arrhythmia resembled mammalian non-respiratory sinus arrhythmia, sick sinus syndrome, and tachycardia-bradycardia syndrome (Goldberger et al., 2017), suggesting a sino-atrial origin.

Nevertheless, changes to ventricular excitation did occur with anoxia exposure at 21 °C. The prolonged QRS duration in anoxia indicates that the spread of depolarization over the ventricle is slowed, despite the doubling of peak ventricular voltage-gated Na^+ current density (I_{Na}) that occurs with 6 h of anoxia exposure at warm temperature (Stecyk et al., 2007). The prolonged QT interval is consistent with the inverse relationship reported between QT interval and f_H in turtles (Kaplan and Schwartz, 1963), including in anaesthetized *T. scripta* (Holz and Holz, 1995), and indicates a longer period of ventricular systole. In concordance, 6 h anoxia exposure at 21 °C results in a 47% increase in ventricular action potential duration (APD) (Stecyk et al., 2007).

The cardiac arrhythmia documented *in vivo* in 21 °C-acclimated anoxic turtles was replicated *in vitro* when 21 °C spontaneously contracting right atrium with electrically coupled ventricular strip preparations were exposed to AA, AAK, AAKCa, and AAKCaADR extracellular conditions. In these instances, like *in vivo*, right atrial contraction was always followed by ventricular contraction. Although, the contraction interval between peak right atrium and peak ventricle contraction was increased. The increased duration between peak right atrium and peak ventricular contraction likely developed due to the negative effects of anoxia *per se*, acidosis, and hyperkalemia on rates of cardiac contraction and relaxation (Garner and Stecyk, 2022). Indeed, the increased duration between peak right atrium and peak ventricular contraction was reversed by heightened adrenergic stimulation, which enhances transarcolemmal Ca^{2+} influx (Frace et al., 1993; Reuter, 1983) and also

counteracts the acidotic impairment of myofilament Ca^{2+} sensitivity (Fanter et al., 2017; Tibbits et al., 1992).

4.2. Cardiac arrhythmia was less prominent during prolonged anoxia exposure at 5 °C

Low temperature is cardioplegic for mammals, but it is crucial for the anoxia tolerance of *T. scripta* (Jackson, 2000). The present study reveals that cold acclimation primes the turtle heart to be more resilient to cardiac arrhythmia induced by prolonged anoxic submergence. Although qualitative evidence of irregular heart rhythm was observed in 5 °C-acclimated anoxic turtles, its prevalence was seldom compared to in 21 °C-acclimated anoxic turtles. Further, the variability of the *in vivo* RR interval did not increase with prolonged anoxia exposure at 5 °C, like it did at 21 °C. Moreover, the variability of the *in vitro* RA-RA contraction interval was unaffected by exposure extracellular to anoxia, acidosis, and hyperkalemia at 5 °C. In aggregate, these findings suggest that the disruption of the intrinsic contractile properties of ventricular tissue of cold-acclimated turtles by hyperkalemia (Garner and Stecyk, 2022; Overgaard et al., 2005) is likely not a factor limiting ventricular contraction rate of cold-acclimated anoxic turtles, at least at the duration of anoxia exposure assessed in the present study. The potential underlying mechanistic reason, as demonstrated in the accompanying study (Garner and Stecyk, 2022), is that high levels of circulating catecholamines (Keiver and Hochachka, 1991; Keiver et al., 1992; Wasser and Jackson, 1991) offset the negative effects of hyperkalemia. Indeed, the AV block that was exhibited by one of the 5 °C preparations during exposure to AA, AAK, and AAKCa extracellular conditions was completely eradicated by the heightened adrenergic stimulation present in the AAKCaADR saline solution. In agreement, pharmaceutical block of adrenergic stimulation in live turtles during prolonged anoxia exposure at 5 °C leads to cardiac arrhythmia (Hicks and Farrell, 2000).

