Effect of temperature and prolonged anoxia exposure on electrophysiological properties of the turtle (*Trachemys scripta*) heart

Jonathan A. W. Stecyk1*, Vesa Paajanen2, Anthony P. Farrell3
& Matti Vornanen2

1Department of Zoology, University of British Columbia, Vancouver, BC, Canada, V6T 1Z4

2Department of Biology, University of Joensuu, Joensuu, Finland
   PO Box 11, 80101

3Faculty of Land and Food Systems and Department of Zoology, University of British Columbia, Vancouver, BC, Canada, V6T 1Z4

*Author for correspondence (e-mail jonathan.stecyk@imbv.uio.no)

Short title: Turtle cardiac electrophysiology
Abstract

Cardiac activity of the turtle (Trachemys scripta) is greatly depressed with cold acclimation and anoxia. We examined what electrophysiological modifications accompany and perhaps facilitate this depression of cardiac activity. Turtles were first acclimated to 21°C or 5°C and held under either normoxic or anoxic (6 h at 21°C; 14 d at 5°C) conditions. Then, we measured cardiac action potentials (APs) using spontaneously contracting whole-heart preparations and whole-cell current densities of sarcolemmal ion channels using isolated ventricular myocytes under appropriate normoxic and anoxic conditions. Compared with 21°C-acclimated turtles, 5°C-acclimated turtles exhibited a less negative resting membrane potential (by 18-29 mV), a 4.7- to 6.8-fold slower AP upstroke rate and a 4.2- to 4.9-fold greater AP duration (APD). Correspondingly, peak density of ventricular voltage-gated Na⁺ ($I_{Na}$) and L-type Ca²⁺ currents and inward slope conductance of inward rectifier K⁺ ($I_{K1}$) channel current were approximately 1/7th ($Q_{10} = 3.4$), 1/13th ($Q_{10} = 5.0$), and half ($Q_{10} = 1.4$) those of 21°C-acclimated ventricular myocytes, respectively. With anoxia at 21°C, peak $I_{Na}$ density doubled and ventricular APD increased by 47%; a change proportional to the reported ~30% reduction of intrinsic heart rate. In contrast, with anoxia at 5°C, ventricular AP characteristics were unaffected, and of the ion currents investigated, only the inward conductance via $I_{K1}$ changed significantly (reduced by 46%). The present findings indicate that cold temperature, more so than prolonged anoxia, results in substantial modifications of cardiac APs and reduction of ventricular ion current densities. These changes likely prepare cardiac muscle for winter anoxia conditions.

Key words: action potential, anoxia, $I_{Ca}$, $I_{K1}$, $I_{Kr}$, $I_{Na}$, K⁺ channel, L-type Ca²⁺ channel, Na⁺ channel, red-eared slider turtle, temperature, thermal acclimation, Trachemys scripta
Introduction

Unlike the vast majority of vertebrate species, the red-eared slider freshwater turtle *Trachemys scripta* is extremely anoxia-tolerant. At warm acclimation temperatures (20°C - 25°C) this animal can survive 12 h – 24 h of anoxic submergence, but when acclimated to 3°C - 5°C, anoxia survival time is extended to approximately 45 d (62, 69). During prolonged anoxia exposure at both warm and cold acclimation temperatures, the heart of the turtle continues its role in internal convection, but without oxygen and at a massively reduced rate. Specifically, it is a profound anoxic bradycardia that largely reduces systemic cardiac and power outputs by 4.5- to 20-fold following 6 h and 14 – 21 d anoxic exposures in warm- and cold-acclimated turtles, respectively (18, 19, 20, 21, 57, 59). Heart rate ($f_H$) in turtles decreases from ~25 min$^{-1}$ to ~10 min$^{-1}$ after just 1 h of anoxia at 21°C - 25°C and from a normoxic rate of ~5 min$^{-1}$ to less than 1 min$^{-1}$ after 24 h of anoxia at 5°C. These reductions in cardiac activity during anoxia reflect and match the reduction of whole-animal metabolic rate and demand for blood flow (18, 24), and also serves as a strategy to ensure that cardiac ATP demand falls well below the cardiac glycolytic capacity to supply ATP (9, 19).

The mechanisms underlying the reduction of cardiac activity during prolonged anoxia are not fully understood. In warm-acclimated turtles, cholinergic cardiac inhibition contributes to ~36-48% of the anoxic bradycardia (20, 21), but α-adrenergic (58) and adenosinergic (59) cardiac inhibitory mechanisms do not. However, in cold-acclimated turtles, autonomic cardiovascular control is blunted during anoxia and does not account for the anoxic bradycardia (20, 57). Similarly, adenosinergic cardiac inhibition is not involved either (59). Instead, the suggestion has been made that intrinsic
electrophysiological changes account for the anoxic depression of cardiac activity in cold-acclimated turtles (20, 41, 56). Certainly, an increased prevalence of atrial-ventricular blocks, a phenomenon in which ventricular contraction rate is less than the atrial contraction rate, in isolated turtle hearts during anoxia exposure (25) suggests a reduced ventricular excitability and/or a delay or blockage of electrical impulses through the atrial-ventricular node. Also, intrinsic electrophysiological modifications are likely involved in the profound depression of cardiac activity with cold-acclimation since cholinergic cardiac inhibition is known to be suppressed (20) and rates of contraction and relaxation are decreased (41).

As a first step to understanding these potential electrophysiological modifications with both cold acclimation and prolonged anoxia exposure, the present study examined hearts from the red-eared slider turtle before and after cold acclimation and before and after prolonged anoxia exposure. Specifically, measurements were made of cardiac action potentials (APs) and current densities of four ventricular sarcolemmal ion channels involved in generating cardiac APs, namely the voltage-gated Na⁺ (I_{Na}), L-type Ca²⁺ (I_{Ca}), inward rectifier K⁺ (I_{K1}) and delayed rectifier K⁺ (I_{Kr}) channels (46). Recordings were acquired from four acclimation groups of turtles under conditions similar to those experienced by the turtle. The four acclimation groups were: 1) 21°C-acclimated, normoxic; 2) 21°C-acclimated, 6 h anoxia exposure; 3) 5°C-acclimated, normoxic; and 4) 5°C-acclimated, 14 d anoxia exposure. This experimental design allowed comparisons for the effects of temperature alone in normoxic turtles and the effects of temperature alone in anoxic turtles, as well as the effects of anoxia with and across the two temperature acclimation groups. In addition to measuring electrophysiological
characteristics under conditions similar to those to which the turtle itself had been acclimated and exposed to, some electrophysiological recordings were also made following an acute change in either the experimental temperature or the perfusate pH. Cardiac APs and ventricular sarcolemmal ion channel currents were re-recorded following an acute temperature change in both 21°C- and 5°C-acclimated, normoxic hearts. This acute procedure was done to explicitly distinguish the effects of cold-acclimation from acute, and perhaps direct effects of ambient temperature on the rate of these physiological processes (termed here direct temperature effects). Specifically, the acute temperature change was from either 5°C to 21°C or from 21°C to 5°C for the AP recordings, and the acute temperature change was to the common temperature of 11°C for the ion channel current recordings. To mimic in vivo blood plasma pH of anoxia-exposed turtles, cardiac APs were re-recorded for anoxia-acclimated hearts following a switch to a combined acidotic and anoxic saline. This was done to investigate the effect of acidosis on turtle cardiac APs given the reported temperature-dependency of its negative inotropic and chronotropic effects on the turtle myocardium (41, 49, 56, 71, 73). For sarcolemmal ion channel current recordings, we focussed on ventricular myocytes since the ventricle is the power generating tissue of the turtle heart. Our prediction was that changes in AP shape and duration induced by cold-acclimation and/or prolonged anoxia exposure would be reflected in changes in ion current densities. Also, since the duration of cardiac contraction and AP duration are closely correlated in other ectothermic vertebrate species (eg., 43, 55), we reasoned that changes in cardiac APDs accompany the known decreases in $f_H$ associated with both cold-acclimation and with prolonged anoxia exposure.
Materials and Methods

Experimental animals

Fifty-six red-eared sliders (*Trachemys scripta*, Gray) with body masses ranging between 124 and 518 g (235 ± 77 g, mean ± S.D) were used in this study. Turtles were obtained from Lemberger Inc. (Oshkosh, WI, USA) and The Charles D. Sullivan Company Inc. (Nashville, TN, USA) and shipped by air to the University of Joensuu, Joensuu, Finland or the University of British Columbia, Vancouver, Canada. The exposure design for the turtles was normoxia and anoxia exposure x 21°C- and 5°C-acclimation. *In vitro* measurements were made at the same temperature as the acclimation temperature (unless stated otherwise; i.e., an acute temperature change) and in the appropriate normoxic or anoxic saline. Turtles studied at 21°C were held indoors in aquaria under a 12 h:12 h L:D photoperiod, had free access to basking platforms and diving water and were fed several times a week with commercial turtle food pellets. The turtles studied at 5°C were kept in aquaria with shallow water (3-4 cm) under a 12 h : 12 h L:D photoperiod in a temperature-controlled room set to 5°C for 5 weeks prior to experimentation to allow adequate time for cold-acclimation (19). Acclimation to 5°C occurred during winter months, and turtles were fasted during the acclimation period. Normoxic turtles were sampled from these conditions. For prolonged anoxia, 21°C turtles were exposed to anoxia for 6 h and 5°C turtles for 14 days. These anoxia durations were utilized to be consistent with previous examinations of the anoxic turtle heart and since anoxic cardiovascular status is relatively stable at these times (19, 20, 56, 57). The anoxic conditions were achieved by individually placing turtles into an enclosed, water-containing plastic chamber that still allowed access to air for 24 h, after which the plastic
chamber was completely filled with water, continuously bubbled with N₂ (water $P_{O_2} < 0.3$ kPa) and access to the water surface denied by means of mesh suspended below the surface of the water. 21°C-acclimated turtles were not comatose following the anoxia exposure, whereas 5°C-acclimated turtles were found to be unresponsive to tactile stimulation. All procedures were in accordance with the animal care guidelines of the University of Joensuu and the University of British Columbia.

