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Does the ventricle limit cardiac contraction rate in the anoxic turtle (*Trachemys scripta*)? I. Comparison of the intrinsic contractile responses of cardiac chambers to the extracellular changes that accompany prolonged anoxia exposure

Molly Garner, Jonathan A.W. Stecyk *

Department of Biological Sciences, University of Alaska Anchorage, Anchorage, AK, 99508, USA

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ABSTRACT

Multiple lines of evidence suggest that an inability of the ventricle to contract in coordination with the pacemaker during anoxia exposure may suppress cardiac pumping rate in anoxia-tolerant turtles. To determine under what extracellular conditions the ventricle could be the weak link that limits cardiac pumping, we compared, under various extracellular conditions, the intrinsic contractile properties of isometrically-contracting ventricular and atrial strips obtained from 21 °C- to 5 °C- acclimated turtles (*Trachemys scripta*) that had been exposed to either normoxia or anoxia (16 h at 21 °C; 12 days at 5 °C). We found that combined extracellular anoxia, acidosis, and hyperkalemia (AAK), severely disrupted ventricular, but not right or left atrial, excitability and contractibility of 5 °C anoxic turtles. However, combined hypercalcemia and heightened adrenergic stimulation counteracted the negative effects of AAK. We also report that the turtle heart is resilient to prolonged diastolic intervals, which would ensure that contractile force is maintained if arrhythmia were to occur during anoxia exposure. Finally, our findings reinforce that prior temperature and anoxia experiences are central to the intrinsic contractile response of the turtle myocardium to altered extracellular conditions. At 21 °C, prior anoxia exposure preconditioned the ventricle for anoxic and acidosis exposure. At 5 °C, prior anoxia exposure evoked heightened sensitivity of the ventricle to hyperkalemia, as well as all chambers to combined hypercalcemia and increased adrenergic stimulation. Overall, our findings show that the ventricle could limit cardiac pumping rate during prolonged anoxic submergence in cold-acclimated turtles if hypercalcemia and heightened adrenergic stimulation are insufficient to counteract the negative effects of combined extracellular anoxia, acidosis, and hyperkalemia.

1. Introduction

The red-eared slider freshwater turtle (*Trachemys scripta*) is amongst the champions of vertebrate anoxia survival. At warm acclimation temperatures (20–25 °C), *T. scripta* can tolerate ~24 h of anoxic submergence, whereas at the cold acclimation temperatures (3–5 °C) at which it overwinters, anoxic survival extends to 6–7 weeks (Ultsch, 1985, 2006; Warren et al., 2006). The heart of *T. scripta* continues to beat during prolonged anoxia exposure at both warm and cold acclimation temperatures to ensure the exchange of metabolites and waste products among tissues (Stecyk et al., 2008). However, cardiac activity is markedly reduced with anoxia exposure, largely due to a pronounced bradycardia, as a strategy to match cardiac ATP demand to the limited ATP supply available from anaerobic glycolysis (Farrell and Stecyk, 2007).

Beyond alterations to autonomic cardiac control (Hicks and Farrell, 2000b; Hicks and Wang, 1998), changes in the extracellular milieu that depress intrinsic heart rate (Farrell et al., 1994; Nielsen and Gesser, 2001; Stecyk and Farrell, 2007; Wasser et al., 1990a, 1990b), and a re-setting of the intrinsic firing rate of the cardiac pacemaker to a reduced level (Stecyk and Farrell, 2007; Stecyk et al., 2007, 2021) during anoxia exposure, multiple lines of evidence suggest that an inability of the ventricle to contract in coordination with the atria (i.e., ventricular bradycardia) may also suppress the cardiac pumping rate of anoxic turtles. Firstly, *T. scripta* forced to exercise while breathing hypoxic air exhibited a pronounced atrioventricular block, whereupon ventricular contraction only followed every sixth atrial contraction

* Corresponding author. Department of Biological Sciences, University of Alaska Anchorage 3211 Providence Drive Anchorage, AK, 99508, USA.
E-mail address: jstecyk@alaska.edu (J.A.W. Stecyk).

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List of abbreviations

5Anx	5 °C-acclimated turtles exposed to anoxia for 12 days
5Norm	5 °C-acclimated turtles maintained in normoxia
21Anx	21 °C-acclimated turtles exposed to anoxia for 16 h
21Norm	5 °C-acclimated turtles maintained in normoxia
AA	combined anoxia and acidosis extracellular conditions (saline solution)
AAK	combined anoxia, acidosis, and hyperkalemia extracellular conditions (saline solution)
Control Anx	control anoxic extracellular conditions (saline solution); combined anoxia, acidosis, hyperkalemia, hypercalcemia, and heightened adrenaline concentration
Control Norm	control normoxic extracellular conditions (saline solution)
F_{max}	maximal developed force
F_C	developed force of 'control' contraction at steady-state pacing frequency
F_T	developed force of extrasystolic test pulse
L_{max}	strip length at which developed force was maximal
MRP	mechanical refractory period
NCX	Na^+-Ca^{2+}-exchanger
$Rate_{50\%\ relax}$	average rate of 50% relaxation
$Rate_{rise}$	average rate of contraction
SR	sarcoplasmic reticulum
τ	rate constant of force restitution
$T_{0.5R}$	time-to-half relaxation
T_{DC}	duration of contraction
T_{PF}	time-to-peak force
Δt	test interval duration

(Farmer and Hicks, 2002). Secondly, atrioventricular block, whereupon ventricular contraction followed only every second or third atrial contraction, occurred during *in vitro* exposure of electrically coupled atrium and ventricular preparations from the anoxia-tolerant Western painted turtle (*Chrysemys picta belli*) to anoxia at warm temperatures (Jackson, 1987). Finally, the contraction frequency of *T. scripta* spontaneously contracting right atrium preparations exposed to extracellular conditions that mimicked the extracellular milieu of anoxic turtles (27 beats min^{-1} at 21 °C; 2.1 beats min^{-1} at 5 °C)(Stecyk and Farrell, 2007) is not as slow as the *in vivo* heart rate of live, atropinized (to block vagal cholinergic inhibition) turtles during prolonged anoxia exposure (16.7–19.7 beats min^{-1} at 22–25 °C; 1.2 beats min^{-1} at 5 °C)(Hicks and Farrell, 2000b; Hicks and Wang, 1998), as measured from blood flows and pressures in major systemic blood vessels (i.e., from ventricular contraction frequency).

Theoretically, the inability of the turtle ventricle to contract following atrial contraction under oxygen limiting conditions could arise from a disruption of cardiac electrical conduction at the atrioventricular node, reduced excitability of the ventricular myocardium, and/or limitations of the intrinsic contractile properties of the ventricular myocardium that serve to disproportionately lengthen its contraction cycle compared to the atria. The previous finding that ventricular strips from *C. picta belli* acclimated to cold, anoxic conditions could contract up to 8 beats min^{-1} in anoxic saline, despite exhibiting a pronounced decrease in contractility, implicates disruption of cardiac electrical conduction as a primary mechanism limiting ventricular contraction rate in anoxia (Overgaard et al., 2005). Nevertheless, it remains unknown if the extracellular changes that accompany prolonged anoxia exposure, namely acidosis, hyperkalemia, hypercalcemia, and increased adrenergic stimulation, either individually or in combination, induce confounding effects that alter the ability of the ventricle to contract in coordination with the atria. Indeed, no study has comprehensively and systematically compared the intrinsic contractile responses of *T. scripta* ventricle, right atrium, and left atrium to altered extracellular conditions. Here, we address this information gap and investigate if and under what extracellular conditions reduced excitability and/or limitations of the intrinsic contractile properties of the turtle ventricle could be the weak link that limits cardiac pumping *in vivo* during anoxic submergence. The accompanying study (Garner et al., 2022), assesses *in vivo* the prevalence of cardiac arrhythmia during anoxia exposure and *in vitro* if disruption of cardiac electrical conduction at the atrio-ventricular node contributes to the inability of the turtle ventricle to contract following atrial contraction under oxygen limiting conditions.

Our approach was to compare (1) mechanical refractory period (MRP) to assess myocardial excitability and determine the shortest stimulus interval at which the cardiac chambers could produce a measurable contraction, (2) the rate constant of force restitution (τ) to assess the ability of the myocardium to recover contractile strength following extrasystolic stimulation, (3) maximal developed force, (4) duration of contraction, (5) average rates of contraction and relaxation, and (6) post-rest potentiation to evaluate the effect of extended diastole on myocardial force generation of *T. scripta* isometrically-contracting ventricular, right atrial, and left atrial strips under various extracellular conditions. To factor in the effect of prior acclimation temperature and oxygenation state, strips were obtained from 21 °C- or 5 °C-acclimated turtles that had been exposed to either normoxia or anoxia (16 h at 21 °C; 12 days at 5 °C). We hypothesized that combined anoxia and acidosis, as well as combined anoxia, acidosis, and hyperkalemia, would negatively affect intrinsic contractile properties in strips from all cardiac chambers, but that the negative effects would be more pronounced in the ventricle than the atria. We further posited that combined hypercalcemia and increased adrenaline would attenuate the negative effects, but that the atria would be more responsive to combined hypercalcemia and increased adrenaline than the ventricle. Finally, based on the previous findings that acclimation temperature and prior anoxia exposure alter the intrinsic contractile response of the turtle heart to extracellular changes (Overgaard et al., 2005; Ruhr et al., 2019; Stecyk and Farrell, 2007), that adrenergic stimulation is attenuated in turtles exposed to acute and chronic anoxia (Hicks and Farrell, 2000b; Hicks and Wang, 1998; Stecyk et al., 2004) and that ventricular cell surface β-adrenoceptor density is reduced by ~40% in anoxic turtles (Hicks and Farrell, 2000b), we predicted tissue preparations from anoxia-exposed animals to be more resilient to changes in the extracellular milieu that negatively affect cardiac contractility, but less responsive to hypercalcemia and heightened adrenergic stimulation.

2. Material and methods

2.1. Experimental animals and experimental exposure groups

All animal husbandry and experimental procedures were in accordance with protocols (1362273, 1362274) approved by the University of Alaska Anchorage (UAA) Institutional Animal Care and Use Committee. We utilized a total of 45 red-eared slider turtles (*Trachemys scripta*) of both sexes and with a mass of 250 ± 83.7 g (mean ± SD). Turtles were obtained from a commercial supplier (Niles Biological, Sacramento, CA, USA) and air freighted to UAA. All animals were initially maintained at 21 °C and under a 12h:12 h L:D photoperiod in 437 L polypropylene aquaria that contained basking platforms, heat lamps for thermoregulation and water deep enough for the turtles to swim in freely. Turtles were fed commercial turtle pellets on alternating days.

