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Review

Cardiac survival in anoxia-tolerant vertebrates: An electrophysiological perspective ☆

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ABSTRACT

Certain vertebrates, such as freshwater turtles of the genus *Chrysemys* and *Trachemys* and crucian carp (*Carassius carassius*), have anoxia-tolerant hearts that continue to function throughout prolonged periods of anoxia (up to many months) due to successful balancing of cellular ATP supply and demand. In the present review, we summarize the current and limited understanding of the cellular mechanisms underlying this cardiac anoxia tolerance. What emerges is that cold temperature substantially modifies cardiac electrophysiology to precondition the heart for winter anoxia. Intrinsic heart rate is slowed and density of sarcolemmal ion currents substantially modified to alter cardiac action potential (AP) characteristics. These changes depress cardiac activity and reduce the energetic costs associated with ion pumping. In contrast, anoxia *per se* results in limited changes to cardiac AP shape or ion current densities in turtle and crucian carp, suggesting that anoxic modifications of cardiac electrophysiology to reduce ATP demand are not extensive. Additionally, as knowledge of cellular physiology in non-mammalian vertebrates is still in its infancy, we briefly discuss the cellular defense mechanisms towards the acidosis that accompanies anoxia as well as mammalian cardiac models of hypoxia/ischemia tolerance. By examining if fundamental cellular mechanisms have been conserved during the evolution of anoxia tolerance we hope to have provided a framework for the design of future experiments investigating cardiac cellular mechanisms of anoxia survival.

Contents

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List of Abbreviations

$[Ca^{2+}]_i$	intracellular Ca^{2+} transient
ATP	adenosine triphosphate
AP	action potential
E–C	coupling excitation–contraction coupling
f_H	heart rate
I_{Ca}	L-type Ca^{2+} current
I_{Na}	voltage-gated Na^+ current
I_{K1}	inward rectifier K^+ current
I_{Kr}	delayed rectifier K^+ current
$I_{K,ATP}$	ATP-sensitive K^+ channel current
NHE	Na^+/H^+-exchanger
NCX	Na^+/Ca^{2+}-exchanger
pH_e	extracellular pH
pH_i	intracellular pH
PO	cardiac power output
PO_{sys}	systemic cardiac power output
Q	cardiac output
Q_{sys}	systemic cardiac output
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase
SR	sarcoplasmic reticulum
TnC	troponin C
Vm	resting membrane potential

1. Introduction: the anoxia disaster

The vast majority of vertebrate species are highly susceptible to oxygen deprivation, with anoxic (zero oxygen) survival times rarely exceeding a few minutes. Fatality in anoxia-intolerant vertebrates is primarily ascribed to cellular disruption in tissues with a high demand for metabolic energy (i.e, adenosine triphosphate; ATP), such as the heart. As a muscular pump, the heart's ATP requirement greatly exceeds that of most non-muscular tissues. Beyond the continual ATP supply required for myocytes to contract and relax and generate mechanical work, ATP is also essential for repeated action potential (AP) generation, intracellular Ca^{2+} homeostasis and membrane ion transport (i.e., the supporting, non-contractile physiological processes; Aho and Vornanen, 1997; Rolfe and Brown, 1997; Huss and Kelly, 2005; Taha and Lopaschuk, 2007).

In the presence of oxygen, the high myocardial ATP demand is supported by aerobic metabolism, which yields 29 mol ATP for 1 mol glucose (Brand, 2003). However, without oxygen, the sole route for ATP production, anaerobic glycolysis, yields approximately 1/14th the ATP of oxidative phosphorylation per molecule of glucose (Brand, 2003). Oxygen-deprived cardiomyocytes can solve this problem using at least one of three strategies: 1) increase glycolytic output (Pasteur effect); 2) decrease mechanical work; and 3) lower ATP demand by decreasing the supporting, non-contractile energy consuming physiological processes.

Anoxia-intolerant vertebrates characteristically respond to anoxia by attempting to defend cellular ATP levels by increasing glycolytic output. However, because the cardiac ATP demand typically outstrips that provided by even the maximum anaerobic glycolytic supply, the ensuing mismatching of ATP supply to ATP demand quickly initiates a catastrophic sequence of events that results in cardiomyocyte death by necrosis, cardiac failure, and ultimately, organismal death (Hochachka, 1986; Boutilier 2001). Some anoxia-sensitive vertebrates counteract this problem by reducing cardiac ATP demand thereby extending hypoxic survival time. This depression of cardiac work is commonly achieved by inhabiting or seeking a colder environment (Tattersall and Boutilier 1997) and increasing cardiac parasympathetic nervous system activity (Farrell 2007).

An additional contributing factor to anoxic cardiac failure is the accumulation of protons from anaerobic metabolism (acidosis). Acidosis dramatically decreases the ability of the heart to pump blood by reducing contractile force and promoting fatal ventricular arrhythmias (Williamson et al., 1976; Gesser and Jørgensen, 1982; Orchard and Kentish 1990). Also, increased transcription of death promoting genes by hypoxia-inducible factor-1 (HIF-1α) can cause apoptotic cardiomyocyte death during oxygen deprivation and acidosis (Hochachka et al., 1996; Graham et al., 2004).

A relatively few vertebrate species can survive without oxygen for hours, days and even months. Among them, the freshwater turtle (genera *Chrysemys*, *Trachemys* and *Chelydra*) and the crucian carp (*Carassius carassius*) are undoubtedly the most impressive examples. These species overwinter in ice-covered ponds (Blazka 1958; Ultsch and Jackson 1982; Jackson and Ultsch 1982; Herbert and Jackson 1985a,b; Reese et al., 2002), which become progressively hypoxic, and ultimately anoxic, as thick ice coverage inhibits both photosynthesis and oxygen diffusion from the air (Holopainen and Hyvärinen 1985;

Vornanen and Paajanen 2004). Under these conditions, the heart of turtle and crucian carp continues to function.

The underlying cellular mechanisms that permit maintenance of cardiac function in the anoxic turtle and carp have received little attention and remain poorly understood, with most studies concentrating on *in vivo* and in *vitro* cardiac responses to anoxia. Even though cardiac cellular physiology of non-mammalian vertebrates is still in its infancy, this review synthesizes the available data on intrinsic electrophysiological changes that occur in the hearts of the anoxic freshwater turtle and crucian carp. In contrast, the cellular strategies of anoxic survival in brain and liver of turtles and carp have received more attention, revealing that although the two species have a similar anoxic survival time, they utilize contrasting strategies (see below and reviews by Lutz and Nilsson, 1997; Nilsson and Lutz, 2004; Bickler and Buck, 2007). Therefore, one purpose of the current review is to ascertain whether the apparent contrasting anoxic survival strategies at the whole animal level are apparent at the level of the cardiac myocyte. Another objective is to delineate the role of cold exposure in preserving cardiac function during anoxia. Previous studies of cellular anoxia tolerance in liver and brain have not factored in temperature because most were performed at warm temperatures and with relatively short anoxic periods. However, cold temperature is clearly crucial for anoxic survival as evidenced by anoxia survival being months for cold-acclimated turtles and carp, but only 1–2 days at room temperature (Lutz et al., 2003). In the final sections of the review, we discuss cellular defense mechanisms towards the acidosis that accompanies prolonged anoxia exposure, which if left unchecked will debilitate cardiac performance. In closing, we highlight important similarities and differences between mammalian cardiac models of hypoxia/ischemia tolerance and the hearts of truly anoxia-tolerant vertebrates. In this way, we hope to provide a framework for the design of future experiments to investigate cardiac cellular mechanisms of anoxia survival.