*Action potential recordings from intact cardiac tissue*

Intracellular APs were measured from all three cardiac chambers (right atrium, left atrium and ventricle) of a spontaneously beating whole-heart preparation. The heart was accessed through removal of a 2 cm x 2 cm piece of the plastron using a bone saw following euthanasia by decapitation, which for anoxia-acclimated turtles occurred underwater in the plastic containers. The chambers of the excised heart were then medially opened, spread and gently fixed with insect pins to the Sylgard-coated bottom of a 10 ml, water-jacketed tissue chamber filled with physiological saline containing (in mmol l⁻¹) 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgSO₄, 1 NaH₂PO₄, 10 N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES) and 5 glucose, as well as a physiological relevant tonic (1 nmol l⁻¹) adrenaline concentration (glucose and adrenaline were added immediately prior to use). The desired temperature of the saline (see below) was maintained with a circulating water bath and saline was bubbled continuously with either O₂ (for normoxia-acclimated hearts) or N₂ (for anoxia-acclimated hearts; saline $P_{O_2}$ was ~2 kPa). Saline pH was adjusted (Teopal P600, Teo-Pal, Espoo, Finland) to 7.75 with NaOH at 21°C and allowed to change with temperature. Thus, pH was ~7.95 at 5°C.
Saline was refreshed every 30 min throughout the 4 – 6 h recording period to avoid potential build-up of anaerobic waste products with anoxia-acclimated hearts as well as adrenaline degradation. Saline for anoxia-acclimated hearts was pre-bubbled with N\textsubscript{2}.

Hearts were allowed to stabilize to the experimental conditions for 30 - 45 min before recordings were made. For normoxia-acclimated hearts, APs and spontaneous \( f_{\text{H}} \) were recorded first at the acclimation temperature of the animal (i.e., 21\textdegree C or 5\textdegree C) and then the saline was acutely changed either from 5\textdegree C to 21\textdegree C, or from 21\textdegree C to 5\textdegree C and hearts allowed 45 - 60 min to stabilize before APs and spontaneous \( f_{\text{H}} \) were re-recorded for that preparation. For anoxia-acclimated hearts, APs and spontaneous \( f_{\text{H}} \) were recorded only at the acclimation temperature of the animal, but were also recorded following a switch to acidotic saline (pH 7.25 at 21\textdegree C and pH 7.55 at 5\textdegree C; still continuously bubbled with N\textsubscript{2}) that mimicked \textit{in vivo} blood plasma pH of turtles exposed to anoxia for 6 h (warm-acclimated) or 14 days (cold-acclimated) (63, 72). A 25 - 30 min stabilization period was allowed following the switch to the acidotic saline before APs and spontaneous \( f_{\text{H}} \) were re-recorded for that preparation.

Cardiac APs were recorded using high-resistance, sharp microelectrodes (6 – 30 M\textOmega when filled with either 3.0 mol l\textsuperscript{-1} or 0.3 mol l\textsuperscript{-1} KCl), fabricated from borosilicate glass with an internal filament (World Precision Instruments, 1BBL, Sarasota, FL, USA) and using a L/M-3P-A vertical puller (List Medical, Darmstadt, Germany). 0.3 mol l\textsuperscript{-1} KCl was utilized in some experiments to ensure that observed changes in resting membrane potential with temperature were not an artifact of the 3.0 mol l\textsuperscript{-1} KCl. Microelectrode signals were amplified using a high-impedance amplifier (KS-700, World Precision Instruments, Sarasota, FL, USA), digitized at a sampling rate of 2 kHz.
(Digidata 1200, Axon Instruments, Union City, CA, USA) and recorded to computer using Axotape 2.2 acquisition software. Spontaneous $f_H$ was calculated from the peak-to-peak intervals of right atria contraction force, which was obtained via attachment of one edge of the right atria to a force transducer (FT03, Grass Instruments, West Warwick, RI, USA) by a small metal hook and braided silk thread. The contraction force signal was amplified (7D, Grass Instruments, West Warwick, RI, USA), routed through the digitizer and stored to computer at a sampling rate of 200 Hz. APs and contraction force recordings were analyzed off-line using Clampfit 9.2 (Axon Instruments, Foster City, CA, USA).

Whole-cell voltage-clamp from isolated myocytes

Whole-cell voltage-clamp experiments were performed on individual ventricular myocytes to assess the effect of cold temperature acclimation and prolonged anoxia acclimation on ion current density of $I_{Na}$, $I_{Ca}$, $I_{K1}$ and $I_{Kr}$.

Myocyte isolation

Single ventricular myocytes were enzymatically isolated by adapting an established isolation protocol for teleost fish (51, 65). The turtle heart was accessed as describe above, excised and a cannulated through the left aortic arch into the ventricle. The heart was then perfused retrograde at room temperature ($21 \pm 1^\circ C$) from a height of 50 cm, first with a nominally Ca$^{2+}$-free, low Na$^+$ isolation saline solution (containing in mmol l$^{-1}$: 100 NaCl, 10 KCl, 4 MgSO$_4$, 1 NaH$_2$PO$_4$, 1.2 KH$_2$PO$_4$, 50 taurine, 20 glucose and 10 HEPES, with pH adjusted to 6.9 at 21$^\circ$C with KOH) for 10 min, and then for 20
min with fresh isolation solution supplemented with the proteolytic enzymes collagenase (1.5 mg ml\(^{-1}\); Type IA) and trypsin (1 mg ml\(^{-1}\); Type IX) as well as with 1.5 mg ml\(^{-1}\) of fatty acid-free bovine serum albumin. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The isolation solutions were continuously bubbled with O\(_2\) for normoxia-acclimated hearts or N\(_2\) for anoxia-acclimated hearts and the enzyme supplemented isolation solution recycled using a peristaltic pump and retained following perfusion. The ventricle was then dissected from the atria and the sinus venosus, minced with scissors in fresh isolation solution, and transferred to the retained enzyme-supplemented isolation solution. Ventricular tissue was gently stirred with a small magnetic bar at room temperature with periodic trituration through the opening of a Pasteur pipette for 10 - 20 min or until individual viable myocytes were observed by light microscopy. The solution was then left to settle and myocytes re-suspended in fresh isolation solution and stored at 6ºC. Cells were usually recorded from within 4 – 6 h, as done previously for recordings from anoxia-acclimated crucian carp (*Carassius carassius*) cardiomyocytes (42, 67). However, in some instances, cells were stored for up to 12 h due to the extreme difficulty in obtaining reliable measurements from viable cells.

**Experimental procedures**

Electrophysiological measurements and analysis of sarcolemmal current densities were achieved using established methods and solutions for teleost fish that were adapted for the turtle (14, 15, 42, 51, 55, 65, 66). Specific details for each current measured are given below, but in all instances, an aliquot of dissociated myocytes was placed into a recording chamber mounted on the stage of an inverted microscope and left to adhere to
the bottom of the chamber. Cells were then superfused at a rate of 1 - 2 ml min⁻¹ with an extracellular saline solution. Temperature of the extracellular solution was regulated either by water bath circuits that chilled or heated the inflow tube carrying the extracellular solution to the recording chamber, or a Peltier device. Thermocouples positioned no less than 5 mm from the cell under investigation were used to continuously monitor temperature. For cells from normoxia-acclimated animals, current density was recorded in the same cell first at the acclimation temperature of the animal (i.e., 21ºC or 5ºC) and then after an acute exposure to the common experimental temperature of 11ºC (temperature change was accomplished within 3 – 5 min). This was done to distinguish cold-acclimation effects from direct temperature effects. For cells from anoxia-acclimated animals, current density was recorded only at the acclimation temperature of the animal, and the extracellular solution was continuously bubbled with N₂ (P O₂ of the extracellular solution in the recording chamber was ~5 kPa).

Patch pipettes were pulled from borosilicate glass without an internal filament and had a resistance of 2 – 4 MΩ when filled with pipette solution. Offset potentials were zeroed just before formation of the GΩ seal, and pipette capacitance was compensated after formation of the GΩ seal. The patch was ruptured by delivering a short voltage pulse (zap) to the cell, and capacitive transients eliminated by iterative adjustments of series resistance and cell capacitance circuits. Mean series resistance was 5.3 ± 4.9 (N=165; mean ± S. D.) and mean cell capacitance was 50.2 ± 1.0 pF (N=165; mean ± S.E.M.).

**Voltage-gated Na⁺ current (I_{Na})**
$I_{Na}$ recordings were made (at the University of Joensuu) with an EPC-9 amplifier in conjunction with Pulse v8.65 software (HEKA, Lambrecht, Germany) and a temperature controlled 500 µl recording chamber (RCP-10T, Dagan, Minneapolis, MN, USA). Patch pipettes were pulled from borosilicate glass (Garner F-78045, Claremont, CA, USA) using a two-stage vertical puller [either a L/M-3P-A (List Medical, Darmstadt, Germany) or PP-83 (Narishige Company, Tokyo, Japan)]. Cells were first superfused with normal K+-based extracellular solution containing (in mmol l$^{-1}$) 125 NaCl, 2.5 KCl, 2 CaCl$_2$, 1 MgSO$_4$, 1 NaH$_2$PO$_4$, 10 HEPES, 5 glucose and 0.01 nifedipine (to block L-type Ca$^{2+}$ channels; 12) where GΩ seal and whole-cell patch clamp recording of the myocytes was established. Internal perfusion of the myocytes with pipette solution (containing in mmol l$^{-1}$: 5 NaCl, 130 CsCl, 1 MgCl$_2$, 5 EGTA, 5 MgATP and 5 HEPES; pH adjusted to 7.4 at 21°C with CsOH) continued for at least 3 min to allow buffering of intracellular Ca$^{2+}$ with EGTA. Then, the extracellular solution was switched to a low-Na$^+$ solution (containing in mmol l$^{-1}$: 19 NaCl, 108.5 CsCl, 1 MgSO$_4$, 1 NaH$_2$PO$_4$, 2 CaCl$_2$, 10 HEPES, 5 glucose and 0.01 nifedipine; pH adjusted to 7.75 at 21°C with CsOH) without inducing contracture in the patched myocyte, as previously accomplished in teleost fish myocytes (15, 16). $I_{Na}$ was elicited in the low-Na$^+$ extracellular solution from the holding potential of -80 mV by 10 ms (21°C) or 30 ms (5°C) depolarizing square pulses to voltages between -100 mV and 60 mV in 10 mV steps that were preceded by a 20 ms pre-pulse to -120 mV to remove inactivation. Sampling rate was 20 kHz and the signal was filtered on-line with a 10 kHz Bessel filter. Voltage off-set caused by series resistance was compensated (70 %; 10 µs). Leak current was estimated from current at
end of depolarizing pulses at -100, -90, -80 and -70 mV and subtracted off-line. The amplitude of $I_{Na}$ was calculated as the peak inward current during the depolarizing pulses.