Turtles were assigned to one of four experimental exposure conditions: 21 °C Normoxia (21Norm); 5 °C Normoxia (5Norm); 21 °C Anoxia

(21Anx); and 5 °C Anoxia (5Anx). 21Norm turtles were sampled from their holding conditions. 5Norm turtles were acclimated to 5 °C as previously described (Sparks et al., 2022; Stecyk et al., 2021). Briefly, cold acclimation occurred by placing turtles in plastic containers containing 3–4 cm of water in a commercial refrigerator (GDM-47-LD, True Manufacturing Co., O'Fallon, MO, USA), during which time the animals were fasted. The cold acclimation period occurred in the autumn or winter months (September–December) and was 5–6 weeks to ensure adequate acclimation of the cardiovascular system to cold temperature (Hicks and Farrell, 2000a).

Anoxia exposures at 21 °C and 5 °C occurred as previously described (Sparks et al., 2022; Stecyk et al., 2021). Briefly, subsets of 21Norm and 5Norm animals were placed individually into enclosed plastic containers that were filled with water. A metal grating suspended below the water surface prevented the turtles from gaining air access and the water was continuously bubbled with 100% N_2 to deplete it of oxygen. Anoxic conditions (0.1 mg O_2 l^{-1}) at both acclimation temperatures were confirmed at the conclusion of the anoxia exposure periods with a fiber optic FDO 925 oxygen probe and Multi 3410 m (WTW, Weilheim, Germany).

The duration of the anoxia exposure was 16 h at 21 °C and 12 days at 5 °C. The anoxia exposure times were selected to fall within the range of anoxia exposure durations employed in prior studies assessing the anoxia tolerance of *T. scripta* and *C. picta belli* (3 h–44 days at warm temperature; 7 days to 13 weeks at cold temperature)(Couturier et al., 2019; Herbert and Jackson, 1985a, b; Melleby et al., 2020; Stecyk et al., 2009; Stecyk et al., 2012; Warren and Jackson, 2007; Warren et al., 2006). Importantly, the anoxia exposure durations were well beyond the period when cardiovascular status transitions to a new, reduced 'steady state' in anoxia (Hicks and Farrell, 2000a; Stecyk et al., 2004; Stecyk et al., 2010). Specifically, *in vivo* heart rate decreases from ~25 to ~10 beats min^{-1} within 1 h of anoxia exposure at 21–25 °C, and from a ~5 beats min^{-1} to less than 1 beat min^{-1} within 48 h of anoxia exposure at 5 °C, whereupon the bradycardia is maintained throughout the duration of the anoxia exposure period.

2.2. Preparation of isometrically-contracting cardiac strips

Turtles were sacrificed by decapitation and the brain destroyed. Anoxia-exposed animals were not permitted to breath atmospheric air prior to decapitation. Within the next 2 min, the heart was accessed by removing the plastron with a bone saw, excised, and washed in ice-cold Control Normoxic (Control Norm; for 21Norm and 5Norm turtles) or Control Anoxic (Control Anx; for 21Anx and 5Anx turtles) physiological saline solution, but without adrenaline (Table 1). The ventricle, right atrium, and left atrium were dissected and then sectioned by razor blade to obtain tissue strips. Ventricles were sectioned on the sagittal plane to obtain dorsal-medial strips because this orientation results in less inter-individual variation (Ball and Hicks, 1996). Similarly, right and left atria were splayed lengthwise to expose the lumen and longitudinally sectioned. One end of each strip was fastened to a fixed arm, near stimulating platinum electrodes, and the other attached with a tissue holder to the end to an isometric force transducer (FT03C; Grass Instruments, West Warwick, RI, USA) and micrometer bracket assembly (World Precision Instruments, Sarasota, FL, USA). Strips were then immersed without any stretch on the tissue in 30 ml water-jacketed tissue baths (Radnoti, Covina, CA, USA) that contained Control Norm or Control Anx solution appropriate for the prior exposure condition of the animal, including adrenaline (Table 1). Temperature was maintained at the acclimation temperature of the animal with a refrigerated re-circulating water bath (VWR, Radnor, PA, USA). After a 30 min stabilization period, slack was removed by adjustment with a micrometer screw, and after an additional 20 min, the strips were electrically stimulated to contract using a SD9 Stimulator (Grass Instruments) that was triggered using LabChart 8 software and a PowerLab 8/35 data acquisition system (AD Instruments, Colorado Springs, CO, USA). The square stimulation waveform employed was ~35 V (1.5x the voltage required to initiate contraction) with a 10 ms pulse duration. Initial stimulation frequency represented *in vivo* heart rate for the exposure condition of the animal (Hicks and Farrell, 2000a, b; Hicks and Wang, 1998; Stecyk et al., 2004) and was 0.37 Hz (= 22 bpm), 0.17 Hz (= 10 bpm), 0.08 Hz (= 5 bpm) and 0.02 Hz (= 1 bpm) for 21Norm, 21Anx, 5Norm, and 5Anx strips, respectively. The strips were then gradually stretched to the length at which developed force was maximal (L_{max}). After an additional 20 min equilibration period, the experimental protocol commenced. Strips that failed to contract regularly by this point were excluded from experimentation and data analysis.

2.3. Experimental protocol

Cardiac strips were sequentially exposed to four different extracellular conditions depending on the prior exposure condition of the animal (Table 1). Following equilibration in Control Norm solution, the conclusion of which was designated Baseline, 21Norm and 5Norm strips

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

Exposure Order	Saline Solution	Acclimation Temperature (°C)	NaCl ($mmol^{-1}$)	KCl ($mmol^{-1}$)	$NaHCO_3$ ($mmol^{-1}$)	$CaCl_2$ ($mmol^{-1}$)	$MgSO^{4-}$ ($mmol^{-1}$)	NaH_2PO_4 ($mmol^{-1}$)	Lactic Acid ($mmol^{-1}$)	Glucose ($mmol^{-1}$)	ADR ($nmol^{-1}$)	Gas Composition (%CO_2/%N_2)	pH
Strips from normoxic turtles ↓; Strips from anoxic turtles ↑	Control Normoxia (Control Norm)	21	100	2.5	25	2	1	1	-	5	1	2/98O_2	~7.80
		5	85	2.5	40	2	1	1	-	5	1	1/98O_2	~7.80
	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	**7**	25	2	3	1	**16**	5	1	3/97	**~7.15**
		5	85	**7**	40	2	3	1	**16**	5	1	2/98	**~7.40**
	Control Anoxia (Control Anx)	21	100	7	25	**8**	3	1	16	5	**60**	3/97	~7.15
		5	85	7	40	**10**	3	1	16	5	**25**	2/98	~7.40

ADR: adrenaline.
Bold text highlights differences in saline solution composition from the preceding solution for the normoxia-acclimated experimental protocol.
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).

were sequentially exposed to solutions that consisted of combined anoxia and acidosis (AA), combined anoxia, acidosis, and hyperkalemia (AAK) and finally combined anoxia, acidosis, hyperkalemia, hypercalcemia, and heightened adrenaline concentration (i.e., Control Anx solution). Conversely, 21Anx and 5Anx preparations were exposed to the extracellular conditions in the reverse order. The conclusion of the equilibration period in Control Anx solution was designated Baseline, and the strips were then sequentially exposed to AAK, then AA, and finally Control Norm saline solutions (Table 1). In all instances, tissues were given 20 min to stabilize following solution change before intrinsic contractile parameters were assessed.

The levels of acidosis, hyperkalemia, hypercalcemia, and adrenaline in the AA, AAK, and Control Anx saline solutions were selected to strike a balance between the extracellular changes that occur *in vivo* in anoxia-tolerant turtles with 16 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). Gas mixtures for the saline solutions were obtained using a Gas Mixing System (version 1.0.0; Loligo Systems, Viborg, Denmark). Solutions were pre-equilibrated prior to use and were bubbled continuously throughout the experimental protocol. Anoxic bath conditions were confirmed with a TROXROB3 robust trace oxygen miniprobe and FireSting fiber-optic oxygen meter (PyroScience GmbH, Aachen, Germany).

Preliminary experiments revealed that tissue strips from normoxic animals exhibited irregular contractions when paced at *in vivo* normoxic heart rate in AAK and Control Anx solutions. Therefore, under these extracellular conditions pacing frequency was reduced to the *in vivo* anoxic heart rate. Conversely, to allow comparison of the contractile properties of strips from normoxia- and anoxia-exposed animals within a saline solution, the pacing rate of 21Anx and 5Anx preparations was increased from the *in vivo* anoxic heart rate to the *in vivo* normoxic heart rate during exposure to AA and Control Norm solutions.

2.4. Data acquisition and characterization of intrinsic contractile properties

Signals from the force transducers were amplified with CPI22 AC/DC strain gage amplifiers (Grass Instruments) and continuously digitized at 100 Hz using a PowerLab 8/35 data acquisition system.

Mechanical restitution was determined as detailed by Aho and Vornanen (1999) (Aho and Vornanen, 1999). Briefly, the steady-state pacing frequency was interrupted with an extrasystolic test pulse (F_T) of progressively shorter intervals until contraction failed to ensue (Fig. S1A). The maximal developed force (F_{max}) of F_T was normalized to the developed force of the preceding 'control' contraction at steady-state pacing frequency (F_C) and plotted against the test interval duration (Δt) to produce a restitution curve, which represents the increase in force of contraction associated with progressively longer extrasystolic intervals (Fig. S1B). Two variables, mechanical refractory period (MRP) and the rate constant of force restitution were used to characterize mechanical restitution (Fig. S1B). MRP is the shortest stimulus interval that produced measurable contractions. The rate constant of force restitution is expressed by the time constant (τ) of the single-exponential equation $y = a(1-e^{-b/t})+c$ that was fit to the restitution curve. τ quantifies the time required for F_T to recover to 63% of F_C and thus reflects the slope of the restitution curve and the ability of the myocardium to return to normal contractile strength following extrasystolic stimulation.

For 21Norm and 21Anx preparations, F_T was progressively shortened in 100 ms increments from the steady-state pacing frequency. For 5Norm and 5Anx preparation in Sim Norm and AA saline solutions, F_T was progressively shortened in 500 ms increments. Due to the extremely long interval of the steady-state pacing frequency utilized for 5Norm and 5Anx preparations exposed to AAK and Control Anx saline solutions (i.e., 60 s), F_T was initially shortened by 5 s increments, down to an interval of 10 s, whereupon F_T was progressively shortened in 500 ms increments. This ensured that the mechanical restitution experimental protocol was not too prolonged whilst still allowing for the collection of enough data points to generate a restitution curve. Ten (at 21 °C) or 3 (at 5 °C) contractions at physiological pacing frequency occurred between F_T pulses to ensure intracellular Ca^{2+} was consistent (as evidenced by consistent F_{max}) for each F_T.