2. Cellular strategies of anoxic survival

2.1. Avoiding the anoxia disaster in brain and liver

Our central thesis for long term anoxic survival is the successful balance of ATP supply to ATP demand, and the ability to dispose of, or tolerate, harmful acidic bi-products from anaerobic metabolism. The turtle and the carp achieve this condition in two very different ways (Lutz and Nilsson, 1997; Nilsson and Lutz, 2004). Turtles enter a hypometabolic state during anoxia by turning down cellular energy requirements to a "pilot light" state of metabolism. By suppressing whole body metabolism (hypometabolism), ATP demand is matched to the reduced ATP supply of anaerobic metabolism. Further, the production of harmful bi-products, such as H^+ and lactate, is retarded. Whole-body metabolic rate during anoxia exposure at 20 °C–24 °C is just 15%–18% of that of normoxic warm-acclimated turtles (Jackson, 1968; Herbert and Jackson, 1985b). For cold-acclimated turtles (3 °C), the decrease in metabolic rate is even greater, decreasing to less than 10% of the normoxic metabolic rate after 12 weeks of anoxia exposure (Herbert and Jackson, 1985b). At the cellular level, the brain and liver of warm-acclimated, anoxic turtles exhibit both a suppression of protein turnover (termed 'translational arrest') and transmembrane ion movement via ion-specific channels and pumps (termed 'channel arrest'). Channel arrest is a hypothesized energy conserving strategy in which the number and/or open probability of functional ion channels is reduced with either oxygen limitation or low temperature to diminish the metabolic cost of ion pumping to maintain membrane integrity (Lutz et al., 1985; Hochachka, 1986). Translational arrest in the turtle has been shown for the heart (Bailey and Driedzic, 1996), hepatocytes (Land et al., 1993) and brain (Fraser et al., 2001), while Na^+, K^+ and Ca^{2+} channel activity is down-regulated in turtle brain and liver tissues (Chih et al., 1989a; Pérez-Pinzón et al., 1992; Buck and Hochachka, 1993; Bickler and Buck, 1998). Additionally, enhanced levels of inhibitory neurotransmitters (Nilsson and Lutz, 1991) reduce the electrical activity and firing frequency of brain cells (termed 'spike arrest') (Chih et al., 1989b; Sick et al., 1993) to further conserve energy.

Anoxic crucian carp, unlike the turtle, remain active, albeit at a reduced level (Nilsson et al., 1993). Metabolic depression occurs, but the reduction is modest compared to the anoxic turtle (Johansson et al., 1995). Crucian carp brain slices show a 30%–40% reduction in ATP turnover after 20 h of anoxia at 12 °C (Johansson et al., 1995), and whole body metabolic rate of *Carassius* is reduced by two-thirds during 3 h of anoxia at 20 °C (van Waversveld et al., 1989). Such small reductions in metabolism do not prevent a Pasteur effect, which is supported by an extremely large liver glycogen store (Holopainen and Hyvärinen, 1985; Hyvärinen et al., 1985) and increased brain blood flow (Nilsson et al., 1994). Further unlike the turtle, there is no evidence of either translational arrest (Smith et al., 1996) or channel arrest in the anoxic crucian carp brain (Johansson and Nilsson, 1995).

Given the strikingly different strategies employed by the freshwater turtle and crucian carp for anoxic brain and liver survival, the cellular mechanisms underlying cardiac anoxia survival are also expected to differ. The following sections review cardiac excitation–contraction (E–C) coupling, highlight the main sources for cardiac ATP demand and present potential electrophysiological mechanisms that could lower ATP demand and thereby afford protection to an anoxic heart. We then present the current understanding of the electrophysiological responses to cardiac anoxia and cold temperature in the freshwater turtle and crucian carp. Throughout, we consider the generality of "channel arrest" as a primary strategy for cardiac ATP conservation during prolonged anoxia survival.

2.2. Cardiac excitation–contraction coupling and ATP demand

E–C coupling refers to the cellular process linking excitation of the cardiac myocyte membrane with contraction of the myofilaments. This process is supported by the contribution and integration of a number of sarcolemmal ionic currents through pore-forming ion channel proteins, namely voltage-gated Na^+ channels (I_{Na}), L-type Ca^{2+} channels (I_{Ca}), delayed-rectifier K^+ channels (I_{Kr}) and inward-rectifier K^+ channels (I_{K1})(Roden et al., 2002), which are briefly outlined in the legend of Fig. 1. In mammalian myocytes, an action potential (AP) excites the sarcolemmal membrane and triggers Ca^{2+} entry via L-type Ca^{2+} channels. Ca^{2+} entry from the L-type Ca^{2+} channels triggers the release of a greater store of Ca^{2+} from the sarcoplasmic reticulum (SR) through Ca^{2+} release channels (termed ryanodine receptors); i.e., Ca^{2+}-induced Ca^{2+}-release (Bers and Despa, 2006). The intracellular Ca^{2+} transient ($[Ca^{2+}]_i$), which controls myofilament contraction by binding to regulatory sites on troponin C (TnC), is the sum of sarcolemmal Ca^{2+} influx and SR Ca^{2+} release. Relaxation occurs when Ca^{2+} is returned to diastolic levels by reuptake into the SR via the SR Ca^{2+}-ATPase (SERCA). Also, Ca^{2+} is extruded across the sarcolemma via forward-mode Na^+/Ca^{2+}-exchanger (NCX) activity, which exchanges one Ca^{2+} ion for three Na^+ ions, and, to a much lesser extent, via the sarcolemmal Ca^{2+}-ATPase. The amplitude and rate of change of $[Ca^{2+}]_i$ largely determines the strength and rate of myocyte contraction.

At the cellular level, Na^+/K^+-ATPase and Ca^{2+}-ATPases are the predominant ATP consuming processes of a quiescent cardiomyocyte. The sarcolemmal Na^+/K^+-ATPase, an electrogenic, transmembrane ATPase, is critical for maintaining a negative resting membrane potential (Vm) and repeatedly restoring the ionic gradients disrupted with each AP by the opening of ion channels (Schramm et al., 1994). Therefore, with higher heart rate (f_H), the ATP requirement of the Na^+/K^+-ATPase is increased, independent of the force of cardiac contraction. For mammals, these ATPases have been estimated to account for 15–25% of the cardiac ATP demand. In comparison, the ATP demands associated with mechanical activity (i.e. those of the actomyosin ATPase) account for 75–85% of the cardiac ATP demand (Schramm et al., 1994; Rolfe and Brown, 1997). A small proportion (~3%) is due to various housekeeping