**L-type Ca$^{2+}$ current ($I_{Ca}$)**

$I_{Ca}$ recordings were made (at the University of British Columbia) with an Axopatch 200B amplifier, a CV 203BU headstage and ClampEx v9.2 software (Axon Instruments, Union City, CA, USA). A RC-26GLP recording chamber (234 µl; Warner Instruments LLC, Hamden, CT, USA) was used and temperature regulated with a PHC-2 Heater/Cooler Jacket in conjunction with a SC-20 Dual In-line Solution Heater/Cooler and a CL-100 Bipolar Temperature Controller (Warner Instruments LLC, Hamden, CT, USA). Pipettes were pulled from borosilicate glass (GC150T-7.5, Harvard Apparatus, St. Laurent, QC, Canada) using a Sutter P-97 puller (Sutter Instrument Company, Novato, CA, USA). Myocytes were superfused with a Cs$^+$-based extracellular solution to eliminate contaminating K$^+$ currents. Extracellular solution contained (in mmol l$^{-1}$): 125 NaCl, 2.5 CsCl, 2 CaCl$_2$, 1 MgSO$_4$, 1 NaH$_2$PO$_4$, 10 HEPES and 5 glucose; pH was adjusted to 7.75 at 21ºC with CsOH. The pipette solution contained (in mmol l$^{-1}$): 130 CsCl, 1 MgCl$_2$, 5 Na$_2$-phosphocreatine, 4 MgATP, 0.03 Na$_2$GTP, 5 EGTA, 15 tetraethylammonium chloride (TEA; to block K$^+$ currents; 23), and 10 HEPES; pH was adjusted to 7.4 at 21ºC with CsOH. L-type Ca$^{2+}$ currents were elicited from the holding potential of -70 mV by 500 ms depolarizing square pulses to voltages between -70 mV and +70 mV in 10 mV steps. A preceding 50 ms or 100 ms pre-pulse to -40 mV was used to inactivate voltage-gated Na$^+$ channels and eliminate fast Na$^+$ currents, as the turtle ventricular myocytes are relatively insensitive to the specific Na$^+$ blocker tetrodotoxin.
Sampling rate was 10 kHz, and signals were low-pass filtered at 2 kHz on-line with the Axopatch amplifier. Signals were analyzed off-line using Clampfit 9.2 software (Axon Instruments, Union City, CA, USA). The amplitude of $I_{Ca}$ was calculated as the difference between peak inward current and the current at the end of the depolarising pulse.

Additionally, since $I_{Ca}$ can run-down over time when measured in whole-cell configuration and the experimental protocol utilized here required repeated measurements of $I_{Ca}$, the magnitude of $I_{Ca}$ run-down was assessed in a separate group of myocytes. This was accomplished by repeatedly measuring $I_{Ca}$ at 3 min intervals over a period of 15 min (Fig. 1).

**Inward rectifier K$^+$ ($I_{K1}$) and delayed rectifier K$^+$ ($I_{Kr}$) currents**

$I_{K1}$ and $I_{Kr}$ recordings were made (at the University of Joensuu) with an Axopatch 1D amplifier, a CV-4 1/100 headstage and ClampEx v8.2 software (Axon Instruments, Union City, CA, USA). A RC-26 recording chamber (150 µl; Warner Instruments LLC, Hamden, CT, USA) was used, and temperature regulated using water bath circuits. Patch pipettes were pulled from borosilicate glass (Garner F-78045, Claremont, CA, USA) using a two-stage vertical puller [either a L/M-3P-A (List Medical, Darmstadt, Germany) or PP-83 (Narishige Company, Tokyo, Japan)]. Cells were superfused with normal K$^+$-based extracellular solution (described above). Pipette solution contained (in mmol l$^{-1}$): 140 KCl, 1 MgCl$_2$, 5 EGTA, 4 MgATP and 10 HEPES; pH was adjusted to 7.4 at 21°C with KOH. $I_{K1}$ was measured relative to zero membrane current at the end of 1000 ms square voltage pulses that were elicited from the holding potential of -80 mV to voltages
between -120 mV and 20 mV in 20 mV steps. No action was taken to abolish the faster 
$I_{Na}$, either by pharmacological blockade or a pre-pulse to -40 mV, during $I_{K1}$ recordings 
since Na$^+$ channel activation and inactivation was completed ~500 ms (at 5ºC) and ~800 
ms (at 21ºC) before the time at which $I_{K1}$ was measured. $I_{Kr}$ was measured as an outward tail current at -40 mV after 4000 ms depolarizing square pulses between -80 mV and 80 
 mV in 20 mV steps elicited from the holding potential of -40 mV. Signals were sampled 
at 2 kHz, low-pass filtered on-line at 2 kHz, and analyzed off-line using Clampfit 9.2 
software (Axon Instruments, Union City, CA, USA).

**Data and statistical analysis**

All results are expressed as means ± S.E.M. The number of observations (N) for 
cardiac AP data was number of turtles (i.e., 4 – 5 at each of the four acclimation groups), 
with AP characteristics from 1 - 6 cells per tissue per animal averaged for each 
individual. AP shape and duration was quantified by measuring resting membrane 
potential (RMP), peak potential and calculating duration to 0 mV (APD$_{0}$), 50% (APD$_{50}$), 
90% (APD$_{90}$) and 100% (APD$_{100}$) repolarization. AP upstroke rate was calculated by 
dividing the difference in RMP and peak potentials by the time-to-peak potential. 
Number of myocytes constitutes N for whole-cell voltage-clamp experiments. Cells were 
obtained from 2 - 10 animals for each exposure condition. Densities of $I_{Na}$, $I_{Ca}$, $I_{K1}$ and 
$I_{Kr}$ are expressed as pA pF$^{-1}$ by dividing measured currents by the cell capacitance. Slope 
conductance (pS pF$^{-1}$) of inward rectifier K$^+$ channel was calculated for the linear region 
of the $I_{K1}$ current-voltage plot by dividing the difference in $I_{K1}$ between -120 mV and - 
100 mV by the change in voltage (i.e., 20 mV). Statistically significant differences in AP
characteristics and ion current densities between either 21°C- and 5°C-acclimated turtles, or normoxia- and anoxia-exposed turtles of the same acclimation temperature were determined with a two-way analysis of variance (ANOVA) or a t-test where appropriate. Two-way repeated measures (RM) ANOVA tests, or paired t-tests where appropriate, were used to compare AP characteristics or ion current densities following an acute temperature change, and introduction of acidic saline (i.e., AP characteristics). In all instances, $P<0.05$ was used as the level of significance. Where appropriate, multiple comparisons were performed using Student-Newman-Keuls tests.

**Results**

*Effect of temperature on spontaneous normoxic $f_H$*

The initial spontaneous $f_H$ of 21°C- and 5°C-acclimated heart preparations were 35.3 ± 1.3 min$^{-1}$ ($N = 5$) and 5.5 ± 0.7 min$^{-1}$ ($N = 5$), respectively. This 6-fold difference in spontaneous $f_H$ with acclimation to 5°C corresponds to a $Q_{10}$ value of 3.2. Acute exposure of 21°C-acclimated hearts to 5°C decreased spontaneous $f_H$ to a rate (3.0 ± 0.7 min$^{-1}$; $N = 3$) statistically similar to the 5°C-acclimated $f_H$. Conversely, acute exposure of 5°C-acclimated hearts to 21°C significantly increased spontaneous $f_H$ to 27.3 ± 0.2 min$^{-1}$ ($N = 3$), but this rate was significantly lower than the 21°C-acclimated $f_H$. These results suggest direct temperature effects rather than temperature acclimation were predominant in setting the spontaneous $f_H$ under normoxic conditions.

*Effect of temperature on cardiac action potentials in normoxia*
Like spontaneous $f_{H}$, the shape and duration of the cardiac AP were considerably modified by temperature. Primarily, after acclimation to 5°C the RMP was significantly less polarized (by 18-26 mV) in all chambers of the heart (Figs. 2, 3AC, 4AC, 5AC). Also, acclimation to 5°C significantly decreased AP upstroke rate by 4.7- to 6.8-fold in all cardiac chambers, which corresponded to $Q_{10}$ values of 3.3 for the right atria, 2.8 for the left atria and 2.6 for the ventricle (Figs. 2, 3AC, 4AC, 5AC; Table 1). Further, AP duration was prolonged at least 4-fold in all cardiac chambers compared with 21°C-acclimated hearts (Figs. 2, 3AC, 4AC, 5AC). Specifically, $APD_{100}$ of 5°C-acclimated hearts was increased by 4.4-fold in the right atria (614.0 ± 39.0 ms to 2680.5 ± 113.5 ms; Fig. 2AC), 4.9-fold in the left atria (590.0 ± 50.5 to 2894.0 ± 198.5 ms; Fig. 3AC) and 4.2-fold in the ventricle (850.0 ± 38.5 ms to 3559.0 ± 529.5 ms; Figs. 2, 5AC). $Q_{10}$ values for the prolongation of AP duration at 5°C (calculated from reciprocal values of $APD_{90}$) were 2.4, 2.6 and 2.6 for the right atria, left atria and ventricle, respectively. These $Q_{10}$ values point to direct temperature effects playing a predominant role in contributing to the prolongation of AP duration with cold acclimation, a conclusion that was confirmed by the results for the acute temperature change.

Acute exposure of 21°C-acclimated heart to 5°C, as well as acute exposure of 5°C-acclimated heart to 21°C (Fig. 6), mimicked many of the AP differences observed between the temperature acclimated hearts. For instance, the AP upstroke rate was reduced with an acute 5°C exposure for all cardiac chambers (Fig. 6; Table 1) such that the AP upstroke rates for the left atria and ventricle were identical to those measured in cardiac tissue acclimated to 5°C. Also, for all cardiac chambers, all of the indices of APD ($APD_{0}$, $APD_{50}$, $APD_{90}$ and $APD_{100}$) were not significantly different to those of the 5°C-
acclimated heart (Fig. 6ACE). Similarly, the ventricular AP shape of 5°C-acclimated heart acutely exposed to 21°C was not statistically significant different to that of the 21°C-acclimated heart (Fig. 6F). Thus, the changes in AP shape and duration associated with cold acclimation were predominantly determined by a direct temperature effect rather than the chronic effect of temperature acclimation.