The isometric contractile performance of the cardiac strips was quantified from the analysis of 3–10 contractions randomly selected from within the last 3 min of exposure to each saline solution. The following parameters were quantified: F_{max}, time-to-peak force (T_{PF}) and time-to-half relaxation ($T_{0.5R}$). Duration of contraction (T_{DC}) was calculated by adding $T_{PF} + T_{0.5R}$ and average rates of contraction ($Rate_{rise}$) and 50% relaxation ($Rate_{50\%\ relax}$) were calculated by dividing F_{max} by T_{PF} and 0.5 x F_{max} by $T_{0.5R}$, respectively (Kubly and Stecyk, 2019; Shiels et al., 2022; Stecyk et al., 2021). F_{max} is expressed as mN mm^{-2}, where mean cross-sectional area was calculated using the strip wet mass, length, and an assumed muscle density of 1.06 g cm^{-3} (Layland et al., 1995). Cross-sectional area of the ventricular ($n = 39$), right atrial ($n = 36$) and left atrial strips ($n = 33$) strips utilized in the experiment was 0.38 ± 0.27, 0.27 ± 0.19 and 0.32 ± 0.19 mm^{-2} (means ± SD), respectively.

Post-rest potentiation was measured by interrupting the steady-state pacing frequency with progressively longer rest periods and normalizing the developed force of the first post-rest contraction to the developed force of the preceding steady-state contraction (Fig. S1C) (Aho and Vornanen, 1999). The rest periods ranged from 5 to 180 s for 21Norm and 21Anx strips exposed to Control Norm and AA, 10–180 s for 21Norm and 21Anx strips exposed to AAK and Control Anx, 15–210 s for 5Norm and 5Anx strips exposed to Control Norm and AA and 65–240 s for 5Norm and 5Anx strips exposed to AAK and Control Anx, after which steady-state stimulation was resumed. Due the extremely long time that would have been required to acquire measurements of both mechanical restitution and post-rest potentiation from a 5Anx strip under each extracellular condition, and to ensure tissue integrity throughout the duration of the experimental protocol, these measurements were conducted on separate strips obtained from different individuals. This accounts for the difference between the number of animals ($N = 45$) and strips ($n = 33$–36 per chamber) utilized.

2.5. Calculations and statistical analysis

Temperature coefficients (Q_{10}) were calculated to quantify the effect of acclimation temperature on cardiac contractile parameters using the formula: $Q_{10} = (R_1/R_2)^{[10/(T1\text{-}T2)]}$, where R_1 and R_2 are rates at temperatures T_1 and T_2 respectively ($T_1 < T_2$) (Aho and Vornanen, 1999). For non-rate processes (i.e., MRP, τ, F_{max}, and T_{DC}), Q_{10} was calculated using reciprocal values (Stecyk et al., 2007, 2020).

One way analysis of variance (ANOVA) was employed to assess whether contractile parameters measured at Baseline differed between tissue type (i.e., ventricle, right atrium, or left atrium) within an acclimation condition (i.e., 21Norm, 21Anx, 5Norm, or 5Anx). Further, *t*-tests were used to assess whether contractile parameters measured at Baseline differed between acclimation temperature (i.e., 21Norm vs 5Norm and 21Anx vs 5Anx) within each chamber. Two-way repeated-measures (RM) ANOVA was employed to evaluate whether the effects of exposure to the various saline solutions on a cardiac contractile parameter differed between exposure conditions (i.e., normoxia- or anoxia-exposed) at each acclimation temperature, per cardiac chamber. For post-rest potentiation, the two-way RM ANOVA assessed, per cardiac chamber, if F_{max} of the first post-interval contraction was greater than the preceding steady state F_{max} and whether the response differed between exposure condition at each rest interval. In all instances, if significant differences ($P < 0.05$) were detected with the ANOVA, Holm-Sidak *post hoc* pair-wise multiple comparisons were conducted. All statistical analysis was conducted with SigmaPlot 12.5 (Systat Software, Inc, San Jose, CA, USA) and all results are presented as means ± 95% confidence interval (CI), unless otherwise noted. *n* represents number of strips and reflects biological replicates. Only one strip per chamber per animal was utilized.

3. Results

3.1. MRP and T_{DC} were longer in ventricle than atria independent of the prior exposure condition of the turtle

When measured in Control Norm saline solution, MRP of 21Norm ventricle was 384 ms longer ($P < 0.05$) than the MRP of 21Norm right and left atria (Fig. 1A). T_{DC} of 21Norm ventricle was also 531–564 ms longer ($P < 0.05$) than T_{DC} of 21Norm right and left atria (Fig. 1D). By comparison, τ, F_{max}, and $Rate_{50\%\ relax}$ did not statistically significantly differ between cardiac chambers of 21Norm turtles (Fig. 1B, C and F).

Cold acclimation in normoxia prolonged ($P < 0.05$) MRP, τ, and T_{DC}, and slowed ($P < 0.05$) $Rate_{rise}$ and $Rate_{50\%\ relax}$ (Fig. 1A–F). Corresponding Q_{10} values ranged between 2.26 and 4.20 (Table 2). Nevertheless, MRP and T_{DC} remained longer ($P < 0.05$) in 5Norm ventricle compared to 5Norm right and left atria, whereas τ, F_{max}, and $Rate_{50\%\ relax}$ did not statistically significantly differ among 5Norm cardiac chambers. The lengthened ($P < 0.05$) MRP and T_{DC} in ventricle compared to right and left atria, but similar τ, F_{max}, and $Rate_{50\%\ relax}$ among chambers, was also evident following prolonged anoxia exposure at warm and cold acclimation temperature (i.e., in 21Anx and 5Anx strips exposed to Control Anx saline solution; Fig. 1G-L).

3.2. Combined anoxia, acidosis, and hyperkalemia lengthened mechanical refractory period

Qualitatively, the effects of altered extracellular conditions on MRP were consistent between exposure groups and tissue type (Fig. 2A–C and G-I). Except for 5Norm right atrium, in which MRP was unaffected by saline solution (Fig. 2H), MRP was unchanged between Control Norm and AA, prolonged ($P < 0.05$) in AAK compared to Control Norm, and the lengthened MRP in AAK alleviated ($P < 0.05$) by the combined hypercalcemia and increased adrenaline concentration present in the Control Anx saline solution. However, quantitatively, the degree to which MRP was lengthened by combined anoxia, acidosis, and hyperkalemia was dependent on the prior exposure condition of the turtle. Compared to in Control Norm, MRP was longer in AAK by 1.1- to 1.4-fold in 21Norm and 21Anx strips (Fig. 2A–C), 1.8- to 4.4-fold in 5Norm strips (Fig. 2G–I), and 4.3- to 8.5-fold in 5Anx atrial strips (Fig. 2H and I). The negative effect of AAK was most severe for 5Anx ventricle (Fig. 2G). For this tissue, 9 of the 11 strips examined ceased to contract during exposure to AAK (Figs. 2G and S1D). The two strips that did contract displayed an F_{max} that was barely distinguishable from noise, making it impossible to reliably assess MRP.

3.3. The effects of altered extracellular conditions and prolonged anoxic submergence on the rate constant of force restitution (τ) were tissue- and temperature-specific

At 21 °C, τ was greater ($P < 0.05$) in Control Anx extracellular conditions compared to Control Norm and AA saline solutions in the ventricle, but not the atria. Prior anoxia exposure at 21 °C increased ($P < 0.05$) τ by 149 ms in the left atrium, but not in the right atrium or ventricle (Fig. 2D–F).

At 5 °C, τ was greater ($P < 0.05$) in Control Anx extracellular conditions compared to Control Norm and AA saline solutions in both atria, but not in the ventricle. Prior anoxia exposure at 5 °C increased ($P < 0.05$) τ by 1166 ms in the ventricle, but not in the atria (Fig. 2J-L).

3.4. The negative effects of anoxia, acidosis, and hyperkalemia on F_{max}, T_{DC}, $Rate_{rise}$, and $Rate_{50\%\ relax}$ were more pronounced in ventricle than atria of 21Norm and 5Norm turtles

In 21Norm ventricle, exposure to AA from Control Norm solution decreased ($P < 0.05$) F_{max} by 52%, shortened ($P < 0.05$) T_{DC} by 14%, and slowed ($P < 0.05$) $Rate_{rise}$ and $Rate_{50\%relax}$ by 50% and 36%, respectively (Fig. 3A, D, G, and J). Subsequent exposure to AAK did not further affect F_{max}, T_{DC}, $Rate_{rise}$ or $Rate_{50\%relax}$. In comparison, 21Norm right atrium F_{max}, $Rate_{rise}$, $Rate_{50\%\ relax}$, and 21Norm left atrium $Rate_{50\%\ relax}$ were unchanged from Control Norm levels upon exposure to AA and subsequently AAK extracellular conditions (Fig. 3B, H, K, and L). Although, 21Norm left atrium F_{max} was reduced ($P < 0.05$) by 46% (Fig. 3C) and $Rate_{rise}$ slowed ($P < 0.05$) by 50% (Fig. 3I) compared to Control Norm during exposure to AAK.

In 5Norm ventricle, F_{max}, T_{DC}, $Rate_{rise}$, and $Rate_{50\%\ relax}$ were not affected by exposure to AA, indicating that cold acclimation preconditioned the tissue for anoxic and acidosis exposure (Fig. 4A, D, G, and J). However, subsequent exposure to AAK decreased ($P < 0.05$) F_{max} by 57%, prolonged ($P < 0.05$) T_{DC} by 41%, and slowed ($P < 0.05$) $Rate_{rise}$ and $Rate_{50\%relax}$ by 62% and 39%, respectively, from Control Norm levels (Fig. 4A, D, G, and J). In comparison, 5Norm right atrium F_{max} and $Rate_{50\%}$ relax (Fig. 4B and K), as well as 5Norm left atrium F_{max}, $Rate_{rise}$, and $Rate_{50\%\ relax}$ (Fig. 4C, I, and L) were unchanged from Control Norm levels by exposure to AA and AAK extracellular conditions. Although, 5Norm right and left atrial T_{DC} were prolonged in AAK compared to Control Norm (Fig. 4E and F).