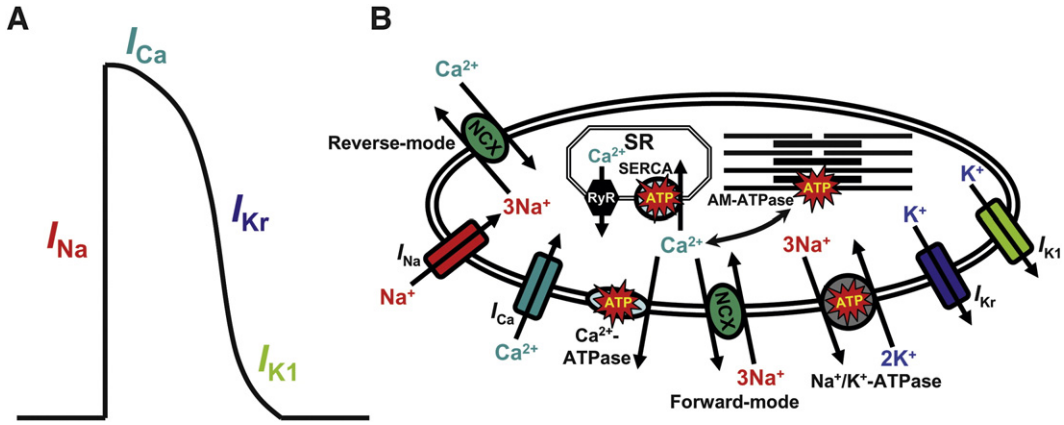

Fig. 1. (A) Action potential and (B) the ion channels, transporters and pumps involved in action potential generation and excitation-contraction coupling of a prototypical cardiomyocyte. Upon excitation of the sarcolemma, voltage-gated Na^+ channels (I_{Na}) open and Na^+ enters the cell, depolarizing the membrane. Depolarization causes L-type Ca^{2+} channels (I_{Ca}) to open, allowing Ca^{2+} into the cell. Ca^{2+} may also enter the cell via reverse-mode Na^+/Ca^{2+} exchanger (NCX) activity. Depending on the species, cardiac chamber and temperature, the increased intracellular Ca^{2+} concentration can trigger Ca^{2+} to be released from the sarcoplasmic reticulum (SR) through Ca^{2+} release channels (RyR). Ultimately, the increase of intracellular Ca^{2+} induces contraction of the myofilaments where ATP is consumed by the actomyosin ATPase (AM-ATPase). Relaxation occurs when Ca^{2+} is returned to resting levels by reuptake into the SR via the SR Ca^{2+} ATPase (SERCA), extrusion from the cell via forward-mode NCX, or via the sarcolemmal Ca^{2+}-ATPase. Repolarization of the membrane is due to outward K^+ currents via delayed-rectifier K^+ channels (I_{Kr}) and inward-rectifier K^+ channels (I_{K1}), the latter of which are important in setting Vm. The Na^+/K^+-ATPase maintains the ion gradients across the sarcolemma. ATP consuming processes are highlighted.

functions such as protein synthesis as well as proton leak (Rolfe and Brown, 1997).

The breakdown of the cardiac ATP budget in the turtle and carp may differ from mammals due to differences in cellular structure and Ca^{2+} management. Teleost and reptilian cardiomyocytes lack T-tubules and their SR is relatively poorly developed (Santer, 1985; Galli et al., 2006a,b). Consequently, I_{Ca} and Ca^{2+} entry via reverse-mode NCX exchange (i.e. one Ca^{2+} in, three Na^+ ions out) are the primary sources for the increase in $[Ca^{2+}]_i$ responsible for initiating contraction (Vornanen et al., 2002a; Galli et al., 2006b). For relaxation, forward-mode NCX exchange and sarcolemmal Ca^{2+}-ATPase remove Ca^{2+}. The relative metabolic cost of the Na^+/K^+-ATPase in fish cardiomyocytes has been estimated as approximately the sum of the mammalian SERCA and Na^+/K^+-ATPase (Aho and Vornanen, 1997). Even so, the energetic cost of mechanical work likely remains the greater fraction of total cardiac ATP expenditure because myofibrillar volume density of cardiomyocytes is similar for both fish and mammals (Santer, 1985; Aho and Vornanen, 1997). As with mammalian hearts, protein synthesis accounts for a small proportion (2%) of cardiac ATP demand in normoxic teleost fish (Houlihan et al., 1988).

2.3. Potential electrophysiological mechanisms to reduce cardiac ATP demand

Beyond strategies to minimise mechanical work (see Farrell and Stecyk, 2007), electrophysiological alterations can contribute to lowering cardiac ATP demand. Analogous to spike arrest in turtle brains, slowing f_H, termed 'action potential arrest' (Paajanen and Vornanen, 2003), reduces the reiterative demands of the ATPases. f_H can be slowed by vagal inhibition and/or modification of the electrophysiological properties of pacemaker, atrial and ventricular myocytes. Cardiac channel arrest could also reduce demand on the heart, and may involve decreases in I_{Na} and I_{K1}, thereby reducing demand on the Na^+/K^+-ATPase. Specifically, inward rectifier K^+ channels represent a K^+ leakage pathway across the sarcolemma (Roden et al., 2002), allowing for continuous K^+ efflux during both diastole and systole and placing continuous demands on the Na^+/K^+-ATPase. Therefore, down-regulation of I_{K1} would limit K^+ leakage and lower ATP demand. Reduced I_{Na} during AP upstroke means less extruded afterwards by the Na^+/K^+-ATPase. Similarly, a decrease in I_{Ca} would lower the demand of the Ca^{2+}-ATPases as well as the Na^+/K^+-ATPase due to its linkage via Na^+ ions to the NCX. Furthermore, a smaller I_{K1} and concurrent increase in Vm to a more positive value could make cardiomyocytes more excitable (i.e., less Na^+ would have to enter the myocyte to reach the AP threshold potential) and save energy. However, Vm must remain below the level at which voltage-gated Na^+ channels are activated, for the danger of increased excitability is re-entry arrhythmias and ventricular fibrillation.

3. Freshwater turtles: reducing cardiac activity during anoxia

3.1. Cardiovascular status and its control during anoxia exposure

The cardiovascular responses to anoxia in the turtle have been recently reviewed (Farrell and Stecyk, 2007; Overgaard et al., 2007). Both warm- and cold-acclimated turtles progressively and considerably depress cardiac power output (*PO*) during anoxia, in line with their reductions in metabolic rate (Jackson, 1968; Herbert and Jackson, 1985b; Hicks and Wang, 1998; Hicks and Farrell, 2000a,b; Stecyk et al., 2004a; Stecyk et al., 2007a; Fig. 2). Systemic cardiac power output (PO_{sys}) is reduced 6.6-fold and 20-fold in warm- and cold-acclimated turtles, respectively. Thus, ATP demand of the anoxic turtle heart lies well below its capability for anaerobic ATP supply (Reeves, 1963; Farrell et al., 1994; Arthur et al., 1997; Farrell and Stecyk, 2007). Central to minimizing ATP demand is a pronounced anoxic bradycardia, with f_H decreasing by 2.5-fold from ~25 min^{-1} to ~10 min^{-1} within 1 h of the commencement of anoxia exposure at 21–25 °C and by 5-fold from ~5 min^{-1} to less than 1 min^{-1} within 48 h of anoxia exposure at 5 °C. Therefore, control of f_H is critical to cardiac energy management, but the control mechanisms differ between cold- and warm-acclimated anoxic turtles. Our current understanding of these mechanisms is summarized in Fig. 3.