Nevertheless, not all changes in AP characteristics with cold acclimation could be attributed solely to a temperature effect, as some changes were not replicated by an acute temperature change. Notably, acute exposure of 21°C-acclimated hearts to 5°C did not result in a statistically significant depolarization of RMP like that associated with 5°C acclimation (Table 1). Even so, acute exposure of all cardiac chambers from 5°C-acclimated hearts to 21°C did result in a significant decrease in RMP to a membrane potential statistically similar the 21°C-acclimated RMP (Table 1). These disparate findings indicate that the mechanism underlying the increase in RMP with cold acclimation can be more quickly reversed than initiated. Further, APD_{90} and APD_{100} of right and left atria were significantly longer in 5°C-acclimated hearts acutely exposed to 21°C than APD_{90} and APD_{100} of 21°C-acclimated atria (Fig. 6BD).

Effect of temperature on ventricular sarcolemmal ion channel current densities

Consistent with the changes in AP characteristics with cold-acclimation, current densities of ventricular sarcolemmal ion channels involved in generating APs, as elicited from square voltage-clamp pulse protocols, were drastically reduced in 5°C-acclimated ventricular myocytes compared to 21°C-acclimated cells (Fig. 7). However, the manner
through which current density is depressed with cold acclimation differed with each channel type (Figs. 8, 9, 10).

Consistent with the reduced AP upstroke rate at 5°C, in 5°C-acclimated ventricular myocytes, $I_{Na}$ density was significantly reduced (Figs. 7A) and the kinetics of sodium channel activation and inactivation slower (Fig. 9) compared to 21°C-acclimated ventricular myocytes. Peak $I_{Na}$ density was 7.3-times less in 5°C-acclimated ventricular myocytes (-1.2 ± 0.1 pA pF⁻¹) than 21°C-acclimated ventricular myocytes (-8.7 ± 0.9 pA pF⁻¹). The $Q_{10}$ values were 3.4 for the decrease in $I_{Na}$, 2.5 for sodium channel activation and 1.9 for $I_{Na}$ inactivation time (the latter were calculated from reciprocal values at -20 mV) (Figs. 8A, 9). When measured at a common temperature of 11°C, peak $I_{Na}$ density did not differ between 5°C-acclimated (-3.3±0.4 pA pF⁻¹; $Q_{10}$ was 5.4 for the acute temperature change) and 21°C-acclimated (-4.1±1.2 pA pF⁻¹; $Q_{10}$ was 2.1 for the acute temperature change) ventricular myocytes (Fig. 8A). Thus, while the density of functional sodium channels on ventricular myocytes has a clear temperature dependency, it was unchanged with cold acclimation.

The reduction in $I_{Ca}$ with acclimation to 5°C (Fig. 7B) was more profound than $I_{Na}$. Peak $I_{Ca}$ density of 5°C-acclimated myocytes (-0.43 ± 0.03 pA pF⁻¹) was approximately 1/13th the peak $I_{Ca}$ density of 21°C-acclimated myocytes (-5.7 ± 0.5 pA pF⁻¹). The $Q_{10}$ for this depression in $I_{Ca}$ with acclimation to 5°C was 5.0. When measured at a common temperature of 11°C, peak $I_{Ca}$ density of 5°C-acclimated myocytes (-1.3 ± 0.09 pA pF⁻¹; $Q_{10}$ was 6.3 for the acute temperature change) remained significantly lower than 21°C-acclimated myocytes (-2.2 ± 0.3 pA pF⁻¹; $Q_{10}$ was 2.5 for the acute temperature change) (Fig. 8B). Therefore, in addition to the negative effect of cold temperature and
unlike $I_{Na}$, a component of the decreased $I_{Ca}$ of 5°C-acclimated myocytes involved the down-regulation of functional L-type Ca$^{2+}$ channels.

The inward $I_{K_1}$ density and the inward slope conductance of inward rectifier K$^+$ channels were significantly reduced with acclimation to 5°C (Fig. 7C), findings consistent with the depolarized RMP and prolonged APD of 5°C-acclimated ventricular tissue. At -120 mV, inward $I_{K_1}$ density of 5°C-acclimated myocytes (-4.6 ± 0.3 pA pF$^{-1}$) was 26% less than the inward $I_{K_1}$ density of 21°C-acclimated myocytes (-6.2 ± 0.7 pA pF$^{-1}$). Inward slope conductance of inward rectifier K$^+$ channels between -120 mV and -100 mV was reduced by almost 50% from 214.7 ± 16.8 pS pF$^{-1}$ at 21°C to 125.3 ± 9.6 pS pF$^{-1}$ at 5°C. When measured at a common temperature of 11°C, 5°C-acclimated ventricular myocytes displayed greater inward $I_{K_1}$ density at -120 mV (-5.5 ± 0.3 pA pF$^{-1}$ compared to -3.3 ± 0.4 pA pF$^{-1}$; Fig. 10), as well as a greater inward rectifier K$^+$ channel slope conductance (191 ± 8.6 pS pF$^{-1}$ compared to 89.8 ± 11.0 pS pF$^{-1}$; Fig. 8C) than 21°C-acclimated ventricular myocytes. The fact that the Q$_{10}$ for the reductions in slope conductance were 1.4 when comparing acclimated hearts, but around 2 for an acute temperature change lends support to the notion that the density of functional inward rectifier K$^+$ channels was up-regulated with cold acclimation (Fig. 8C). In contrast to inward $I_{K_1}$ density, no significant difference in outward $I_{K_1}$ density (i.e., at -80 mV and -60 mV) existed between 5°C-acclimated ventricular myocytes and 21°C-acclimated ventricular myocytes (Fig. 7C). This finding indicates that temperature did not affect $I_{K_1}$ equally at all voltages. However, like inward $I_{K_1}$ and inward slope conductance, outward $I_{K_1}$ density of 5°C-acclimated myocytes was significantly greater than 21°C-acclimated myocytes at -80 mV and -60 mV when measured at the common temperature of 11°C.
(Fig. 10). This finding further supports the notion that the density of functional inward rectifier K\(^+\) channels was up-regulated with acclimation to 5\(^\circ\)C to partially compensate for the negative effect of cold temperature on I\(_{\text{K1}}\).

I\(_{\text{Kr}}\) was minor and no different for 21\(^\circ\)C- and 5\(^\circ\)C-acclimated ventricular myocytes (Fig. 7D), and this situation was unchanged following acute exposure to 11\(^\circ\)C (data not shown).

\textit{Effect of prolonged anoxia on spontaneous f\(_{\text{H}}\)}

Spontaneous f\(_{\text{H}}\) of 5\(^\circ\)C anoxic turtles (3.0 ± 0.7; \(N = 5\)) was significantly 45\% lower than the spontaneous f\(_{\text{H}}\) [5.5 ± 0.7 min\(^{-1}\) (\(N = 5\))] of 5\(^\circ\)C normoxic turtles under normoxic conditions. At 21\(^\circ\)C, spontaneous f\(_{\text{H}}\) of anoxic preparations was 27.4 ± 4.6 min\(^{-1}\) (\(N = 5\)), and although 32\% lower than the 21\(^\circ\)C spontaneous f\(_{\text{H}}\) [35.3 ± 1.3 min\(^{-1}\) (\(N = 5\))] under normoxic conditions, there was no statistically significant difference. These findings are consistent with our other work demonstrating that prolonged anoxia at both 21\(^\circ\)C and 5\(^\circ\)C re-sets intrinsic f\(_{\text{H}}\) to a rate 25\% - 53\% lower than during normoxia (56).

\textit{Effect of prolonged anoxia on cardiac action potentials}

In contrast to the large effects of temperature on AP shape and duration, prolonged anoxia exposure caused few and only small changes in cardiac APs. Further, the effect of anoxia on cardiac APs was cardiac chamber-specific unlike the chamber-independent effects of temperature on AP shape and duration. At 21\(^\circ\)C, APs for the right and left atria were not significantly modified after 6 h of anoxic exposure (Figs. 3AB, 4AB). Likewise, AP shape or duration of all cardiac chambers remained unchanged
following 14 d of anoxia at 5°C (Figs. 2, 3CD, 4CD, 5CD). In contrast, ventricular APD\textsubscript{50}, APD\textsubscript{90} and APD\textsubscript{100} were increased significantly by 39\%, 49\% and to 47\%, respectively, following anoxia exposure at 21°C (Figs. 2, 5AB). Therefore, the prolongation of the ventricular APD after prolonged anoxia at 21°C is of the same magnitude as the reduction in spontaneous \(f_H\).

*Effect of prolonged anoxia on ventricular sarcolemmal ion channel current densities*

Of the four ventricular sarcolemmal membrane currents examined, prolonged anoxia at 21°C only significantly altered \(I_{Na}\). Specifically, peak \(I_{Na}\) density doubled from \(-8.7 \pm 0.9\) pA pF\(^{-1}\) to \(-16.1\pm1.7\) pA pF\(^{-1}\) with 6 h of anoxia at 21°C (Fig. 11A), but without affecting activation and inactivation kinetics of \(I_{Na}\) (Fig. 9). No significant changes in \(I_{Ca}\), \(I_{K1}\) or \(I_{Kr}\) density occurred as a result of 6 h of anoxia exposure at 21°C (Fig. 11BCD).

Similar to findings at 21°C, peak \(I_{Ca}\) density was unaffected by 14 d of anoxia at 5°C (Fig. 12B). However, peak \(I_{Na}\) of anoxic 5°C-acclimated ventricular myocytes (-1.1±0.2 pA pF\(^{-1}\)) was not significantly different compared with normoxic 5°C-acclimated myocytes (-1.2 ± 0.1 pA pF\(^{-1}\); Fig. 12A), unlike at 21°C. When recorded with higher (125 mM) extracellular sodium concentration to enhance \(I_{Na}\) current density, there was still no difference between anoxic (-17.6 ± 2.6 pA pF\(^{-1}\)) and normoxic (-18.0 ± 2.6 pA pF\(^{-1}\)) 5°C-acclimated myocytes. This result excludes the possibility that the measurement of \(I_{Na}\) under reduced sarcolemmal Na\(^+\) gradient resulted in minimal \(I_{Na}\) current density at 5°C and thus an inability to detect small changes in \(I_{Na}\) occurring with prolonged anoxia exposure. At 5°C, and similar to the results at 21°C, prolonged anoxia exposure did not significantly alter the activation and inactivation kinetics of the sodium current (Fig. 9).
Thus, while the effect of prolonged anoxia exposure on $I_{Na}$ density was temperature-dependent, the effect of anoxia on sodium channel activation and inactivation kinetics was temperature-independent.