3.5. Combined hypercalcemia and heightened adrenergic stimulation did not completely offset the negative effects of anoxia, acidosis, and hyperkalemia in 21Norm ventricle

Exposure of 21Norm ventricle to Control Anx saline, which combined anoxia, acidosis, hyperkalemia, hypercalcemia, and elevated adrenaline concentration, did not fully offset the decreased F_{max}, slower $Rate_{rise}$, and shortened T_{DC} measured in AA and AAK extracellular conditions (Fig. 3A, D, and G). Even though all three parameters increased ($P < 0.05$) from AAK extracellular conditions, they remained statistically significantly lower than when measured in Control Norm saline. Only $Rate_{50\%\ relax}$ returned to a level not statistically significantly different than in Control Norm (Fig. 3J). In comparison, in 21Norm right and left atria, F_{max}, T_{DC}, $Rate_{rise}$, and $Rate_{50\%\ relax}$ did not statistically significantly differ between Control Norm and Control Anx extracellular conditions (Fig. 4B, C, E, F, H, I, K, and L).

3.6. Prior anoxia exposure at 21 °C diminished the effects of combined anoxia and acidosis, as well as combined anoxia, acidosis, and hyperkalemia in ventricle, but had minimal effects on the contractile responses of right and left atria to altered extracellular conditions

In contrast to the decreased F_{max} and $Rate_{50\%\ relax}$ of 21Norm ventricle in AA and AAK extracellular conditions, F_{max} and $Rate_{50\%\ relax}$ of 21Anx ventricle were unaffected by exposure to AA and AAK extracellular conditions (Fig. 3A and J). In fact, 21Anx ventricle F_{max} and $Rate_{50\%\ relax}$ remained stable across all four extracellular conditions. 21Anx ventricle T_{DC} was also longer than T_{DC} of 21Norm ventricle in Control Anx, AAK, and AA saline solutions (Fig. 3F), whereas the magnitudes of change in 21Anx ventricle $Rate_{rise}$ in response to altered extracellular conditions was less than that of 21Norm ventricle (Fig. 3G). In comparison, the responses of 21Norm and 21Anx right and left atria to altered extracellular conditions were almost identically (Fig. 3B, C, E, F, H, I, K, and L).

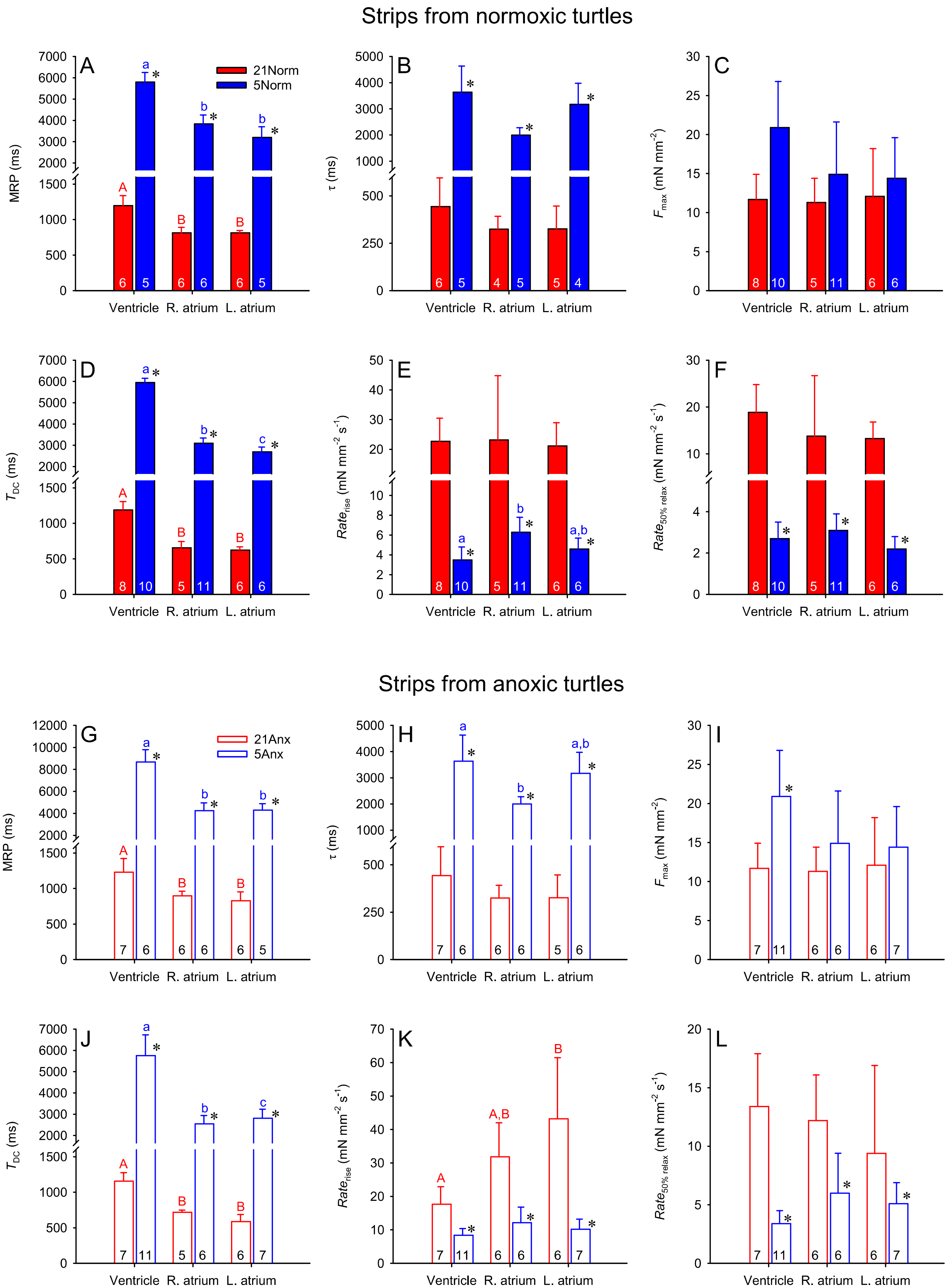

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Fig. 1. Comparison of the intrinsic contractile properties of isometrically-contracting ventricular, right atrial, and left atrial strips of (A–F) 21 °C- and 5 °C-acclimated, normoxia-exposed turtles (21Norm and 5Norm, respectively) at Baseline in Control Normoxia (Control Norm) extracellular conditions and (G–L) 21 °C- and 5 °C- acclimated, anoxia-exposed turtles (21Anx and 5Anx, respectively) at Baseline in Control Anoxia (Control Anx) extracellular conditions. MRP: mechanical refractory period; τ: time course of force restitution; F_{max}: maximal developed force; T_{DC}: duration of contraction; $Rate_{rise}$: average rate of contraction; $Rate_{50\% relax}$: average rate of relaxation. One-way ANOVAs, with Holm-Sidak multiple comparison *post hoc* tests were employed to assess statistically significant differences ($P < 0.05$) between cardiac chambers within an acclimation temperature. Significant differences between chambers of 21 °C-acclimated turtles are demarcated by dissimilar uppercase letters. Significant differences between chambers of 5 °C-acclimated turtles are demarcated by dissimilar lowercase letters. T-tests were employed to assess statistically significant differences ($P < 0.05$) between acclimation temperatures for each contractile parameter. Significant differences are demarcated by an asterisk by the 5 °C value. Values are means ± 95% CI. *n* values are presented at the base of each bar.

Table 2
Q_{10} values for intrinsic contractile parameters of *T. scripta* cardiac tissues.

Parameter	Ventricle	Right Atrium	Left Atrium
MRP	2.68	2.64	2.35
τ	3.83	3.80	4.20
F_{max}	1.22	0.88	1.15
T_{DC}	2.74	2.63	2.49
$Rate_{rise}$	3.22	2.26	2.60
$Rate_{50\% relax}$	3.37	2.54	3.08

Q_{10} values were calculated from 21Norm and 5Norm tissues at Baseline in Control Normoxia. MRP: mechanical refractory period; τ: rate constant of force restitution; F_{max}: maximal developed force; T_{DC}: duration of contraction; $Rate_{rise}$: average rate of contraction; $Rate_{50\% relax}$: average rate of relaxation.

3.7. Prior anoxia exposure at 5 °C enhanced the responsiveness of ventricle and atria to combined hypercalcemia and increased adrenaline concentration, but exacerbated the negative effects of combined anoxia, acidosis, and hyperkalemia in ventricle

In Control Anx saline solution, F_{max}, T_{DC}, and $Rate_{rise}$ of 5Anx ventricle were greater than that of 5Norm ventricle (Fig. 4A, D, and G). Similarly, F_{max}, T_{DC}, $Rate_{rise}$, and $Rate_{50\% relax}$ of 5Anx right and left atria were greater than that of 5Norm atria in Control Anx saline (Fig. 4B, C, E, F, H, I, K, and L). However, in the absence of combined hypercalcemia and increased adrenaline concentration, 5Anx ventricle was extremely susceptible to combined anoxia, acidosis, and hyperkalemia. As noted above, only 2 of 9 5Anx ventricle strips exhibited contractions in AAK saline solution (Fig. S1D). The two strips that contracted exhibited reductions ($P < 0.05$) in F_{max}, $Rate_{rise}$, and $Rate_{50\% relax}$ of 96%, 92%, and 92%, respectively, compared to in Control Norm (Fig. 4A, G, and J). Intrinsic contractile properties of 5Anx right and left atria were also reduced ($P < 0.05$) in AAK compared to Control Anx saline (Fig. 5 B, C, E, F, H, I, K, and L). However, F_{max}, T_{DC}, $Rate_{rise}$, and $Rate_{50\% relax}$ of 5Anx atria in AAK was equivalent to that of 5Norm atria exposed to AAK.

3.8. T. scripta cardiac strips did not display post-rest potentiation or post-rest decay

By and large, ventricle, right atrium, and left atrium F_{max} were stable following extended diastolic intervals across all exposure and extracellular conditions (Fig. 5). Exceptions were the post-rest decay ($P < 0.05$) exhibited by 21Norm ventricle exposed to Control Norm extracellular conditions at the longest diastolic interval of 180 s (Fig. 5A), the post-rest decay ($P < 0.05$) displayed by 21Anx ventricle in Control Norm and AA saline solutions (Fig. 5A and B), and the post-rest potentiation ($P < 0.05$) displayed by 21Norm ventricle and 21Anx left atrium during exposure to Control Anx (Fig. 5D and L).

Of note, post-rest F_{max} of 5Anx strips exposed to AA and Control Norm was maximal at diastolic intervals corresponding to the range of *in vivo* heart rate displayed by cold, anoxic turtles (i.e., 45–90 s), and decreased at shorter stimulation intervals (Fig. 5M, N, Q, R, U, and V). This finding means that the F_{max} values reported for 5Anx tissues in AA and Control Norm solutions (Fig. 5A–C), for which the steady-state pacing rate was set to the *in vivo* normoxic rate, are less than what they would have been if pacing rate was maintained at the slower *in vivo* anoxic rate. Consequently, the effects of AAK and Control Anx relative to Control Norm, as well as comparison between 5Anx and 5Norm tissues in Control Norm and AA are underestimated.