For both warm- and cold-acclimated turtles, a re-setting of intrinsic f_H is an important contributor to the anoxic bradycardia, accounting for ~40–66% (Stecyk and Farrell, 2007). At 21 °C, cholinergic inhibition contributes a further ~36–48% of the anoxic bradycardia (Hicks and Wang, 1998; Hicks and Farrell, 2000b), whereas α-adrenergic (Stecyk et al., 2004a) and adenosinergic (Stecyk et al., 2007a) cardiac inhibitory mechanisms are not involved. In contrast, autonomic cardiovascular control is blunted during anoxia at 5 °C and neither cholinergic nor adenosinergic mechanisms are involved (Hicks and Farrell, 2000b, Stecyk et al., 2004a; Stecyk et al., 2007a).

Available evidence suggests that the re-setting of intrinsic f_H with anoxia exposure is inherent to the electrophysiological properties of the turtle heart, rather than a consequence of changes in the

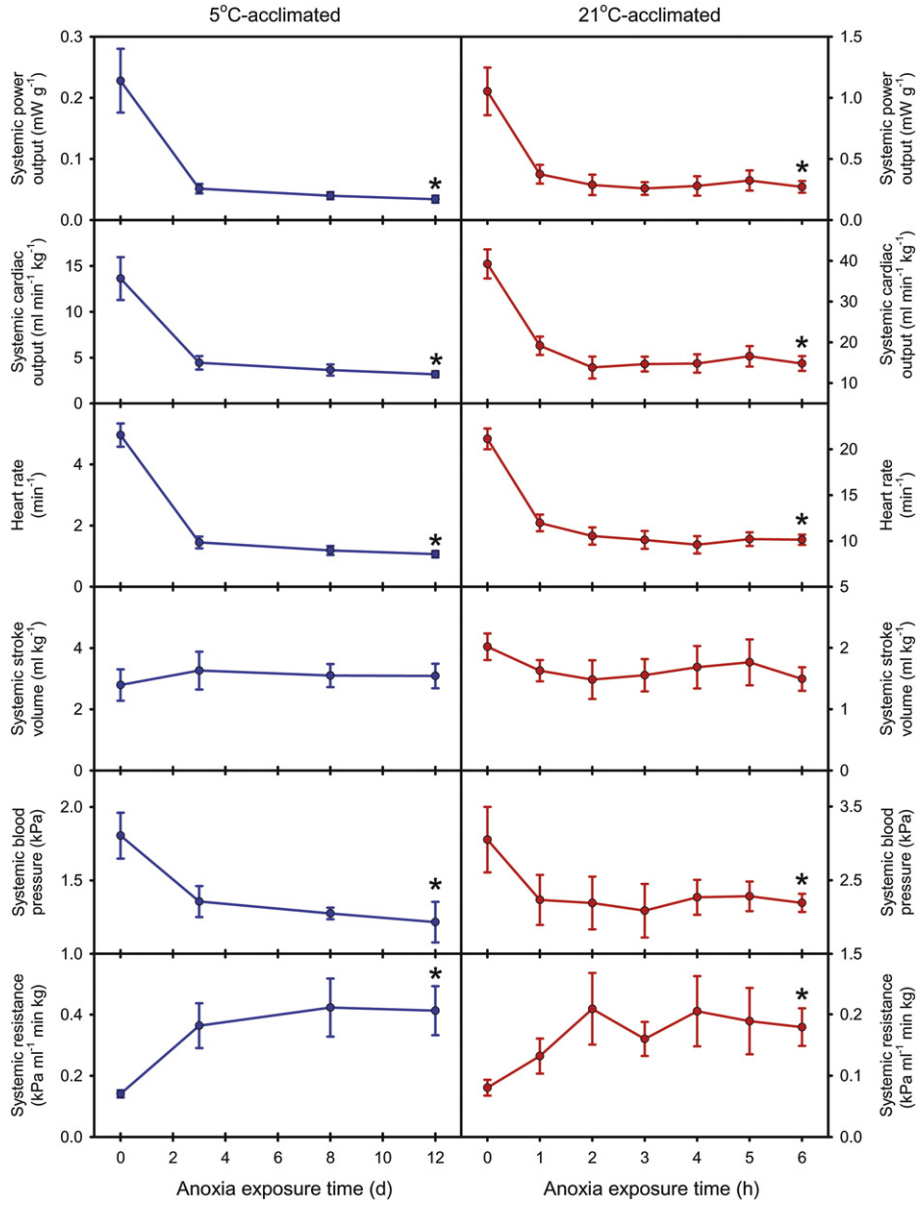

Fig. 2. Chronological changes of cardiovascular status in 5 °C-acclimated (left column) and 21 °C-acclimated (right column) turtles during anoxic submergence. Note the differences in anoxia exposure time (12 days at 5 °C vs. 6 h at 21 °C) and scaling of *y*-axes between acclimation groups. With the onset of anoxia turtles substantially reduce systemic cardiac power output (PO_{sys}), and thus their cardiac ATP demand. The decreased PO_{sys} arises from a decreased systemic cardiac output, which falls because of a large anoxia-induced bradycardia and minor decrease in systemic blood pressure. Concurrently, there is a marked increase in systemic resistance, which indicates vasoconstriction. Significant differences ($P<0.05$) of each final anoxic measurement from normoxic control (time zero) are indicated by asterisks. Values are means ± S.E.M.; $N=6–8$. Data adapted from Stecyk et al. (2004a).

extracellular milieu (i.e. changes in oxygen availability, pH, K^+, Ca^{2+} and adrenaline; see Overgaard et al., 2007 for review). Under identical extracellular conditions, spontaneous contraction of right-atrial preparations obtained from anoxia-exposed turtles (6 h at 21 °C; 14 days at 5 °C) are slower than preparations from normoxia-exposed turtles (Stecyk and Farrell, 2007). While the mechanisms underlying the reduction of intrinsic f_H remain to be discovered, reduced ventricular excitability and/or delay or of electrical impulses through the atrial-ventricular node have been implicated (Jackson, 1987). Pacemaker current may be modified during anoxia in turtles and future electrophysiological studies should address the reduction of intrinsic f_H with anoxia in isolated pacemaker cells.