The effects of prolonged anoxia on $I_{K1}$ were temperature-dependent. Prolonged anoxia at 5°C significantly reduced inward $I_{K1}$ density and inward slope conductance of inward rectifier K$^+$ channels (Fig. 12C), whereas neither effect occurred with anoxia at 21°C (Fig. 11C). $I_{K1}$ density of 5°C anoxia-acclimated myocytes was significantly 33% and 18% lower than $I_{K1}$ density of 5°C normoxia-acclimated myocytes at -120 mV and -100 mV, respectively. Inward slope conductance of inward rectifier K$^+$ channels was significantly reduced by 45% from 125.3 ± 9.6 pS pF$^{-1}$ to 68.3 ± 6.6 pS pF$^{-1}$. In contrast, no statistical differences in outward $I_{K1}$ density occurred with prolonged anoxia exposure at 5°C. $I_{Kr}$ remained minor after prolonged anoxia at 5°C (Fig. 12D).

*The combined effect of acidosis and anoxia on spontaneous $f_H$ and cardiac action potentials*

Spontaneous $f_H$ of anoxia-acclimated spontaneously beating whole-heart preparations was unaffected by acute acidosis exposure. At 21°C, spontaneous $f_H$ of anoxia-acclimated heart preparations prior to acidosis was 21.6 ± 5.2 min$^{-1}$ ($N = 5$) and 19.3 ± 3.5 min$^{-1}$ ($N = 5$) with acidosis. Similarly at 5°C, spontaneous $f_H$ was unchanged prior to (3.8 ± 0.7 min$^{-1}$; $N = 5$) and with acidosis (4.3 ± 0.3 min$^{-1}$; $N = 5$).

In contrast, acute acidosis exposure altered the AP shape and duration depending on the acclimation temperature and cardiac chamber. At 21°C, right atria APD$_0$, APD$_{50}$, APD$_{90}$ and APD$_{100}$ were increased significantly by 18% to 20% with acidosis (Fig. 3B), but left atria and ventricular AP shape and duration were unaffected (Figs. 4B, 5B). At
5ºC, APD50 increased by 18% in the left atria with acidosis (Fig. 4D), but right atria and ventricular AP shape and duration were unaffected (Figs. 3D, 5D).

Discussion

The present study examined if modification of electrophysiological properties of the turtle heart facilitates the down-regulation of cardiac activity that accompanies cold-acclimation and prolonged anoxia exposure. To this end, we compared cardiac APs of spontaneously contracting turtle heart preparations and whole-cell current densities of key sarcolemmal ion channels of ventricular myocytes isolated from turtles that had been acclimated to either 21ºC or 5ºC, and exposed to either normoxia or prolonged anoxia. Our findings revealed that the substantial modifications of cardiac APs and reduction of ion current densities that likely contribute significantly to decreased cardiac activity were more evident with cold temperature - both direct and acclimation effects - than with prolonged anoxia exposure. In view of this finding, we suggest that the reduction in electrophysiological activity of the turtle heart associated with cold exposure is a critical component in the preparation of cardiac muscle so that turtles can successfully over-winter under anoxia conditions.

Critique of methods

Action potential recordings from intact cardiac tissue in vitro

A difficulty with in vitro work is that both the isolation procedure and the test conditions may not exactly mimic the in vivo situation, especially when trying as we did to maintain the anoxic condition of the tissue. Clearly, a caveat with our AP results for anoxia-acclimated turtles is that the recording chamber saline PO2 could not be
completely depleted of oxygen due to practical limitations of the experimental set-up. However, we were greatly encouraged by spontaneous \( f_H \) for the normoxic 21°C- and 5°C-acclimated heart preparations being close to previously reported *in vivo* and *in vitro* intrinsic rates (8, 20, 72). Similarly, the reduced spontaneous \( f_H \) of anoxic 21°C- and 5°C-acclimated heart preparations is qualitatively and quantitatively comparable with previous *in vivo* and *in vitro* work (20, 55). Thus, if there had been a problem with anoxia-acclimated hearts reverting to their normoxic state during isolation and measurement, we would not have detected the decreased intrinsic \( f_H \) or seen the similarity to previous study. Further, the clear, chamber-specific effect of anoxia acclimation on APs at 21°C despite similar measurement conditions argues against the possibility that the lack of effects of anoxia acclimation on cardiac APs at 5°C were a measurement artifact. Therefore, we are confident that our novel recordings of turtle APs for all three cardiac chambers and under all four acclimation conditions were from viable cardiac tissues and representative for the prior exposure history of the animal. Nevertheless, our methods could have underestimated some effects of anoxia.

**Whole-cell patch-clamp in isolated ventricular myocytes**

For turtles, we have provided the first measurements of ventricular sarcolemmal \( I_{Na} \), \( I_{K4} \), and \( I_{Kr} \) at any acclimation temperature or condition (i.e., normoxia or anoxia exposed). Our novel measurements of ventricular \( I_{Ca} \) of warm-acclimated anoxia-exposed turtles and cold-acclimated, normoxia- and anoxia-exposed turtles adds to a very recent report of \( I_{Ca} \) for 20-21°C-acclimated yellow-bellied turtles (*Trachemys scripta scripta*) (12).
Like the anoxia-acclimated turtle AP data, a caveat with our recordings of $I_{Na}$, $I_{Ca}$, $I_{K1}$, and $I_{Kr}$ from anoxia-acclimated turtles is that cardiomyocytes could not be maintained anoxic throughout all steps of the isolation and recording protocols. In particular, cells were not continuously bubbled with N$_2$ during storage at 6°C, were exposed to air when left to settle in the recording chamber, a step that lasted only 1 – 2 min at 21°C and up to 5 min at 5°C, and recording chamber saline $P_{O2}$ could not be completely depleted of oxygen. However, we are confident that our ion channel current recordings from anoxia-acclimated turtles faithfully represent the prior anoxia exposure history of the animal. Firstly, this is because differences in ion channel current densities were indeed found for cardiomyocytes before and after anoxia acclimation, the currents affected by anoxia acclimation were temperature-dependent (i.e., $I_{Na}$ at 21°C and $I_{K1}$ at 5°C), and the directional changes in current amplitude with anoxia exposure were channel-dependent (Figs. 11 and 12). Further, the finding that peak $I_{Ca}$ remained unchanged with anoxia exposure appears legitimate, at least for 5°C-acclimated turtles, since twitch force and time-to-peak force of ventricular tissue from 5°C-acclimated turtles does not differ between normoxia- and anoxia-acclimated animals (41). Finally, given that at the organismal level, the changes cardiovascular status with anoxia take ~ 1 h and ~ 24 h at 21°C and 5°C, respectively (18, 19, 20, 21, 57, 59), it seems highly unlikely that these changes would all be reversed during the relatively short (i.e., 9 min) recording periods when perfusate oxygen levels were not zero.

Beyond the issues noted above for the isolation and test conditions, a limitation of the whole-cell patch-clamp technique is the disruption of the native intracellular milieu by the pipette solution. This disruption affects intracellular ion balance and buffering
capacity, interferes with normal cellular signalling by intracellular pH, second messengers and covalent modification (47), and leads to deterioration (i.e., run-down) of currents over time (34). At the outset of this study, turtle cardiac myocytes had never been investigated with the whole-cell patch-clamp technique. Thus, our intracellular and extracellular solutions were modified from established whole-cell patch-clamp methodologies for teleost fish (15, 16, 42, 51, 55, 65, 66), but were relevant to the freshwater turtle in terms of ionic composition and pH (see 17, 27, 28 for detailed description of turtle blood ionic composition and pH). Specifically, pipette pH was set to 7.4 at 21°C and was 7.6 at 5°C (49, 60, 71). Further, to minimize changes in the amplitude of $I_{Ca}$, a current particularly sensitive to run-down, throughout the duration of the experimental protocol, 5 mmol l$^{-1}$ EGTA was included in the pipette solution (23). In the present study, $I_{Ca}$ was $-5.7 \pm 0.5$ pA pF$^{-1}$ for 21°C-acclimated normoxic turtles and in all experimental groups, this current remained unchanged for at least 9 min with repeated measurements of $I_{Ca}$ (Fig. 1). The magnitude of $I_{Ca}$ and the repeatability of its measurement are comparable to recent reports for cardiomyocytes of yellow-bellied turtles using the perforated-patch technique (12), a technique that overcomes some of the concerns with whole-cell patch-clamp. Therefore, we are confident of our measured currents and that our reported alterations in current densities with acute temperature change, which were all completed within 9 min, are not due to variation in current density over time.

Nevertheless, the possibility exists that our reported current densities differ from the in vivo condition. Clearly, the square voltage-clamp pulses used to characterize $I_{Na}$, $I_{Ca}$, $I_{K1}$ and $I_{Kr}$ do not emulate the change in membrane potential that occurs with an AP.
Further, we did not mimic all the changes in intracellular and extracellular environment that accompanies prolonged anoxia exposure (for detailed descriptions see 17, 27, 28, 29, 49, 60, 71). For instance, adrenergic stimulation of cardiomyocytes likely exists at a tonic level under normoxia and perhaps at an increased level under anoxia because circulating catecholamines are greatly elevated (31, 32, 70). Adrenaline increases the open probability of L-type Ca\(^{2+}\) channels (45), but was not utilized during measurements of \(I_{Ca}\). Since this study was a first step in comparing electrophysiological properties of 21ºC- and 5ºC-acclimated turtles exposed to normoxia and anoxia, future studies, some of which have already commenced, utilizing physiologically relevant AP pulse-protocols and faithfully mimicking the extracellular changes in pH and adrenaline that accompany prolonged anoxia, are logical next steps for research. Finally, it should be noted that the 20 ms pre-pulse to -120mV from the holding potential of -80 mV utilized to remove steady-state inactivation of voltage-gated Na\(^+\) channels for measurement of \(I_{Na}\) may not have been sufficient to fully recover channels from inactivation at 5ºC (15). This could partially contribute to the small \(I_{Na}\) reported for cold-acclimated turtles.

Modification of turtle heart electrophysiology by temperature and anoxia

The coordinated pumping action of the turtle heart first involves production of APs in pacemaker cells, which set the spontaneous rhythm of cardiac contraction through a synchronized propagation of excitation throughout the atria and ventricle. The generation of the AP and its expression throughout the heart requires the integrated activity of a number of sarcolemmal ionic currents. Thus, the potential exists for numerous types of cardiac control mechanisms that are intrinsic to the sarcolemma and
that could modify cardiac performance in response to a change in whole-body blood flow demand, extracellular conditions and ambient temperature.