4. Discussion

4.1. Extracellular hyperkalemia, in combination with anoxia and acidosis, disrupts ventricular contraction of cold, anoxic turtles, but the negative effect is alleviated by combined hypercalcemia and heightened adrenergic stimulation

Our primary research objective was to determine if and under what conditions the ventricle may be the weak link that limits contractile frequency *in vivo* during anoxia submergence. In this regard, our most relevant discovery is that combined anoxia, acidosis, and hyperkalemia arrested ventricular contraction of 5Anx turtles and could limit cardiac pumping rate *in vivo* during prolonged anoxic submergence at cold acclimation temperature. By comparison, under comparable extracellular conditions, the right and left atrium continued to contract, and the cardiac pacemaker continues to fire (Stecyk and Farrell, 2007). The finding is consistent with a report from 70 years ago that at warm temperature (25 °C) and in normoxia, the mechanical and electrical activity of the turtle (*Chelydra serpentina*) ventricle is less tolerant to extracellular hyperkalemia than the atria and sinus venosus (Butcher et al., 1952). The present finding is also in agreement with the strong negative inotropy and irregular contractions induced by hyperkalemia in ventricular strips of cold-acclimated *C. picta belli* (Overgaard et al., 2005). Indeed, hyperkalemia reduces cardiomyocyte resting membrane potential (Nielsen and Gesser, 2001), which in mammals decreases the opening probability of voltage-gated fast Na^+ channels, thereby decreasing cardiac excitability and slowing cardiac conduction (Weiss et al., 2017). Hyperkalemia also shortens cardiac action potential duration and disrupts transarcolemmal Ca^{2+} influx and efflux, leading to decreased inotropy (Bouchard et al., 2004; Nielsen and Gesser, 2001). Notably, in fish exposed to acute warming, reduced ventricular excitability causes atrioventricular block and ventricular bradycardia (Haverinen and Vornanen, 2020). The temperature induced atrioventricular block arises from a mismatch of the background inward rectifier K^+ (I_{K1}) and voltage-gated fast Na^+ (I_{Na}) currents, which increases the excitation threshold of the ventricle (Vornanen, 2020). Future electrophysiological studies are required to confirm if an analogous mechanism is at play in the cold-acclimated, anoxic, acidotic, and hyperkalemic turtle heart. Indirect evidence that a similar mechanism may be at play stems from the finding that the negative effects of hyperkalemia on the normoxic turtle heart are antagonized by an increase in extracellular Na^+ concentration (Butcher et al., 1952).

Combined anoxia, acidosis, and hyperkalemia also negatively affected the intrinsic contractile properties of 21Norm, 21Anx and 5Norm cardiac chambers. Indeed, decreases in ventricular force to almost zero under high concentration (12.5 $mmol^{-1}$) of K^+ have previously been reported for warm-acclimated (25 °C) *T. scripta* ventricle (Nielsen and Gesser, 2001). Whereas the lower level of hyperkalemia (7 $mmol^{-1}$) employed in conjunction with anoxia and acidosis in our study did not abolish F_{max}, it prominently prolonged MRP, indicating disruption of cardiac excitation-contraction coupling or cardiomyocyte excitation (Wohlfart and Noble, 1982). Nevertheless, unlike for 5Anx

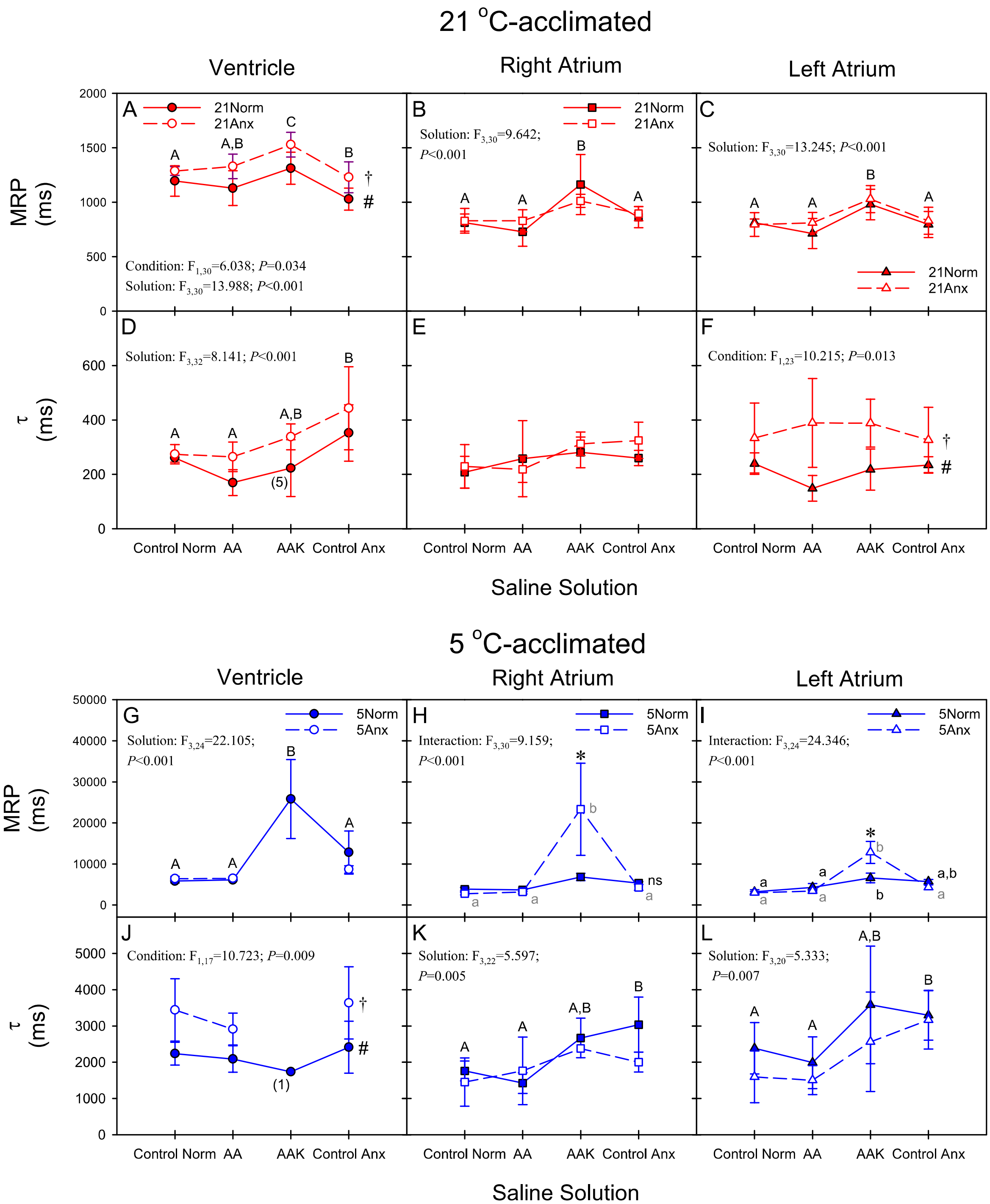

Fig. 2. Comparison of the effects of exposure to various extracellular conditions on (A-C and G-I) MRP: mechanical refractory period and (D-F and J-L) the time course of force restitution (τ) of isometrically-contracting ventricular, right-atrial, and left-atrial strips from (A–F) 21 °C-acclimated, normoxia-exposed (21Norm) and anoxia-exposed (21Anx) turtles and (G–L) 5 °C-acclimated, normoxia-exposed (5Norm) and anoxia-exposed (5Anx) turtles. Saline solution exposure order was from left-to-right for tissues from 21Norm and 5Norm animals and from right-to-left for tissues from 21Anx and 5Anx animals. Control Norm: Control normoxia; AA: combined anoxia and acidosis; AAK: combined anoxia, acidosis, and hyperkalemia; Control Anx: Control anoxia (see Table 1). 2-way repeated-measures ANOVAs, with Holm-Sidak multiple comparison *post hoc* tests, were employed to assess statistically significant differences ($P < 0.05$) among exposure groups and saline solutions for each contractile variable. Statistically significant main effects or a statistically significant interaction are detailed in each panel, inclusive of the F ratio, degrees of freedom, and *P* value. Dissimilar uppercase letters demarcate a main effect of and differences ($P < 0.05$) among saline solutions. Dissimilar symbols († and #) indicate a main effect of and a difference ($P < 0.05$) between exposure groups. A significant interaction ($P < 0.05$) between saline solution and exposure group is demarcated with asterisks and dissimilar lowercase letters. An asterisk indicates a significant difference ($P < 0.05$) between exposure groups within a saline solution. Dissimilar lowercase letters signify significant differences ($P < 0.05$) between saline solutions within an exposure group (black font for 21Norm and 5Norm; grey font for 21Anx and 5Anx). ns = no statistically significant differences among saline solutions. Values are means ± 95% CI. *n* values are presented in Fig. 1 unless noted in parenthesis to the left of a data point (black font for 21Norm and 5Norm; grey font for 21Anx and 5Anx).