3.2. Turtle cardiac electrophysiology

Two studies (Galli et al., 2006b; Stecyk et al., 2007b) have examined the electrophysiological properties of turtle ventricular cardiomyocytes. Like other ectotherms, the turtle cardiomyocyte is spindle-shaped (being ~190 μm in length and 5–7 μm in width and depth), lacks T-tubules, has a high surface area-to-volume ratio (18:1) and a small cell volume (~2 pl) (Galli et al., 2006b; Fig. 4.). Patch-clamping experiments have shown the majority of Ca^{2+} used for contraction originates via the L-type Ca^{2+} channel (i.e., I_{Ca}) and the density and kinetics of the warm-acclimated turtle I_{Ca} is similar to that reported for other ectothermic species (Galli et al., 2006b). The NCX contributes a significant amount of activator Ca^{2+} for contraction, while SR contribution is negligible, as revealed by comparing the $[Ca^{2+}]_i$ amplitude before and after pharmacological blockade of SR ryanodine receptors (Galli et al., 2006b). Thus, at least for warm-acclimated turtles, ATP costs associated with the removal of Ca^{2+} from the cytoplasm may be greater compared to mammals where the SR and NCX compete for the removal of Ca^{2+} during relaxation (see Fig. 1). This is because trans-sarcolemmal Ca^{2+} cycling is energetically less favourable than SR Ca^{2+} cycling and puts higher demands on the Na^+/K^+-ATPase (Vornanen et al.,

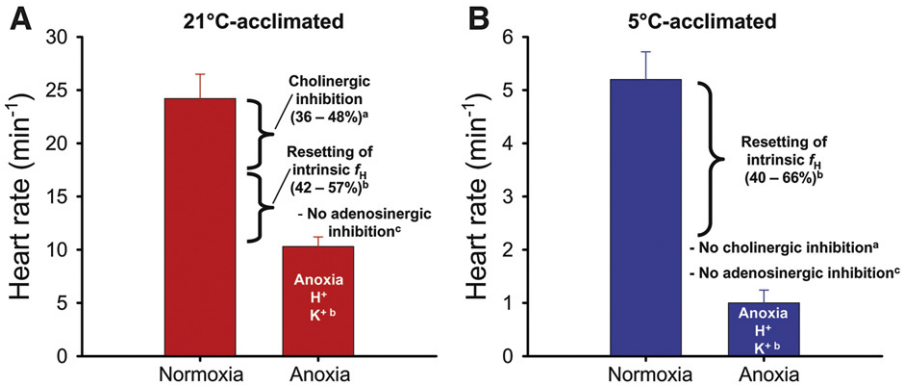

Fig. 3. The current understanding of the mechanisms contributing to the anoxic bradycardia for (A) warm- and (B) cold-acclimated turtles. Data adapted from [a]Hicks and Farrell (2000b), [b]Stecyk and Farrell (2007), [c]Stecyk et al. (2007a).

2002a). The SR Ca^{2+}-ATPase pumps two Ca^{2+} ions per ATP consumed, whereas NCX Ca^{2+} extrusion only transfers one Ca^{2+} per ATP consumed by the Na^+/K^+-ATPase to create the Na^+ gradient. It is unknown if SR Ca^{2+} cycling is enhanced in cold-acclimated or anoxic turtles, as it is known to be in cold-acclimated rainbow trout (*Oncorhynchus mykiss*; Vornanen et al., 2002a).

A striking and intriguing difference between turtle cardiomyocytes and those of other vertebrates is that I_{Kr} is non-existent in turtle ventricular myocytes isolated from warm- and cold-acclimated turtles, regardless of whether they were normoxia- or anoxia-exposed (Stecyk et al., 2007b). In stark contrast, I_{Kr} is the predominant ventricular repolarizing current in mammals (Roden et al., 2002). In the cold stenothermic burbot (*Loto lota*), I_{Kr} is much larger than I_{K1} (Shiels et al., 2006), and I_{Kr} increases markedly with cold acclimation in rainbow trout atria and ventricle (Vornanen et al., 2002b). However, these interspecific differences in I_{Kr} are apparently unrelated to anoxia tolerance as I_{Kr} is present in crucian carp ventricular myocytes and up-regulated with cold-acclimation (M. Vornanen, personal communication). Whether this phenomenon is unique to turtles or common to reptilian species remains to be discovered.

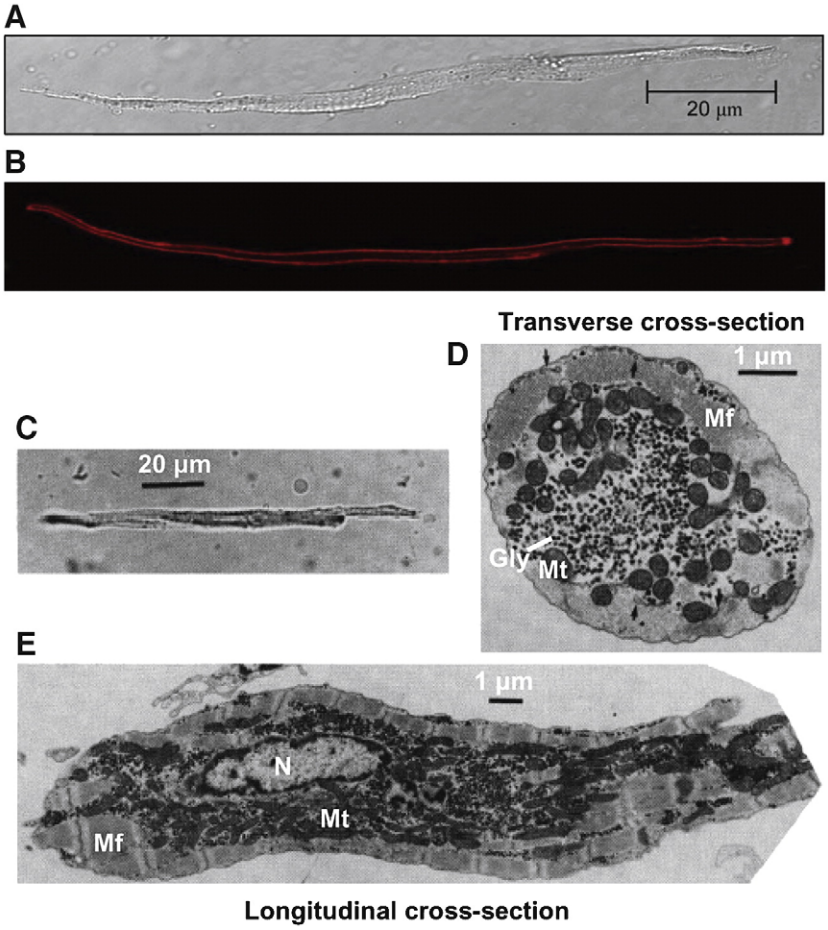

Fig. 4. Morphology of turtle and crucian carp ventricular cardiomyocytes. (A) Light and (B) confocal microscopy images of live turtle ventricular cardiomyocytes. Scale bar applies to both images. (D) Morphology of live ventricular cardiomyocyte of the crucian carp. (E) Electron micrograph of a transversely cut crucian carp cardiomyocyte showing peripheral myofibrils (Mf), centrally located mitochondria (Mt) and abundant glycogen (Gly). (F) Electron micrograph of a longitudinally cut crucian carp cardiomyocyte. Note the single nucleus (N). Images from Vornanen (1997) and Galli et al. (2006b).

The effects of prolonged anoxic exposure (6 h at 21 °C; 14 days at 5 °C) on turtle cardiac electrophysiology are minor (Stecyk et al., 2007b; Fig. 5; Table 1). APs of the right and left atria were unaffected by 6 h anoxic exposure at 21°C and all cardiac chambers were unaffected after 14 days of anoxia at 5 °C (Fig. 5). Only ventricular AP duration changed with acute anoxia exposure at 21°C, increasing by 47%, and this change was proportional to a ~30% reduction in spontaneous contraction frequency (Stecyk and Farrell, 2007).