The present study focused on the effects of cold temperature acclimation and prolonged anoxia exposure on turtle cardiac APs and our goal was to elucidate the contribution of sarcolemmal ion channels to the known reduction in cardiac activity. Four key sarcolemmal ion channel currents were studied in ventricular myocytes. $I_{Na}$, a fast inward Na$^+$ current via voltage-gated Na$^+$ channels, is the first current to be activated in atrial and ventricular cells, determines the amplitude and slope of the AP upstroke (10), is linked to excitation-contraction coupling of cardiac myocytes via the sarcolemmal Na$^+$/Ca$^{2+}$ exchange (3), and allows for subsequent activation of other ion channels involved in AP generation (33). $I_{Ca}$, the inward Ca$^+$ current via voltage-gated L-type Ca$^{2+}$ channels, is responsible for the plateau phase of the AP (2), and is the predominant source of free intracellular Ca$^{2+}$ needed to bind to the myofilaments and trigger contraction in the turtle heart (11, 12). $I_{K1}$, an outward K$^+$ current via inward rectifier K$^+$ channels, is primarily responsible for maintaining a stable RMP and terminal repolarization of the AP, but not thought to be present during AP plateau (35, 48). $I_{Kr}$, a repolarizing K$^+$ current, gradually develops during the plateau phase of the AP, conducts outward current at more positive voltages than the $I_{K1}$, and thus is important in balancing $I_{Ca}$ and contributing to the plateau of the cardiac AP (46).

**Modification of turtle heart electrophysiology with cold temperature**

*In vivo* cardiac activity of cold-acclimated freshwater turtles is substantially lower than that of warm-acclimated turtles, with a 5- to 15-fold decrease in $f_H$ following
acclimation to 5°C from 21°C-22°C driving similar reductions in $Q_{sys}$ and $PO_{sys}$ (19, 57, 59). Similarly, spontaneous $f_H$ is 6- to 7-times lower in \textit{in vitro} heart preparations from 5°C-acclimated turtles compared with 21°C-acclimated turtles (56). The present findings not only confirm these findings for spontaneous $f_H$, but also indicate that, at least for ventricular tissue, cold temperature can induce changes in the density of sarcolemmal ionic currents in response to direct temperature effects as well as acclimation. These changes are presumed to serve to alter cardiac AP characteristics and contribute to the depression of cardiac activity \textit{in vivo}.

To summarize these temperature effects for normoxic turtles, 5°C-acclimated turtle heart exhibited depolarization of RMP by 18 mV to 26 mV, 4.7- to 6.8-fold decreases in AP upstroke rate and a prolongation of APD by 4.2- to 4.9-fold in all cardiac tissues examined (Figs. 2, 3AB, 4AB, 5AB). In the ventricle, the increased RMP and APD are consistent with the 50% reduction of $I_{K1}$ conductance (Fig. 7C), whereas the decreased AP upstroke rate is consistent with the 7-fold reduction in peak density of $I_{Na}$ (Fig. 7A). The 13-fold reduction in ventricular $I_{Ca}$ of 5°C-acclimated turtles (Fig. 7B) is consistent with reductions in twitch force and time to peak force of 5°C-acclimated turtle ventricular tissue compared to ventricular tissue from 21°C-acclimated turtles (41).

By making additional measurements of ventricular sarcolemmal current densities after acutely switching the temperature to a common temperature of 11°C, direct temperature effects could be clearly dissected from those due to cold temperature acclimation. Specifically, we found that $I_{Na}$ decreased (also reflected in the decreased AP upstroke rate of 5°C-acclimated turtles) predominantly due to a direct temperature effect (Figs. 5AB, 8A), whereas density of functional L-type Ca$^{2+}$ channels was down-regulated
as part of the cold acclimation (Fig. 8B). These findings contrast in one respect with the
effect of cold acclimation in anoxia-tolerant crucian carp (Carassius carassius) where
functional sodium channels were reduced (15), but share a common response to cold
acclimation of active down-regulation of functional L-type Ca\(^{2+}\) channels (67). It
remains to be clarified if the cold acclimation induced change in ventricular \(I_{Ca}\) for turtles
is due to temperature-dependent changes in channel phosphorylation, transcription,
translation, rate of protein degradation, or trafficking of channels to the sarcolemmal
membrane. Further, we found that density of functional inward rectifier K\(^{+}\) channels was
up-regulated with cold acclimation to partially compensate the negative direct effect of
cold temperature on \(I_{K1}\), despite an overall decrease in \(I_{K1}\) conductance (Figs. 8C). This
active regulation of inward rectifier K\(^{+}\) channel density with cold temperature acclimation
is consistent with the indication from AP recordings that the increase in RMP to less
negative values with cold acclimation is not solely a result of direct temperature effects
(Table 1). Again, the possible mechanisms underlying the cold acclimation induced
change in ventricular \(I_{K1}\) and subsequently RMP, such as changes in channel
phosphorylation, transcription, translation, rate of protein degradation, or trafficking of
channels to the sarcolemmal membrane, remain to be clarified. However, it is tempting
to speculate that the known suppression of turtle cholinergic cardiac tone with cold
acclimation (20) plays an important role. In rainbow trout atrial myocytes, acetylcholine
activates inwardly rectifying K\(^{+}\) currents and stabilizes RMP at more negative voltages
(38). Therefore, if a similar phenomenon exists for turtle cardiomyocytes, it is
conceivable that the absence of turtle cardiac cholinergic tone with cold acclimation
allows for the decreased $I_{K1}$, the less negative RMP, as well as the potential for control of $I_{K1}$ by other mechanisms.

The reduced ventricular sarcolemmal ion currents and the prolongation of cardiac APs of 5°C-acclimated turtles compared to 21°C-acclimated turtles are consistent with the concept of inverse thermal compensation as a strategy to cope with cold temperature. Due to the inhibitory effect of cold temperature on rates of physiological processes, ectothermic vertebrates exhibit a variety of strategies to cope with the cold. Some ectotherms show physiological compensation that allows for the continuation of an active lifestyle at cold temperature. At the cardiac level, established compensatory changes include increased relative ventricular mass (14), increased myofibrillar ATPase activity and decreased refractoriness of the heart (1), proliferation of the sarcoplasmic reticulum (5), modulation of Ca$^{2+}$ cycling (50, 51, 52, 53), increased $I_{Na}$ (15), and alterations in K$^+$ conductances that shorten APD (42, 66). However, for a freshwater turtle that becomes inactive during a prolonged period of winter anoxia, physiological processes must be primed to conserve fuel and make compensatory changes maladaptive (22). Accordingly, these organisms reduce activity, routine metabolic rate and subsequently cardiac activity with cold exposure in anticipation of winter anoxia conditions (18, 26). Similarly, cold-acclimated crucian carp reduced $f_H$ (37, 64), reduced the rate of cardiac contraction (61), decreased the maximal conductance of $I_{Na}$ by 4.4-fold (15) and decreased the peak $I_{Ca}$ density by 6.1-fold (67). Therefore, similar to the findings for crucian carp, the reduced peak density of 5°C-acclimated turtle ventricular $I_{Na}$ and $I_{Ca}$ as well as inward slope conductance of inward rectifier K$^+$ channels suggest inverse thermal compensation at the electrophysiological level, which would provide for energetic savings through reducing
the cost of ion pumping, one of the largest energy consuming processes of cells (22). In
principle, decreased $I_{Na}$ and $I_{Ca}$ would reduce demands on the Na$^+$/K$^+$-ATPase, which
extrudes a proportion of the Na$^+$ that enters the myocyte during AP upstroke and also via
the sarcolemmal Na$^+$/Ca$^{2+}$ exchange. Further, the current required to trigger an AP is
likely to be less with a smaller $I_{K1}$ and less negative RMP, making myocytes more readily
excitability at cold temperature.

Nevertheless, still needed to confirm these suggestions are investigations of direct
and acclimatory temperature effects on $I_{Na}$ and $I_{Ca}$ with pulse protocols representative of
physiological APs and in-depth examination of sodium and calcium channel
activation/inactivation kinetics. This is because of the possibility that the prolonged AP
and slower activation/inactivation kinetics at cold temperature could allow channels to be
open longer, resulting in an increase in total charge transferred despite the decreased peak
densities. The possibility is certainly in evidence for atrial myocytes of rainbow trout
(*Oncorhynchus mykiss*), a cold-active species that exhibits positive thermal
compensation; the charge carried by $I_{Ca}$ while temperature dependent with square pulses
becomes temperature-independent when stimulated with physiologically relevant AP
pulse protocols (51). Of course, the reductions in peak $I_{Na}$ and $I_{Ca}$ density with cold
acclimation in the turtle are much larger than the proportional prolongation of APD,
which suggests that these reductions should indeed reduce total charge transferred and
contribute to energy conservation. Further, tonic adrenergic stimulation, which likely
occurs *in vivo*, influences $I_{Ca}$ (45) and certainly plays a role in cold rainbow trout (54),
could be important during cold acclimation in turtles. Future studies investigating the
role of adrenaline on $I_{Ca}$ would thus be very insightful. Similarly, future investigation on
the effect on temperature on Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) current is needed to clarify the apparent juxtaposition of decreased \(I_{\text{Ca}}\) and prolonged ventricular APD at 5ºC and determine if reversed NCX current maintains the long AP in the cold.

A surprising discovery in the present study is that \(I_{\text{Kr}}\) was not a predominant current in turtle ventricular myocytes under any experimental condition (Figs. 7D, 11D, 12D). In contrast, \(I_{\text{Kr}}\) is the predominant repolarizing current in mammalian (46) and fish cardiomyocytes. In fact, cold acclimation markedly increases \(I_{\text{Kr}}\) in rainbow trout (66), and in the burbot (\textit{Lota lota}), a cold stenothermic fish, \(I_{\text{Kr}}\) is much larger than \(I_{\text{K1}}\) (55). This interspecific difference in \(I_{\text{Kr}}\) does not appear to be related to the different cold survival strategies among these species because \(I_{\text{Kr}}\) is present in the crucian carp and is up-regulated with cold acclimation (M. Vornanen, personal communication). Whether this phenomenon is unique to turtles remains to be determined.

Finally, the longer APD\(_{90}\) and APD\(_{100}\) of atrial tissue, but not ventricular tissue, of 5ºC-acclimated turtles when acutely warmed (Fig. 6BD) suggests that the density of K\(^+\) currents and/or the effect of cold acclimation on K\(^+\) currents differs among cardiac tissue types. A similar phenomenon was reported for rainbow trout where substantial differences in \(I_{\text{K1}}\) exist between atrial and ventricular myocytes (66). Again, future studies are needed to clarify this possibility.