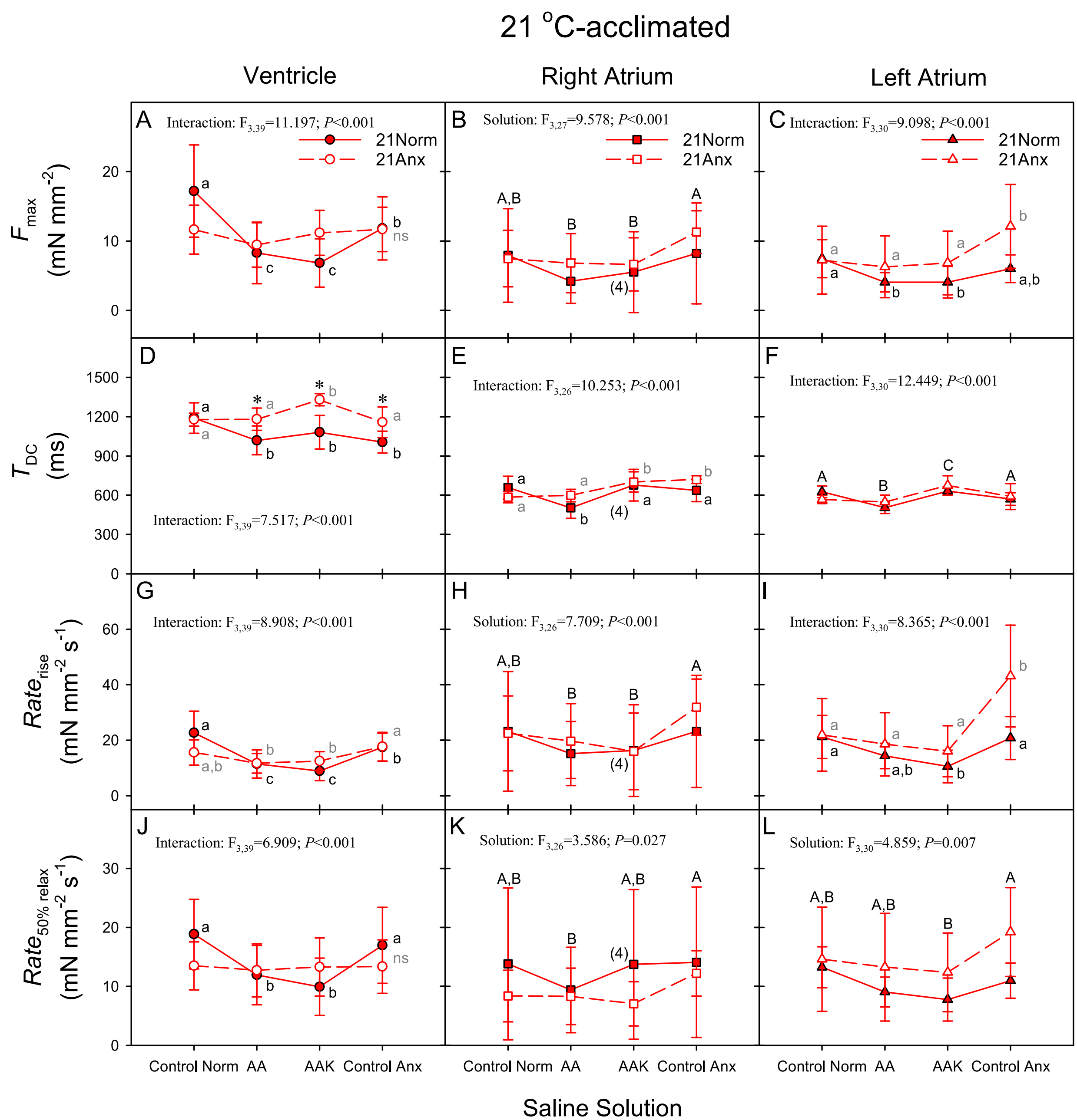

Fig. 3. Comparison of the effects of exposure to various extracellular conditions on (A–C) F_{max}: maximal developed force, (D–F) T_{DC}: duration of contraction, (G–I) $Rate_{rise}$: average rate of contraction, and (J–L) $Rate_{50\%\ relax}$: average rate of relaxation of isometrically-contracting ventricular, right-atrial, and left-atrial strips from 21 °C-acclimated, normoxia-exposed (21Norm) and anoxia-exposed (21Anx) turtles. Saline solution exposure order was from left-to-right for 21Norm tissues and from right-to-left for 21Anx tissues. Control Norm: Control normoxia; AA: combined anoxia and acidosis; AAK: combined anoxia, acidosis, and hyperkalemia; Control Anx: Control anoxia (see Table 1). 2-way repeated-measures ANOVAs, with Holm-Sidak multiple comparison *post hoc* tests, were employed to assess statistically significant differences ($P < 0.05$) among exposure groups and saline solutions for each contractile variable. Statistically significant main effects or a statistically significant interaction are detailed in each panel, inclusive of the F ratio, degrees of freedom, and *P* value. Dissimilar uppercase letters demarcate a main effect of and differences ($P < 0.05$) among saline solutions. Dissimilar symbols († and #) indicate a main effect of and a difference ($P < 0.05$) between exposure groups. A significant interaction ($P < 0.05$) between saline solution and exposure group is demarcated with asterisks and dissimilar lowercase letters. An asterisk indicates a significant difference ($P < 0.05$) between exposure groups within a saline solution. Dissimilar lowercase letters signify significant differences ($P < 0.05$) between saline solutions within an exposure group (black font for 21Norm; grey font for 21Anx). ns = no statistically significant differences among saline solutions. Values are means ± 95% CI. *n* values are presented in Fig. 1 unless noted in parenthesis to the left of a data point (black font for 21Norm; grey font for 21Anx).

ventricle, the maximum contraction rate in combined anoxia, acidosis, and hyperkalemia for 21Norm, 21Anx, and 5Norm ventricle, right atrium, and left atrium, calculated from MRP, was considerably faster than the *in vivo* heart rate exhibited by 21 °C- and 5 °C-acclimated anoxic turtles (Fig. 6). Thus, the combined effects of extracellular anoxia, acidosis, and hyperkalemia, at least at the levels employed in the present study, does not appear to be a factor limiting cardiac pumping rate in warm-acclimated, anoxic turtles. Nevertheless, a caveat is that the magnitude of hypercapnic acidosis utilized for the 21 °C experiments was less than occurs *in vivo* with prolonged anoxia exposure (Warren and Jackson, 2007). Consequently, intracellular pH may have been artificially higher in the tissue strips than in cardiomyocytes *in vivo*, which

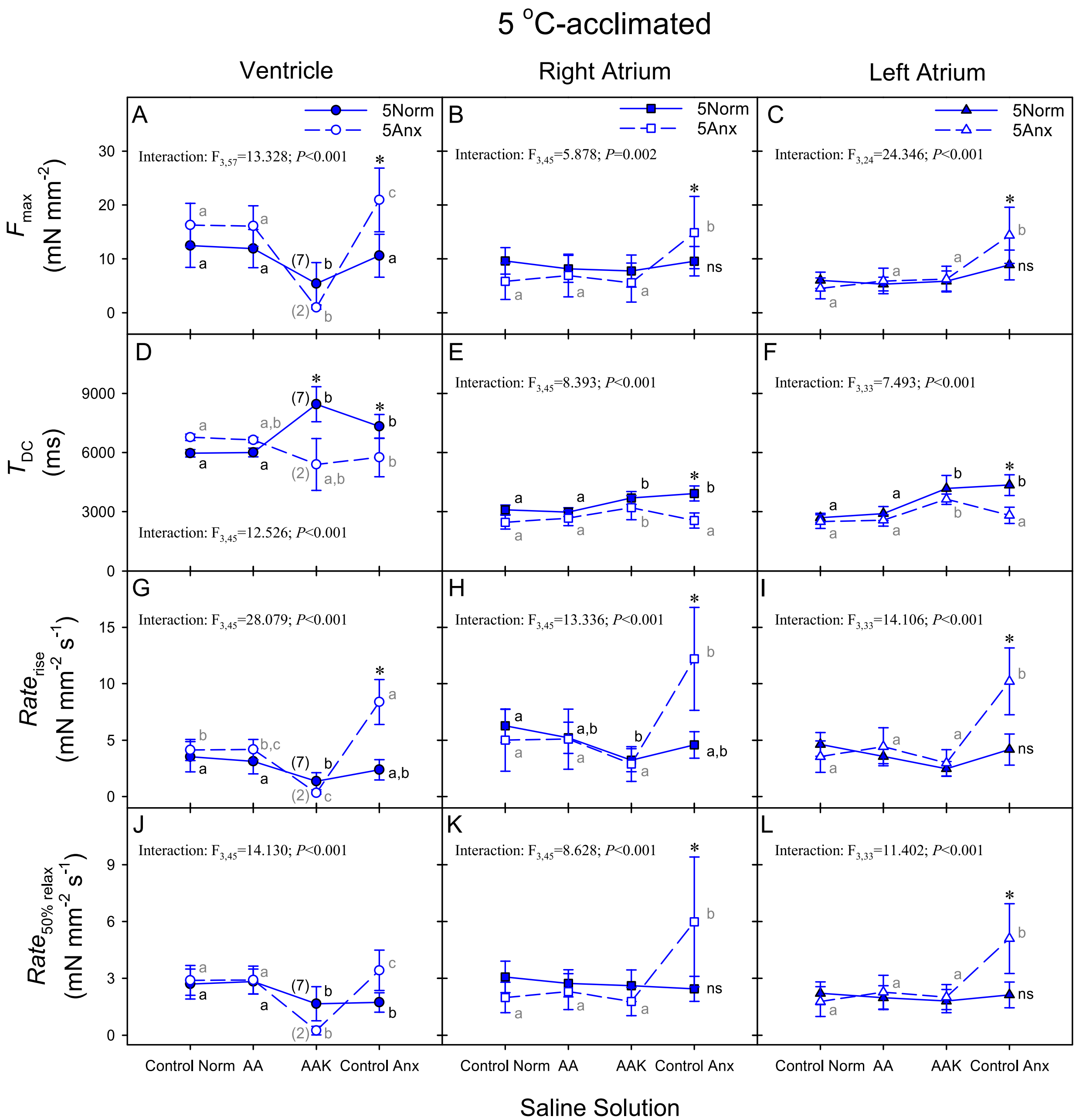

Fig. 4. Comparison of the effects of exposure to various extracellular conditions on (A–C) F_{max}: maximal developed force, (D–F) T_{DC}: duration of contraction, (G–I) $Rate_{rise}$: average rate of contraction, and (J–L) $Rate_{50\%\ relax}$: average rate of relaxation of isometrically-contracting ventricular, right-atrial, and left-atrial strips from 5 °C-acclimated, normoxia-exposed (5Norm) and anoxia-exposed (5Anx) turtles. Saline solution exposure order was from left-to-right for 5Norm tissues and from right-to-left for 5Anx tissues. Control Norm: Control Norm: Control normoxia; AA: combined anoxia and acidosis; AAK: combined anoxia, acidosis, and hyperkalemia; Control Anx: Control anoxia (see Table 1). 2-way repeated-measures ANOVAs, with Holm-Sidak multiple comparison *post hoc* tests, were employed to assess statistically significant differences ($P < 0.05$) among exposure groups and saline solutions for each contractile variable. Statistically significant interactions are detailed in each panel, inclusive of the F ratio, degrees of freedom, and P value. An asterisk indicates a significant difference ($P < 0.05$) between exposure groups within a saline solution. Dissimilar lowercase letters signify significant differences ($P < 0.05$) between saline solutions within an exposure group (black font for 5Norm; grey font for 5Anx). ns = no statistically significant differences among saline solutions. Values are means ± 95% CI. n values are presented in Fig. 1 unless noted in parenthesis to the lower left of a data point (black font for 5Norm; grey font for 5Anx).

could mean that the effect of combined anoxia, acidosis, and hyperkalemia at 21 °C was underestimated.