With few differences in AP shape and duration, few differences in the underlying sarcolemmal ion currents would be expected with anoxia exposure. Of four ventricular membrane currents examined to date (I_{Na}, I_{Ca}, I_{Kr} and I_{K1}; Stecyk et al., 2007b), only I_{Na} was altered (a doubling) with anoxia at 21 °C (Table 1). With 14 days of anoxia at 5 °C, only I_{K1} changed (a 18–33% decrease), which contributed to a 45% reduction in the inward slope conductance of inward rectifier K^+ channels (Table 1). The increase in I_{Na} density indicates an up-regulation, as opposed to channel arrest, of functional voltage-gated Na^+ channels with warm anoxia. This may help to maintain myocyte excitability and compensate for the depressive effect of increased extracellular K^+ concentration on I_{Na} (Jackson and Heisler, 1982; Jackson and Ultsch, 1982; Herbert and Jackson, 1985a). A similar increase in I_{Na} is likely unnecessary with anoxia at 5 °C because of the decrease in I_{K1} at this temperature. 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

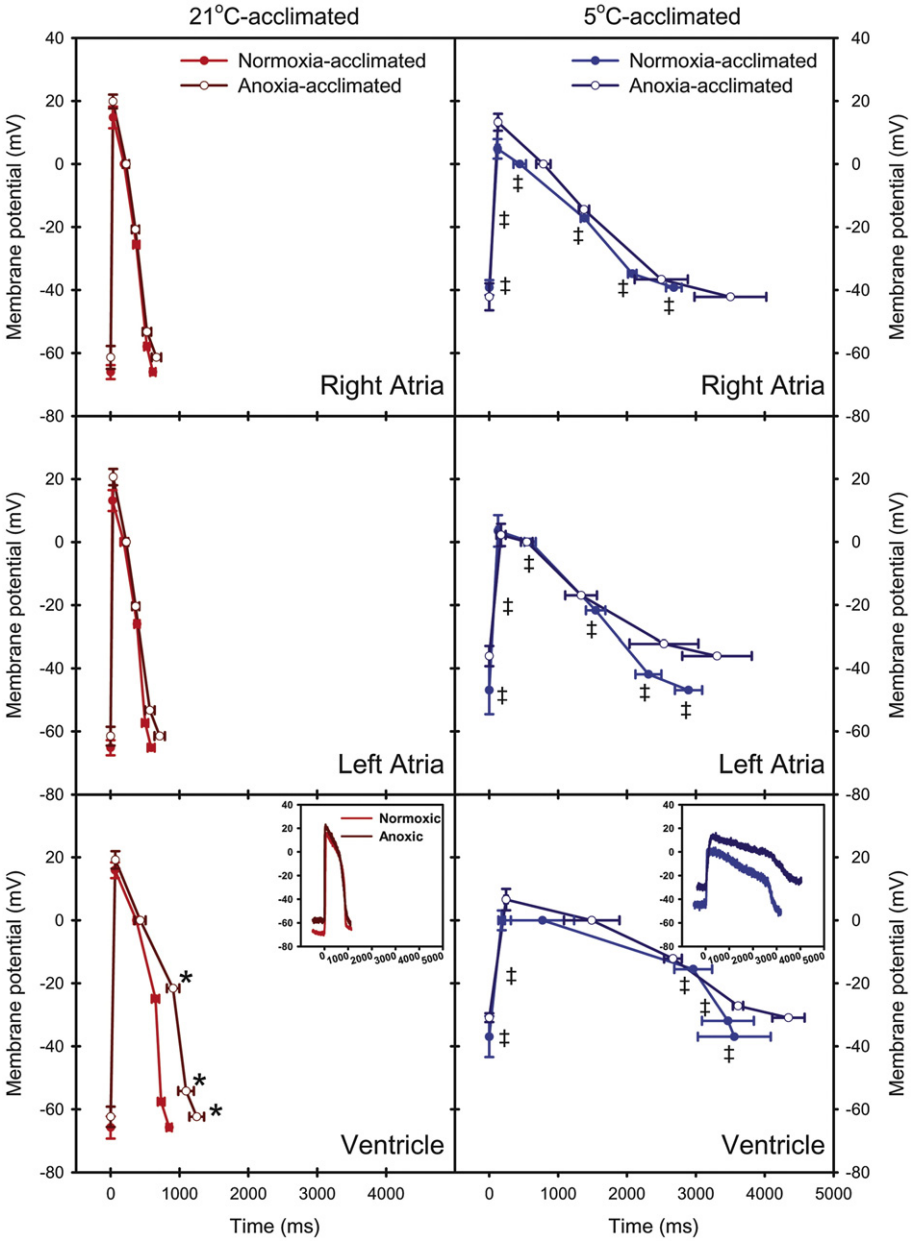

Fig. 5. Graphical representation and representative recordings (insets) of right atrial, left atrial and ventricular action potentials (AP) recorded from spontaneously contracting heart preparations from 21 °C normoxia-acclimated, 21 °C anoxia-acclimated (6 h anoxia exposure), 5 °C normoxia-acclimated and 5 °C anoxia-acclimated (14 days anoxia exposure) turtles. Action potential shape and duration was quantified by measuring resting membrane potential (Vm), peak potential and calculating duration to 0 mV, 50%, 90% and 100% repolarization. Anoxia exposure has minimal effect on turtle cardiac APs. At 21°C, APs of the right and left atria are not modified by anoxia exposure, but the duration of ventricular AP increases by 47%. APs of all cardiac chambers remain unchanged following 14 days of anoxia at 5 °C. In contrast to the effects of prolonged anoxia exposure, the shape of turtle cardiac APs is substantially modified by cold acclimation. Vm becomes less negative, AP upstroke rate decreases and AP duration prolonged. Asterisks signify statistically significant changes ($P<0.05$) in AP shape for a specific tissue following anoxia exposure. Double crosses (‡) signify statistically significant changes ($P<0.05$) in AP shape for a specific tissue following cold acclimation. Values are means±S.E.M.; $N=4$–5 turtles. Data adapted from Stecyk et al. (2007b).

Table 1
Effects of anoxia exposure, cold acclimation and anoxia combined with acidosis on the electrophysiological properties of the turtle and crucian carp heart