Given these important direct and acclimation temperature effects, we can return to the quantitative issue of how these effects might account for the depression of cardiac activity in cold-acclimated turtles. Even the longest tissue APD\(_{100}\), i.e., 3559.0 ms measured in the ventricle, would allow a \(f_H\) of \(~17\) min\(^{-1}\) if no refractory period existed. Therefore, APD is unlikely to represent a major restriction on the spontaneous \(f_H\) of \(~5\)
min\(^{-1}\). Since cholinergic cardiac inhibition is not involved in the depression of cardiac activity with cold acclimation (20), the decrease in contraction frequency must be due to changes in cardiac refractoriness and other mechanisms such as intercellular electrical coupling and/or pacemaker mechanisms. In ventricular tissue of the anoxia-tolerant crucian carp, the ventricular refractory period increases by 6-fold with cold acclimation (61). If refractory period of ventricular tissue increased in cold-acclimated turtles by an amount similar to that observed with crucian carp, an intrinsic \(f_{\text{I}}\) of approximately 4 - 5 min\(^{-1}\) at 5ºC (56; present study) could be fully accounted for. Indeed, depolarization of RMP at 5ºC (Figs. 2, 5AC) should theoretically result in slower recovery of voltage-gated Na\(^+\) channels from inactivation and an increased refractory period. However, unless properties of turtle voltage-gated Na\(^+\) channels are different from those of fish and mammals, the depolarized RMP would inactivate practically all voltage-gated Na\(^+\) channels. Future studies are needed to resolve these various possibilities.

**Modification of turtle cardiac electrophysiology with prolonged anoxia exposure**

In contrast to the large effects of temperature on AP shape and duration, prolonged anoxia exposure resulted in few and only small changes in cardiac APs. Nevertheless, the 47% increase in ventricular APD\(_{100}\) with prolonged anoxia exposure at 21ºC (Fig. 5AB) closely matches the reduction of ventricular contraction rate of ~30%. Thus, for warm-acclimated turtles, a prolongation of APD is proportional to the reduction in spontaneous cardiac contraction frequency with anoxia. At 5ºC, 14 d of anoxia exposure did not affect AP characteristics of any cardiac chamber (Figs. 2, 3CD, 4CD, 5CD). Therefore, the re-setting of intrinsic \(f_{\text{I}}\) with prolonged anoxia at cold acclimation
temperatures (56; present study) results primarily from either a marked prolongation of the pacemaker potential, increased contraction and relaxation times, increased muscle refractoriness, or some combination. However, time-to-peak twitch force and time-to-relaxation were similar at 5ºC for normoxia- and anoxia-acclimated turtle ventricular strips (41). Thus, intrinsic mechanisms related to increased refractoriness of the heart, modification of intercellular electrical coupling and modification of pacemaker mechanisms and/or other extrinsic modifiers of sarcolemmal ion currents must account for the reduction in intrinsic $f_H$ with prolonged, cold anoxia exposure. The present findings exclude the prospect of acidosis as one of these possibilities because exposure of anoxia-acclimated heart preparations to an acidosis equivalent to the in vivo situation (i.e., pH of 7.55 at 21ºC or pH of 7.25 at 5ºC) did not produced any marked, chamber-independent alterations of APs (Fig. 3BD, 4BD, 5BD).

Some changes in ventricular sarcolemmal currents were induced by prolonged anoxia exposure without affecting APs. For instance, peak $I_{Na}$ density doubled with 6 h of anoxia exposure at 21ºC, but not at 5ºC (Fig. 11A). This finding suggests that an up-regulation of functional voltage-gated Na$^+$ channels with anoxia in warm-acclimated, but not cold-acclimated turtles is needed to maintain myocyte excitability and perhaps compensate the depressive effect of an increased extracellular K$^+$ concentration (17, 27, 28) on $I_{Na}$. However, the temperature-dependent effect of anoxia exposure on $I_{Na}$ may be due to the temperature-dependent effect of prolonged anoxia on $I_{K1}$ (Figs. 11C, 12C). The ability of $I_{Na}$ to depolarize the membrane is dependent on repolarizing currents such as $I_{K1}$ that overlap $I_{Na}$ at the voltage range of AP onset and thus decrease the net depolarizing current (13). Thus, the reduced $I_{K1}$ density and conductance with a 14-d
anoxic exposure at 5°C would mean that less Na⁺ current is needed to trigger an AP. Consequently, an increased $I_{Na}$ with anoxia at this temperature would not be necessary. In contrast, the lack of change in $I_{K1}$ density with prolonged anoxia at 21°C seems to necessitate an increased $I_{Na}$ to maintain myocyte excitability.

The reason why changes in current densities did not affect AP shape for anoxia-acclimated turtle hearts is unclear. Too low a statistical power to distinguish minor differences in AP shape is a possibility. For instance, the final repolarization of 5°C anoxia-acclimated ventricular APs appears to be slightly prolonged (but not statistically significant) compared to that of APs from 5°C normoxia-acclimated ventricles (Fig. 5CD) and consistent with reduction in $I_{K1}$ with prolonged anoxia at 5°C. Another difference is that saline solutions utilized with whole-heart preparations contained 1 nM adrenaline, whereas no adrenaline was present for the recording of current densities. Finally, the discrepancy could be due to the prolonged time between myocyte isolation and current recordings for anoxia-acclimated myocytes, which occurred due to the extreme difficulty in obtaining reliable recordings. Even so, our current density findings are consistent with previous studies. For example, the constancy of peak $I_{Ca}$ density with a 14-d anoxic exposure (Fig. 12B) is consistent with the lack of change in twitch force, time-to-peak force and relaxation time of ventricular tissue from 5°C anoxia-acclimated turtles compared with 5°C normoxia-acclimated animals (41).

Perspective: Prevalence of channel arrest in the turtle heart

Prolonged anoxia tolerance requires a matching of ATP demand to the reduced ATP supply available from anaerobic metabolism. Channel arrest is a proposed
mechanism through which ATP supply and ATP demand could be matched during anoxia through the reduction in the density and/or activity of ion channels to decrease the energetic cost of ion pumping (22). Such a strategy has been demonstrated in freshwater turtle brain and liver, where Na⁺, K⁺ and Ca²⁺ channel activity are all down-regulated with anoxia (4, 6, 7, 44). Given the profound reduction in turtle cardiac activity with prolonged anoxia and the high demand for ATP to support cardiac contraction, the turtle heart would appear to be an ideal, but previously uninvestigated tissue to study channel arrest.

For warm-acclimated turtles, present findings indicate that channel arrest during prolonged anoxia exposure is not a ubiquitous means of energy conservation. For the ventricle, there were no significant changes in ventricular $I_{Ca}$, $I_{K1}$ and $I_{Kr}$ densities (Fig. 11BCD). Similarly, inward slope conductance of inward rectifier K⁺ channels was unaffected by prolonged anoxia at 21°C. Further, the doubling of $I_{Na}$ with anoxia exposure at 21°C (Fig. 11A) directly opposes the concept of cardiac channel arrest.

However, our findings for ventricular $I_{Na}$ and $I_{K1}$ of 5°C-acclimated anoxia exposed turtles are consistent with the channel arrest hypothesis. Firstly, the up-regulation of $I_{Na}$ that occurred with 6 h of anoxia exposure at 21°C did not occur with a 14-d anoxic exposure at 5°C. Theoretically, since a proportion of the Na⁺ that enters the myocyte during AP upstroke is actively extruded from the cell, the lack of up-regulation of $I_{Na}$ with anoxia at 5°C could lead to reduced demands on the Na⁺/K⁺-ATPase and conserve ATP during anoxia. Secondly, inward $I_{K1}$ density was reduced by 33% at 120 mV, 18% at -100 mV, and inward slope conductance of inward rectifier K⁺ channels diminished by almost half with prolonged anoxia at 5°C (Fig. 12C). In principle, the
down-regulation of $I_{K1}$ density and conductance with prolonged anoxia could also serve to limit K$^+$ leakage and lower ATP demand. Inward rectifier K$^+$ channels allow for continuous K$^+$ efflux from resting cardiac myocytes and also contribute to phase-3 repolarization of the cardiac AP (46). Therefore, during both diastole and systole, $I_{K1}$ creates a K$^+$ leakage pathway across the sarcolemma and consequently places demands on the Na$^+$/K$^+$-ATPase. Reducing this K$^+$ leak current would thus conserve ATP.

As a point of comparison, there is no evidence for cardiac channel arrest in crucian carp during prolonged, cold anoxia exposure. $I_{K1}$ conductance, inward rectifier K$^+$ channel activity and the number of Ca$^{2+}$ channels remains unchanged in the crucian carp heart with prolonged anoxia at 4ºC (42, 67). However, crucian carp do not exhibit the massive reduction in cardiac activity that is associated with prolonged anoxia in the turtle. In fact, the crucian carp can maintain cardiac activity near normoxic levels for at least 5 d in the complete absence of oxygen (58) and the normoxic cardiac ATP demand is likely within the cardiac glycolytic capability (9). Further unlike the turtle, no channel arrest occurs in the brain of crucian carp during anoxia exposure (30, 68). This difference in the utilization of channel arrest to conserve energy during anoxia between the turtle and crucian carp is most likely related to the differing anoxia-survival strategies exhibited by these two organisms (9, 36). Briefly, in contrast to the turtle that enters a comatose-like state during anoxia, crucian carp do not become comatose (39) and continue to swim, albeit at a reduced level compared to normoxia (40).

**Concluding remarks**
In summary, we compared cardiac APs from spontaneously contracting whole-heart preparations as well as $I_{Na}$, $I_{Ca}$, $I_{K1}$ and $I_{Kr}$ of ventricular myocytes obtained from either 21°C- or 5°C-acclimated, normoxia- or anoxia-exposed turtles. Our results revealed that both direct and acclimatory cold temperature effects modify turtle cardiac electrophysiology that serve to decrease cardiac activity with cold acclimation and also pre-condition the heart for winter anoxia conditions. Specifically, exposure to cold results in an extensive prolongation of cardiac APDs. Further, decreased peak densities of $I_{Na}$ and $I_{Ca}$ and decreased conductance of $I_{K1}$ will conserve ATP by reducing the cost of ion pumping. In contrast, prolonged anoxia exposure at 5°C had only few changes on cardiac APs and ventricular whole-cell ion current densities. This finding contrasts the effect of prolonged anoxia at 21°C, where an increase in ventricular APD is proportional to the decrease in spontaneous $f_{H}$, and indicates that the re-setting of intrinsic $f_{H}$ to a reduced level that occurs with prolonged anoxia exposure in the turtle involves mechanisms other than an increase in cardiac cycle length. Nevertheless, present findings do suggest the occurrence of some forms of channel arrest in turtle cardiac tissue during prolonged anoxia exposure at cold temperature.