In all cardiac tissue types examined, regardless of experimental exposure condition and including 5Anx ventricle, the negative effect of combined anoxia, acidosis, and hyperkalemia on MRP occurred only in the absence of combined hypercalcemia and heightened adrenergic stimulation. Indeed, hypercalcemia and adrenaline are integral for permitting continued cardiac performance in the anoxic turtle (Hicks and Farrell, 2000b; Jackson, 1987, 2000; Nielsen and Gesser, 2001; Overgaard et al., 2005, 2007; Yee and Jackson, 1984) via the enhancement of Ca^{2+} influx through sarcolemmal L-type Ca^{2+} channels (Frace et al., 1993; Reuter, 1983) and myofilament Ca^{2+} sensitivity (Fanter et al., 2017). Prolongation of action potential plateau duration by adrenergic stimulation via inhibition of delayed rectified K^+ (I_{Kr}) current, as occurs in the rainbow trout (*Oncorhynchus mykiss*) heart (Abramochkin et al., 2022), could also offset the shortening of action

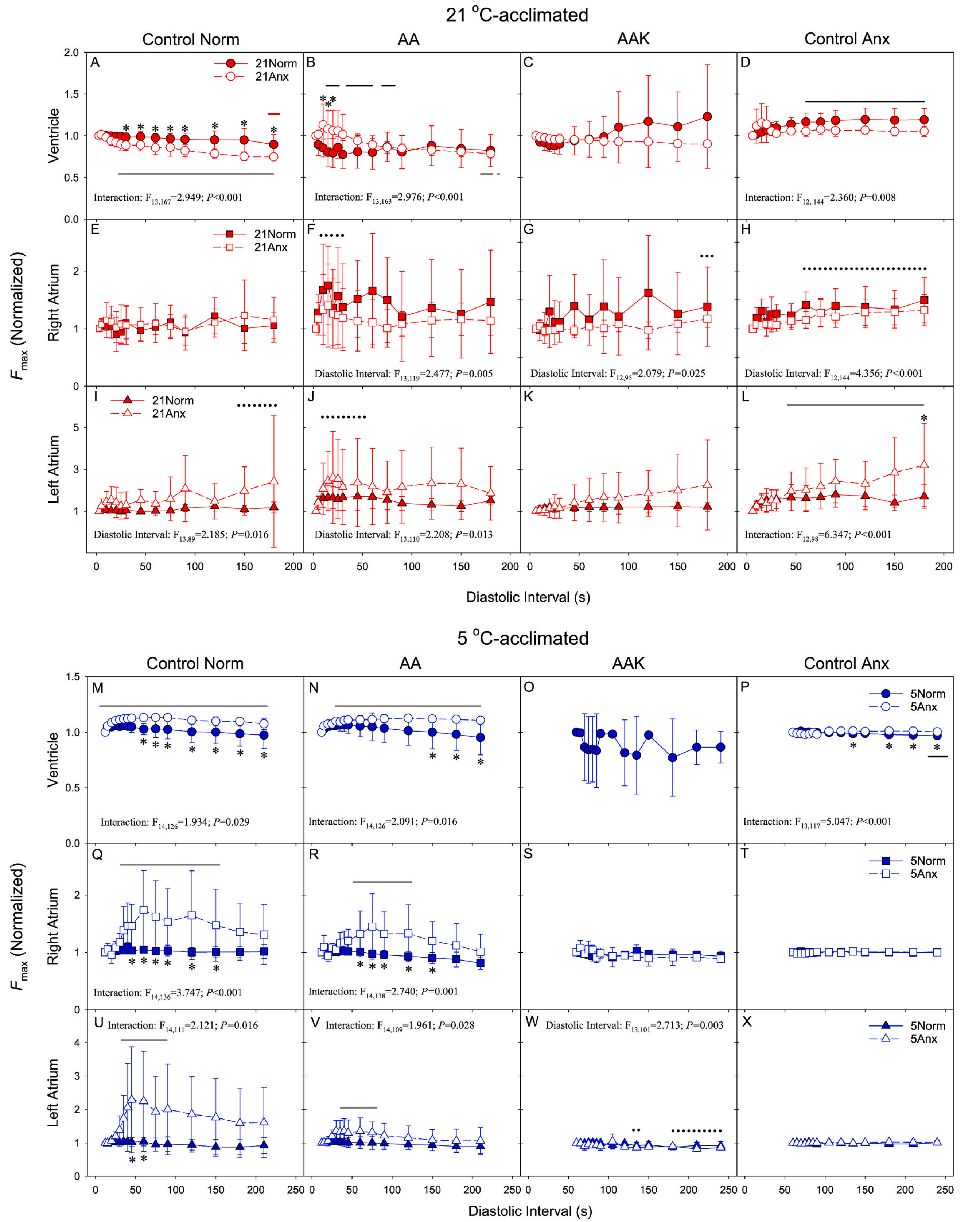

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Fig. 5. Comparison of the effects of exposure to various extracellular conditions on the post-rest potentiation of isometrically-contracting ventricular, right-atrial, and left-atrial strips from (A–L) 21 °C normoxia-exposed (21Norm) and anoxia-exposed (21Anx) turtles and (M–X) 5 °C normoxia-exposed (5Norm) and anoxia-exposed (5Anx) turtles. Saline solution exposure order was from left-to-right for tissues from 21Norm and 5Norm animals and from right-to-left for tissues from 21Anx and 5Anx animals. Control Norm: Control normoxia; AA: combined anoxia and acidosis; AAK: combined anoxia, acidosis, and hyperkalemia; Control Anx: Control anoxia (see Table 1). 2-way repeated-measures ANOVAs, with Holm-Sidak multiple comparison *post hoc* tests, were employed to assess statistically significant differences ($P < 0.05$) between normoxia- and anoxia-exposed tissues, as well between post-rest contractions and the preceding steady-state contraction within each exposure group. Statistically significant main effects or a statistically significant interaction are detailed in the panels, inclusive of the F ratio, degrees of freedom, and P value. A dotted line indicates a main effect of diastolic interval ($P < 0.05$) and demarcates the post-rest contractions with a different ($P < 0.05$) F_{max} than that of the preceding steady-state contraction. A significant interaction ($P < 0.05$) between the effect of diastolic interval on post-rest F_{max} and exposure group is demarcated with asterisks and solid lines. An asterisk indicates a significant difference ($P < 0.05$) between exposure groups at a given diastolic interval. A solid line demarcates the diastolic intervals at which the post-rest F_{max} differed ($P < 0.05$) from the preceding, control steady state F_{max} (black line for 21Norm and 5Norm; grey line for 21Anx and 5Anx). Values are normalized to the steady-state contraction and are means ± 95% CI. n values are presented in Figs. 1–4.

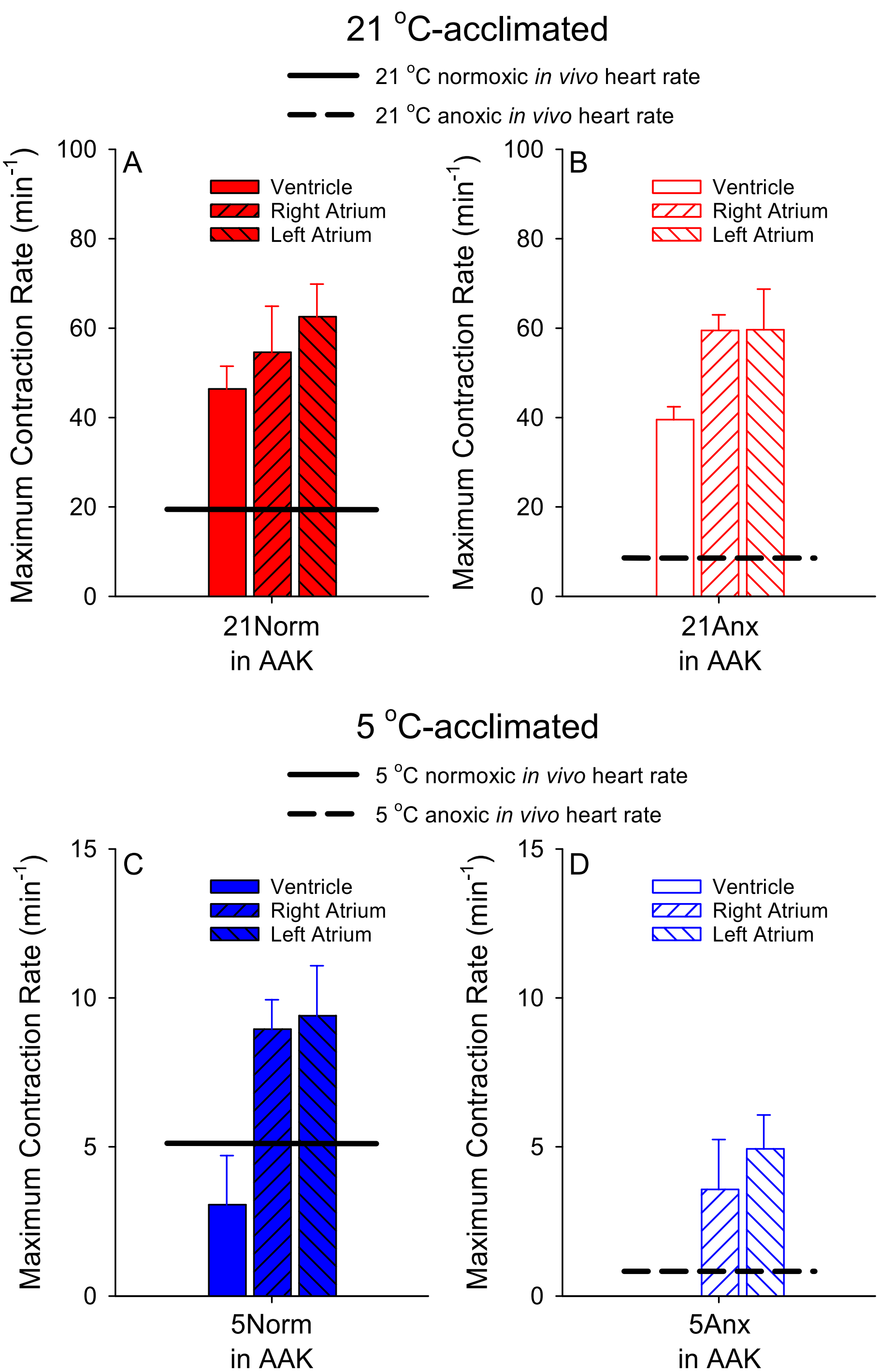

Fig. 6. Theoretical maximum contraction rate of isometrically-contracting ventricular, right-atrial, and left-atrial strips from (A) 21 °C-acclimated, normoxia-exposed (21Norm), (B) 21 °C-acclimated, anoxia-exposed (21Anx), (C) 5 °C- acclimated, normoxia-exposed (5Norm), and (D) 5 °C- acclimated, anoxia-exposed (5Anx) turtles exposed to combined anoxia, acidosis, and hyperkalemia (AAK) extracellular conditions (see Table 1). Maximum contraction rates were calculated from mechanical refractory period (MRP). In vivo heart rate of normoxic and anoxic turtles at each acclimation temperature are indicated by the solid and dashed horizontal lines, respectively, and are from Stecyk et al. (2004) (Stecyk et al., 2004). n values are presented in Fig. 1.