Tissue		Red-eared slider turtle (*Trachemys scripta*)				Crucian carp (*Carassius carassius*)	
		Effect of anoxia	Effect of anoxia	Effect of anoxia cold-acclimation	Effect of anoxia+acidosis	Effect of anoxia***	Effect of cold-acclimation
		(6 h at 21 °C)	(14 days at 5 °C)	(Q_{10} from 21 °C to 5 °C)	(at 5 °C or 21 °C)		(from 18 °C to 4 °C)
Whole heart	Intrinsic f_H	−0.3x	−0.5x	−6.4x (Q_{10}=3.2)	nc	nc (5 days at 8 °C)[d]	−4.3x (Q10=2.9)[a, **]
Ventricular cardiomyocytes	Peak I_{Na}	+2x	nc	−7.3x (Q_{10}=3.4)	nd	nd	−4.8x (Q10=3.0)[f]
	Peak I_{Ca}	nc	nc	−13.2x (Q_{10}=5.0)	nd	nc (seasonally captured fish)[e]	−6.1x (Q10=3.6)[e]
	Inward I_{K1}	nc	−0.33x (at −120 mV) −0.18x (at −100 mV)	−0.26x (at −120 mV)	nd	nc (20 days at 4 °C)[c]	nd
	I_{K1} inward slope conductance	nc	−0.45x	−0.5x (Q_{10}=1.4)	nd	nc (20 days at 4 °C)[c]	nd
Ventricle	Vm	nc	nc	+28.9 mV	nc	less polarized[b]	nd
	AP upstroke rate	nc	nc	−4.7x (Q_{10}=2.6)	nc	nc[b]	nd
	APD	+1.5x	nc	+4.2x (Q_{10}=2.6)*	nc	+1.15x[b]	nd
Right atria (atria for crucian carp)	Vm	nc	nc	+26.8 mV	nc	less polaraized[b]	nd
	AP upstroke rate	nc	nc	−6.8x (Q_{10}=3.3)	nc	nc[b]	nd
	APD	nc	nc	+4.4x (Q_{10}=2.4)*	+1.2x (21 °C; APD_{50})	nc[b]	n/a
Left Atria	Vm	nc	nc	+19.4 mV	nc	n/a	n/a
	AP upstroke rate	nc	nc	−5.2x (Q_{10}=2.8)	nc	n/a	n/a
	APD	nc	nc	+4.9x (Q_{10}=2.6)*	+1.2% (5 °C; APD_{50})	n/a	nd

AP: action potential; APD: action potential duration; APD_{50}: time to 50% repolarization f_H: heart rate; nc: no change; nd: not determined; Vm: resting membrane potential.
*Q_{10} values calculated for time to 90% repolarization.
**Difference between 15 °C- and 5 °C-acclimated fish.
***Anoxia achieved via metabolic inhibition by cyanide for action potential data.
Turtle data from Stecyk et al. (2007b); [a]Crucian carp data from Matikainen and Vornanen (1992), [b]Vornanen and Tuomennoro (1999), [c]Paajanen and Vornanen (2003), [d]Stecyk et al. (2004b), [e]Vornanen and Paajanen (2004), [f]Haverinen and Vornanen (2004) and [g]Paajanen and Vornanen (2004).

thus decrease the net depolarizing current (Golod et al., 1998). Therefore, the reduced I_{K1} density and conductance with a 14 days anoxic exposure at 5 °C would mean less Na^+ current is needed to trigger an AP.

Collectively, these findings do not lend any support to channel arrest being a ubiquitous means of energy conservation for anoxic survival in excitable tissues. The doubling of I_{Na} with anoxia exposure at 21 °C is the direct opposite of the down-regulation of Na^+, K^+ and Ca^{2+} channel activities in turtle brain and liver during acute experimental anoxia (Chih et al., 1989a; Pérez-Pinzón et al., 1992; Buck and Hochachka, 1993; Bickler and Buck, 1998). However, the reduced I_{K1} with anoxic exposure at 5 °C turtles can be considered consistent with the channel arrest hypothesis, as can the unchanged I_{Na} with anoxia at 5 °C. The down-regulation of I_{K1} density and conductance with prolonged anoxia may reduce demands on the Na^+/K^+-ATPase by reducing K^+ leakage across the sarcolemma (Roden et al., 2002). However, the density of ventricular Na^+/K^+-ATPase pump increases by approximately 30% with prolonged, cold anoxia (Overgaard et al., 2005), which appears counterproductive for energy conservation. It is unknown if the efficiency and activity of this pump changes with cold anoxia. Thus, in contrast to the anoxic turtle brain and liver, which utilizes the channel arrest strategy, a reduction in cardiac mechanical work (bradycardia) may provide the main means to survive cardiac anoxia, without the necessity to alter membrane ion gradients. Further studies are necessary to clarify this contention.

4. Crucian carp: sustaining cardiac activity during anoxia

4.1. Cardiovascular status and its control during anoxia exposure

Unlike the turtle, the crucian carp remains active during prolonged anoxia, prevents lactate from accumulating by converting it to ethanol and also maintains routine cardiac performance, (see Shoubridge and Hochachka, 1980; van Waarde, 1991; Nilsson, 2001; Stecyk et al., 2004b; Farrell and Stecyk, 2007). Nevertheless, the immediate and acute cardiovascular response to anoxia in the turtle and crucian carp (as well as the common carp, *Cyprinus carpio*), like other fishes, is a substantial bradycardia (Vornanen, 1994a; Vornanen and Tuomennoro, 1999; Stecyk and Farrell, 2002, 2006; Farrell, 2007). However, by 48 h of anoxia at 8 °C, cardiac output (Q), f_H, PO and stroke volume in crucian carp are all returned to control normoxic levels, where they remain stable for at least 5 days (Stecyk et al., 2004b)(Fig. 6). The crucian carp's ability to maintain routine cardiac activity during anoxia implies that routine cardiac ATP demand is matched by ATP supply and it is believed this is possible because cardiac ATP demand of their routinely low PO is below their maximum glycolytic potential (Farrell and Stecyk, 2007). This cardiac ATP conservation strategy comes about through a low arterial blood pressure (Stecyk et al., 2004b). Maintaining Q when there is no need to move oxygen in the blood has been proposed (Stecyk et al., 2004b; Farrell and Stecyk, 2007) to be essential for shuttling ethanol to the gills for excretion and distributing glucose from the crucian carp's large liver glycogen stores (Holopainen and Hyvärinen, 1985; Hyvärinen et al., 1985).

The crucian carp's maintenance of Q during anoxia diametrically contrasts with the profound reduction of Q in anoxic turtles, and if it is a corollary to their anoxic waste management strategy of converting lactate to ethanol, it should not come as a surprise that neural control systems remain functional: the autonomic control of the heart and peripheral circulation remains intact (Vornanen and Tuomennoro, 1999; Stecyk et al., 2004b) and the brain remains functional (Lutz and Nilsson, 1997; Nilsson, 2001). The retention of integrated and fine cardiovascular control in anoxic crucian carp clearly contrasts with the blunting of autonomic cardiovascular control in cold, anoxic turtles. Nevertheless, it remains to be determined if this level of control is maintained beyond 5 days of anoxia and at colder acclimation temperatures. Like the turtle, there is no adenosinergic inhibition of the anoxic crucian carp heart. Despite adenosine having weak negative chronotropic and inotropic effects on normoxic carp cardiac tissue *in vitro* (Vornanen and Tuomennoro, 1999), pharmacological blockade of adenosine receptors had no significant effect on cardiac activity during either short-term anoxia at 22 °C (Vornanen and Tuomennoro, 1999) or 5 days of anoxia at 8 °C (Stecyk et al., 2007a).