The resilience to cardiac arrhythmia during anoxia exposure at 5 °C in *T. scripta* mirrors that of cold-acclimatized crucian carp (*Carassius carassius*), another anoxia-tolerant species (Tikkanen et al., 2017). In the fish, cold acclimatization pre-conditions the heart against cardiac arrhythmia through modulation of sarcolemmal L-type Ca^{2+} current (I_{CaL}) and sarcoplasmic reticulum Ca^{2+} cycling. Similarly, cold-acclimation induces alterations to ventricular transarcolemmal Ca^{2+} flux (Stecyk et al., 2021), including a 13-fold reduction in peak ventricular I_{CaL} density (Stecyk et al., 2007), in *T. scripta*. Moreover, 5 °C-acclimated turtles had a longer QRS duration and QT interval in normoxia compared to 21 °C-acclimated animals. The prolongation of QT interval with cold acclimation in normoxia is consistent with the increased duration of PR, RT, and RR intervals with decreasing body temperature in five species of turtles, including *T. scripta*, regardless of the temperature at which the animals were acclimated (Risher and Claussen, 1987), as well as the effect of cold acclimation on ECG parameters of toad (*Bufo raddei*) and lizard (*Eremias multiocellata*) (Liu and Li, 2005). The prolonged QT interval also aligns with the 4.2-fold prolongation of ventricular APD that occurs with cold acclimation in *T. scripta* (Stecyk et al., 2007), whereas the prolonged QRS duration coincides with the peak density of ventricular I_{Na} in 5 °C-acclimated *T. scripta* being ~1/7th of that at 21 °C (Stecyk et al., 2007).

4.3. HRV analysis supports increased parasympathetic activity during anoxia exposure

HRV describes the variations between RR intervals and is used as a quantitative marker of cardiac autonomic nervous system in vertebrates, ranging from fish to mammals (Campbell et al., 2005; Haworth et al., 2014; Hoshi et al., 2013; Hsu et al., 2012; Stecyk et al., 2020; Tikkanen et al., 2017). The increased SD1/SD2 ratio that occurred with anoxia exposure at 21 °C and 5 °C suggests a change in sympathovagal balance towards increased vagal activity, whereas the return of the SD1/SD2 ratio to normoxic levels upon reoxygenation, suggests reversal of the

parasympathetic drive. The finding aligns well with the increase in vagal activity that causes the rapid onset of Right-to-Left shunt and bradycardia during diving, apnea, and anoxia exposure in *T. scripta* acclimated to warm temperature (Hicks and Farrell, 2000; Hicks et al., 1996; Hicks and Wang, 1998). Interestingly, cardiac arrhythmias, including atrioventricular blocks are often the result of parasympathetic innervation of the heart and activation of the vagus nerve (Chen et al., 2018). Thus, enhanced vagal drive may be a factor contributing to the arrhythmia observed during anoxia exposure at 21 °C, in addition to the extrinsic effects of anoxia *per se*, acidosis, and hyperkalemia. However, the finding contrasts with the suppression of cardiac cholinergic inhibition with cold acclimation in *T. scripta*, explicated from the lack of a statistically significant effect of atropine infusion on f_H of 5 °C-acclimated turtles under either normoxic or anoxic conditions (Hicks and Farrell, 2000).

4.4. Concluding remarks and future directions

The present study provides novel information on *T. scripta* cardiac electrical activity and the prevalence of abnormalities of cardiac electrical activity and excitation in normoxia, during prolonged anoxia exposure, and subsequent reoxygenation at both warm and cold acclimation temperatures. Our *in vivo* and *in vitro* results revealed that sino-atrial cardiac arrhythmia was prominent in 21 °C-acclimated anoxic turtles, for which prolonged anoxia exposure is not a natural occurrence, but that is negotiable due to constitutive and expressed physiological factors. Yet, the arrythmia ceased with reoxygenation. The finding indicates that the *T. scripta* heart recovers from disruptions to cardiac electrical activity and excitation. By comparison, in mammals, abnormal ECG manifestations are often considered pathological. Moreover, our findings revealed that cold acclimation primes the turtle heart to be resilient to the cardiac arrhythmia induced by prolonged anoxic submergence. Future investigations into the effects of anoxia exposure and extracellular changes on the electrophysiological properties of turtle cardiac pacemaker, nodal, and conduction system cells (Burggren, 1978; Robb, 1952) would lend mechanistic insight into the ionic basis of the cardiac arrhythmia, as well as the differential responses displayed by warm- and col-acclimated anoxic turtle hearts.

CRediT authorship contribution statement

Molly Garner: Investigation, Formal analysis, Writing – original draft. **Riley G. Barber:** Investigation, Formal analysis. **Jace Cussins:** Investigation, Formal analysis, Writing – original draft. **Diarmid Hall:** Investigation, Formal analysis. **Jessica Reisinger:** Investigation, Formal analysis. **Jonathan A.W. Stecyk:** Conceptualization, Formal analysis, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

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