potential duration induced by hyperkalemia, leading to increased transarcolemmal Ca^{2+} influx and cardiac inotropy. Thus, ventricular bradycardia in cold-acclimated anoxic turtles may only occur if extracellular hypercalcemia and/or adrenergic stimulation is insufficient to overcome the negative effects of extracellular hyperkalemia in combination with anoxia and acidosis. This postulation is supported by the findings of the accompanying study (Garner et al., 2022). *In vitro* experiments with spontaneously contracting right atrium with electrically coupled ventricle strip preparations highlighted the importance of hypercalcemia and adrenergic stimulation in counteracting atrioventricular block. Moreover, *in vivo* electrocardiogram recordings revealed the heart of 5 °C-acclimated *T. scripta* to be resilient to cardiac arrhythmia during prolonged anoxia exposure when circulating levels of Ca^{2+} (Herbert and Jackson, 1985b; Jackson and Heisler, 1982) and adrenaline are elevated (Keiver and Hochachka, 1991; Keiver et al., 1992; Wasser and Jackson, 1991).

4.2. Turtle cardiac contractile force is resilient to prolonged diastolic intervals

Our study provides novel evidence that the anoxic turtle heart is protected from force degradation with extended diastole, either intrinsically at cold acclimation temperature, or by combined hypercalcemia and heightened adrenergic stimulation at warm acclimation temperature. Except for the minor post-rest decay exhibited by 21Norm ventricle in Control Norm extracellular conditions, a finding in-line with previous study (Galli et al., 2006a), ventricular and atrial F_{max} were stable following extended diastolic intervals across all exposure and extracellular conditions. Consequently, the turtle heart should be able to maintain contractile force should atrioventricular block and/or cardiac arrhythmia occur during prolonged anoxia exposure.

The lack of post-rest decay displayed by turtle cardiac tissues is consistent with the minimal contribution of sarcoplasmic reticulum (SR) Ca^{2+} to cardiac contraction in warm-acclimated, normoxic *T. scripta* (Galli et al., 2006a, 2006b). By comparison, post-rest decay of cardiac contractility in the rabbit heart, which relies heavily on SR Ca^{2+} for contraction, is attributed to the leak of Ca^{2+} from sarcoplasmic reticulum stores and its extrusion from the cardiomyocyte by the Na^{+}-Ca^{2+}-exchanger (NCX) (Sutko et al., 1986). However, the post-rest potentiation displayed by 21Norm and 21Anx tissues in Control Anx extracellular conditions suggests that adrenergic stimulation may enhance role of the SR in turtle cardiac contraction under these conditions. Indeed, in some mammalian hearts, post-rest potentiation is attributed to a larger fractional release of Ca^{2+} from the SR (Bouchard and Bose, 1989; Lewartowski and Zdanowski, 1990), which can be modulated by adrenergic stimulation (Valdivia, 2014). Likewise, β-adrenergic stimulation triggers SR Ca^{2+} release in rainbow trout cardiomyocytes under stressful conditions, leading to increased contractility (Cros et al., 2014). Investigation into whether the apportionment of transarcolemmal and SR Ca^{2+} reported for ventricular contraction of warm-acclimated, normoxic turtles (Galli et al., 2006b) is altered with anoxia exposure at warm and cold temperature, cold acclimation in normoxia, differs between *T. scripta* cardiac chambers as occurs in other ectothermic vertebrates (Shiels and Galli, 2014), or is modulated by adrenergic stimulation would be informative avenues of future research.

4.3. Prior temperature and anoxia experiences determine the intrinsic contractile response of the turtle myocardium to altered extracellular conditions

The preconditioning of the 21 °C turtle ventricle by prior anoxia exposure to tolerate combined anoxia and acidosis is consistent with previous findings for *T. scripta* pacemaker (Stecyk and Farrell, 2007) and ventricle (Stecyk et al., 2021). Combined, the past and present results suggest that prolonged anoxia exposure induces cellular modifications that counteract the negative inotropic and chronotropic effects of anoxia *per se* and acidosis. Possibilities worthy of future investigation include adjustments to intracellular Ca^{2+} storage and release (Gesser and Jørgensen, 1982; Jackson, 1987), mechanisms of intracellular pH regulation (Shi et al., 1997), and myofilament Ca^{2+} sensitivity (Ruhr et al., 2019).

The pronounced effect of combined hypercalcemia and heightened adrenergic stimulation on F_{max} and contraction kinetics in strips from 5 °C-acclimated turtles that had been exposed to anoxia was surprising and contrary to our prediction. The disparate results between the present finding and the prior reports of reduced ventricular cell surface β-adrenoreceptor density in *T. scripta* exposed to anoxia exposure at warm (22 °C) and cold (5 °C) temperatures (Hicks and Farrell, 2000b) and blunted cardiovascular responses to exogenous adrenaline in live, anoxic *T. scripta* (Hicks and Farrell, 2000b; Hicks and Wang, 1998; Stecyk et al., 2004) could reflect that the exogenous adrenaline was acting on already high concentrations of circulating catecholamines (Keiver and Hochachka, 1991; Keiver et al., 1992; Wasser and Jackson, 1991). The differential results could also reflect that the ventricular strips utilized in the present study were obtained from the dorsal side of the heart, which exhibits a stronger response to adrenaline as compared to strips from the ventral side of the heart (Ball and Hicks, 1996). Additionally, the discrepancy could reflect a positive and counteracting effect of hypercalcemia, not adrenaline, on intrinsic contractile properties. Indeed, the negative chronotropic effect of combined anoxia, acidosis, and hyperkalemia on spontaneously contracting right-atrial preparations from 5 °C-acclimated normoxic turtles is completely counteracted by hypercalcemia, whereas subsequent heightened adrenergic stimulation has no further positive chronotropic effect (Garner et al., 2022; Stecyk and Farrell, 2007). On the other hand, increased extracellular Ca^{2+} does not induce positive inotropy in ventricular strips of cold-acclimated normoxic or anoxic turtles at 5 °C (Overgaard et al., 2005). Unfortunately, the simultaneous manipulation of extracellular Ca^{2+} and adrenaline concentrations in the present study, which was necessitated to ensure tissue integrity for the duration of the already lengthy (~7 h) experimental protocol, precludes resolution of the individual and mechanistic effects of hypercalcemia and heightened adrenergic stimulation. Nevertheless, the present results are consistent with the finding that cold-acclimated anoxic turtles injected with nadolol, a β-adrenergic antagonist, exhibited cardiac arrhythmias, indicating that adrenergic stimulation is vital for continued cardiac activity in the cold, anoxic turtle (Hicks and Farrell, 2000b).

4.4. Additional evidence that cold acclimation primes the turtle heart for winter anoxia

Cold acclimation in normoxia is crucial for priming physiological processes of the anoxia-tolerant turtle for winter anoxia exposure (Hochachka, 1986; Jackson, 2000). Cold acclimation induces whole-body metabolic depression (Jackson and Ultsch, 2010; Ultsch, 1985) and brain function remodeling (Couturier et al., 2019; Hogg et al., 2014; Lari and Buck, 2021). At the level of the heart, reduced cardiac function (Hicks and Farrell, 2000a; Stecyk and Farrell, 2007; Stecyk et al., 2007, 2008, 2021) with cold acclimation in normoxia is accompanied by substantial reduction in the density of sarcolemmal ion currents (Stecyk et al., 2007), prolongation of action potential duration (Stecyk et al., 2007), downregulation of transarcolemmal Ca^{2+} flux (Stecyk et al., 2021), modification of cardiac gene expression (Melleby et al., 2020; Sparks et al., 2022; Stecyk et al., 2012), and reduced density of ventricular Na^{+}/K^{+}-ATPase (Overgaard et al., 2005). The present study adds to this body of knowledge by revealing that τ lengthened with cold acclimation with Q_{10} values greater than 3. The decrease in the rate of force restitution signifies that the rate of cardiac contractile recovery from the inactivation of the molecular mechanisms that initiate contraction is slowed in *T. scripta* heart at cold acclimation temperature. Expectantly, the finding mirrors the prolongation of τ in the heart of the anoxia-tolerant crucian carp (*Carassius carassius*) with cold acclimation

(Tiitu and Vornanen, 2001), for which cold acclimation is also integral for prolonging anoxic survival (Vornanen et al., 2009), but contrasts the increased rate of force restitution displayed by the cold-active and anoxia-sensitive rainbow trout heart with cold acclimation (Aho and Vornanen, 1999). The further prolongation of τ in ventricle of 5 °C-acclimated, anoxic *T. scripta* aligns with the marked depression of cardiac performance that occurs with prolonged anoxia exposure at 5 °C (Stecyk et al., 2008) and suggests an anoxia-induced slowing of ventricular ion channel gating transitions beyond that induced by acclimation to cold temperature in normoxia.

4.5. Concluding remarks

The present study is the first to comprehensively explore the effects of acclimation temperature, prolonged anoxia exposure, and extracellular conditions on mechanical restitution, and post-rest potentiation of the cardiac myocardium of the anoxia-tolerant freshwater turtle. It is also the first to factor in the effects of prior anoxia exposure at both warm and cold temperature, as well as acclimation to cold temperature in normoxia, on the effects of extracellular changes on the intrinsic contractile properties of turtle right and left atrium. Overall, our findings show that although combined extracellular anoxia, acidosis, and hyperkalemia induces negative effects in all *T. scripta* cardiac chambers, they are less pronounced in the atria than the ventricle. Our findings also reveal that disruption of ventricular contraction by the combined negative effects of anoxia, acidosis, and hyperkalemia could limit cardiac pumping rate *in vivo* during prolonged anoxic submergence at 5 °C. However, our results also reinforce that combined extracellular hypercalcemia and heightened adrenergic stimulation are important for counteracting the negative effects of combined anoxia, acidosis and hyperkalemia on the excitability and contractility of ventricle of cold-acclimated, anoxic turtles.

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CRediT authorship contribution statement

Molly Garner: Investigation, Formal analysis, Writing – original draft. **Jonathan A.W. Stecyk:** Conceptualization, Formal analysis, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphys.2022.07.001.

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