Acknowledgements

This research was supported by Natural Sciences and Engineering Research Council of Canada research grants to A. P. F. and J. A. W. S., a University of British Columbia graduate fellowship to J. A. W. S. and Research Council of Academy of Finland funding to M. V. Special thanks to Dr. Holly Shiels for her advice and innumerable
recommendations, as well as to Jaakko Haverinen for his instruction on how to record APs.
References


4. **Bickler PE and Buck LT.** Adaptations of vertebrate neurons to hypoxia and anoxia: maintaining critical Ca\textsuperscript{2+} concentrations. *J. Exp. Biol.* 201: 1141-1152, 1998.


55. Shiels HA, Paajanen V and Vornanen M. Sarcolemmal ion currents and sarcoplasmic reticulum Ca\(^{2+}\) content in ventricular myocytes from the cold stenothermic fish, the burbot (*Lota lota*). *J. Exp. Biol.* 209: 3091-3100, 2006.


**Figure Legend**

**Fig. 1.** Time-dependent changes in peak amplitude of L-type Ca\(^{2+}\) current (\(I_{Ca}\)) of (A) 21°C-acclimated and (B) 5°C-acclimated turtle cardiac myocytes. 5 mmol l\(^{-1}\) EGTA was included in the pipette solution. Statistically significant changes (\(P<0.05\); Friedman RM ANOVA on ranks, Dunn’s post-test) from control (\(t = 0\)) for each experimental group are indicated by asterisks. Values are means ± S.E.M. For 21°C experiments, \(N=7\) (normoxia-acclimated), 12 (anoxia-acclimated), 7 (11°C acute exposure) unless otherwise indicated in parenthesis. For 5°C experiments, \(N=5\) (normoxia-acclimated), 8 (anoxia-acclimated), 3 (11°C acute exposure) unless otherwise indicated in parenthesis.

**Fig. 2.** Representative recordings of ventricular action potentials from (A) 21°C normoxia- and anoxia-acclimated turtles and (B) 5°C normoxia- and anoxia-acclimated turtles.

**Fig. 3.** Graphical representation of right atrial action potentials from (A) 21°C normoxia-acclimated, (B) 21°C anoxia-acclimated, (C) 5°C normoxia-acclimated and (D) 5°C anoxia-acclimated turtles. The effect of acute exposure to combined anoxia with acidosis on action potential shape is also depicted in panels B and C. Statistically significant changes (\(P<0.05\)) in action potential shape following acclimation to 5°C (panel C) are indicated by the symbol ‡. Statistically significant changes (\(P<0.05\)) in action potential shape between anoxia-acclimation and acute exposure to combined anoxia with acidosis
(panels B and D) are indicated by the symbol †. Values are means ± S.E.M. \( N = 4 - 5 \) turtles.

**Fig. 4.** Graphical representation of left atrial action potentials from (A) 21°C normoxia-acclimated, (B) 21°C anoxia-acclimated, (C) 5°C normoxia-acclimated and (D) 5°C anoxia-acclimated turtles. The effect of acute exposure to combined anoxia with acidosis on action potential shape is also depicted in panels B and C. Statistically significant changes \( (P<0.05) \) in action potential shape following acclimation to 5°C (panel C) are indicated by the symbol ‡. Statistically significant changes \( (P<0.05) \) in action potential shape between anoxia-acclimation and acute exposure to combined anoxia with acidosis (panels B and D) are indicated by the symbol †. Values are means ± S.E.M. \( N = 4 - 5 \) turtles.

**Fig. 5.** Graphical representation of ventricular action potentials from (A) 21°C normoxia-acclimated, (B) 21°C anoxia-acclimated, (C) 5°C normoxia-acclimated and (D) 5°C anoxia-acclimated turtles. The effect of acute exposure to combined anoxia with acidosis on action potential shape is also depicted in panels B and C. Statistically significant changes \( (P<0.05) \) in action potential shape following acclimation to 5°C (panel C) are indicated by the symbol ‡. Statistically significant changes \( (P<0.05) \) in action potential shape following anoxia acclimation (panels B and D; 6 h at 21°C and 14 d at 5°C) are indicated by asterisks. Statistically significant changes \( (P<0.05) \) in action potential shape between anoxia-acclimation and acute exposure to combined anoxia with acidosis (panels B and D) are indicated by the symbol †. Values are means ± S.E.M. \( N = 4 - 5 \) turtles.
Fig. 6. Graphical representation of action potentials from right atria (A,B), left atria (C, D) and ventricle (E, F) of 21°C and 5°C normoxia-acclimated turtles recorded at the acclimation temperature of the animal and following acute exposure to 5°C (for 21°C-acclimated hearts) or 21°C (for 5°C-acclimated hearts). Statistically significant changes \((P<0.05)\) in action potential shape between acclimated and acutely-exposed hearts are indicated by asterisks. Values are means ± S.E.M. \(N=4–5\) turtles.

Fig. 7. Mean current-voltage relationships and representative original recordings (insets) of (A) \(I_{Na}\), (B) \(I_{Ca}\), (C) \(I_{K1}\) and (D) \(I_{Kr}\) for ventricular myocytes isolated from 21°C and 5°C normoxia-acclimated turtles. Values are means ± S.E.M.

Fig. 8. Effect of temperature on (A) peak \(I_{Na}\) density, (B) peak \(I_{Ca}\) density and (C) inward slope conductance of \(I_{K1}\) in ventricular myocytes isolated from 21°C and 5°C normoxia-acclimated turtles. Recordings were first made at the acclimation temperature of the animal and secondly at a common temperature of 11°C. Significant differences \((P<0.05)\) between 21°C- and 5°C-acclimated turtles at 11°C are indicated by an asterisk. \(Q_{10}\) for acute and acclimation-induced changes are indicated by black and grey arrows/text, respectively. Values are means ± S.E.M.

Fig. 9. (A) Inactivation time and (B) time to peak current of \(I_{Na}\) recorded from ventricular myocytes isolated from 21°C- and 5°C-acclimated, normoxia- and anoxia-
exposed turtles. Values are means ± S.E.M. N= 11 (21°C normoxia-acclimated), 6 (21°C anoxia-acclimated), 9 (5°C normoxia-acclimated) and 5 (5°C anoxia acclimated).

**Fig. 10.** Mean current-voltage relationship of $I_{K1}$ of 21°C and 5°C normoxia-acclimated ventricular myocytes recorded at the common temperature of 11°C. Values are means ± S.E.M. N= 8 (21°C-acclimated) and 15 (5°C-acclimated).

**Fig. 11.** Mean current-voltage relationships and representative original recordings (insets) of (A) $I_{Na}$, (B) $I_{Ca}$, (C) $I_{K1}$ and (D) $I_{Kr}$ for ventricular myocytes isolated from 21°C normoxia- and anoxia-acclimated turtles. Values are means ± S.E.M.

**Fig. 12.** Mean current-voltage relationships and representative original recordings (insets) of (A) $I_{Na}$, (B) $I_{Ca}$, (C) $I_{K1}$ and (D) $I_{Kr}$ for ventricular myocytes isolated from 5°C normoxia- and anoxia-acclimated turtles. Values are means ± S.E.M.
Table 1. Resting membrane potential and action potential upstroke rate in turtle heart.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Condition</th>
<th>RMP (mV)</th>
<th>AP upstroke (mV ms⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right Atria</td>
<td>21°C-acclimated</td>
<td>-66.0 ± 2.2ᵃ</td>
<td>2.7 ± 0.4ᵃ</td>
</tr>
<tr>
<td></td>
<td>Acute exposure to 21°C</td>
<td>-70.0 ± 5.5ᵃ</td>
<td>2.6 ± 0.1ᵃ</td>
</tr>
<tr>
<td></td>
<td>Acute exposure to 5°C</td>
<td>-58.5 ± 0.9ᵃ</td>
<td>1.2 ± 0.2ᵇ</td>
</tr>
<tr>
<td></td>
<td>5°C-acclimated</td>
<td>-39.2 ± 2.4ᵇ</td>
<td>0.4 ± 0.1ᶜ</td>
</tr>
<tr>
<td>Left Atria</td>
<td>21°C-acclimated</td>
<td>-65.3 ± 2.4ᵃᵇ</td>
<td>3.1 ± 0.3ᵃ</td>
</tr>
<tr>
<td></td>
<td>Acute exposure to 21°C</td>
<td>-68.9 ± 3.4ᵃ</td>
<td>3.5 ± 0.4ᵃ</td>
</tr>
<tr>
<td></td>
<td>Acute exposure to 5°C</td>
<td>-54.3 ± 1.5ᵇᶜ</td>
<td>0.7 ± 0.1ᵇ</td>
</tr>
<tr>
<td></td>
<td>5°C-acclimated</td>
<td>-46.9 ± 7.6ᶜ</td>
<td>0.6 ± 0.2ᵇ</td>
</tr>
<tr>
<td>Ventricle</td>
<td>21°C-acclimated</td>
<td>-65.8 ± 3.5ᵃ</td>
<td>1.4 ± 0.2ᵃ</td>
</tr>
<tr>
<td></td>
<td>Acute exposure to 21°C</td>
<td>-68.5 ± 1.9ᵃ</td>
<td>1.2 ± 0.1ᵃ</td>
</tr>
<tr>
<td></td>
<td>Acute exposure to 5°C</td>
<td>-51.2 ± 9.1ᵃᵇ</td>
<td>0.2 ± 0.1ᵇ</td>
</tr>
<tr>
<td></td>
<td>5°C-acclimated</td>
<td>-36.9 ± 6.5ᵇ</td>
<td>0.3 ± 0.1ᵇ</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.; N = 3 (acute exposure) or 5 (acclimated). Dissimilar letters indicate statistical significance within a tissue type.
A peak (pA pF^-1)

-10
-8
-6
-4
-2
0

5°C-acclimated
11°C acute (5°C)
21°C-acclimated
11°C acute (21°C)

Q10 = 5.4
Q10 = 3.4
Q10 = 2.1

B

* Q10 = 5.0
Q10 = 2.5

C

* Q10 = 1.4
Q10 = 2.4

Conductance (pS pF^-1)

60
80
100
120
140
160
180
200
220

Q10 = 1.4
Q10 = 2.4

Temperature (°C)