4.2. Crucian carp cardiac electrophysiology

Studies of the electrophysiological properties of the crucian carp heart by Matti Vornanen's group at the University of Joensuu, Finland have contributed immensely to explaining how the crucian carp heart

prepares for and survives winter anoxia. The gross morphology of crucian carp cardiomyocytes is generally similar to other teleost and reptilian species, including the freshwater turtle (Vornanen, 1997, 1998; Fig. 4). Crucian carp ventricular cardiomyocytes are relatively narrow (6 μm in width, approximately 100 μm in length), have a volume of 1.5 pl and a membrane capacitance of approximately 20 pF. They lack a T-tubular system and possess SR alongside the peripheral myofibrils located just beneath the sarcolemma, which accounts for ~40% of cell volume. However, compared with cardiomyocytes of the anoxia-sensitive and more active rainbow trout, the volume density of centrally located mitochondria is approximately half (~22% of cell volume). Instead, the crucian carp's cardiomyocytes contain large stores of glycogen, the density of which increases further during the autumn (Vornanen, 1994a), but no fat stores. Therefore, crucian carp cardiomyocytes, like the animal itself, are well provisioned for carbohydrate-based, anaerobic production of ATP.

E–C coupling in crucian carp cardiomyocytes is almost exclusively dependent upon transsarcolemmal Ca^{2+} influx and virtually

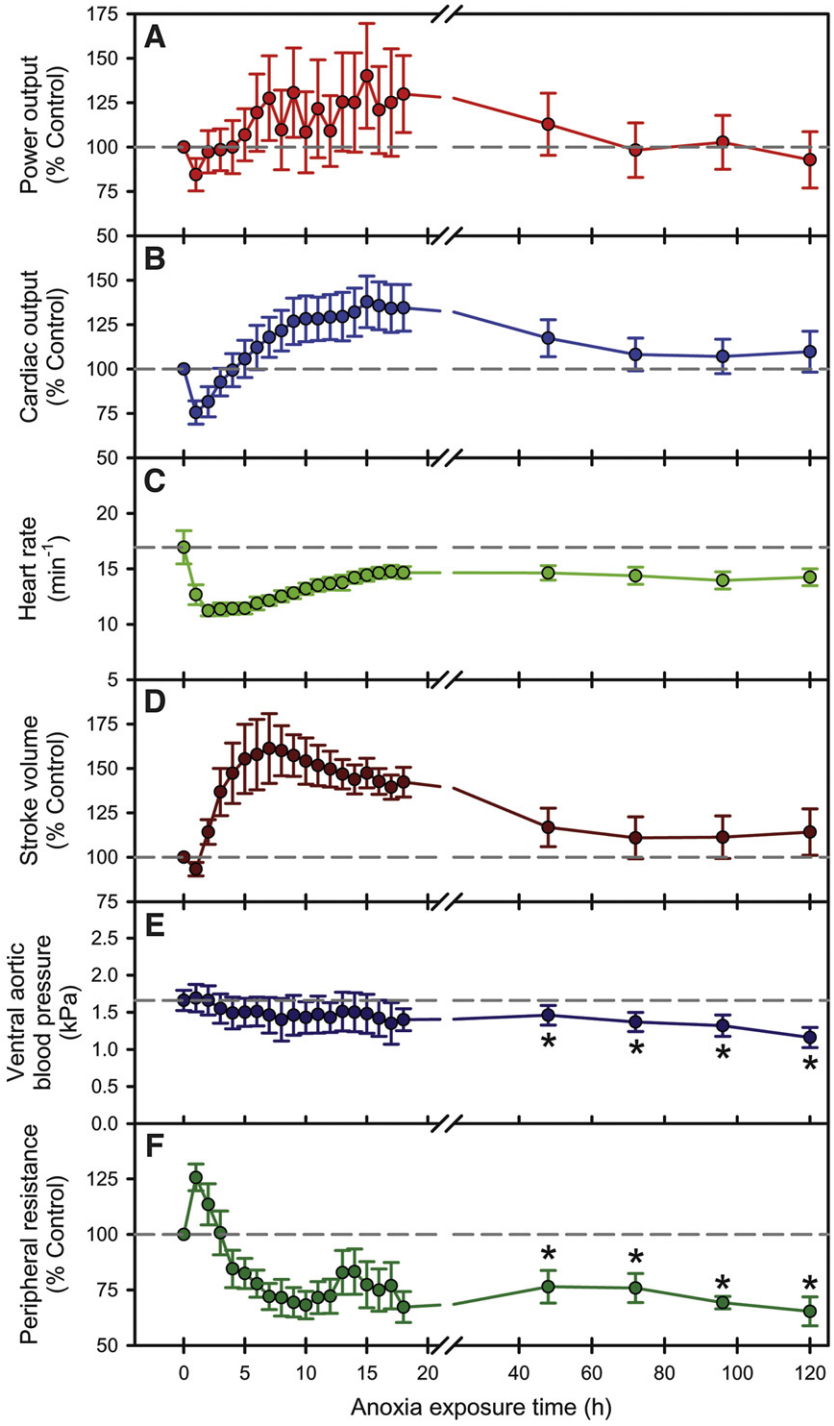

Fig. 6. Chronological changes of cardiovascular status in 8 °C-acclimated crucian carp during 5 days of anoxia exposure. In contrast to the turtle, the crucian carp maintains cardiac activity at normoxic control levels with anoxia exposure, although ventral aortic blood pressure and peripheral resistance decrease with prolonged exposure. Significant differences ($P<0.05$) between normoxic control (time zero) and hours 48, 72, 96 and 120 are indicated by asterisks. The dashed line indicates control normoxic values. Values are means ± S.E.M.; $N=6–18$. Data adapted from Stecyk et al. (2004b).

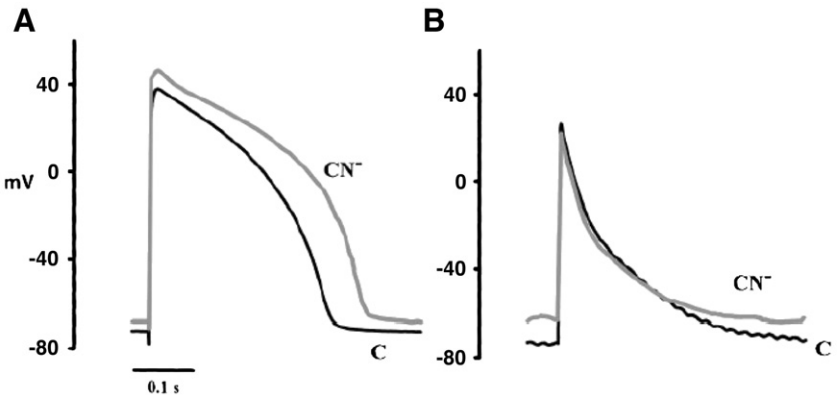

Fig. 7. Effects of metabolic inhibition by cyanide (CN^-) on action potential shape of enzymatically isolated crucian carp (A) ventricular and (B) atrial myocytes. Data from Vornanen and Tuomennoro (1999).

independent of SR Ca^{2+} release (Vornanen 1989, 1996, 1997, 1998, 1999; Tiitu and Vornanen, 2001). Crucian carp ventricular myocytes also have a large reverse-mode NCX Ca^{2+} current, which contributes up to 50% of $[Ca^{2+}]_i$ and is capable of initiating contraction (Vornanen, 1999). Thus, sarcolemmal Ca^{2+} influx through L-type Ca^{2+} channels and reverse-mode NCX can completely explain contractile activation in ventricular myocytes of crucian carp, similar to the turtle.

While inhibition of SR release channels has little to no effect on ventricular contraction, SR Ca^{2+} release channels do exist, but in low abundance compared with rainbow trout (Tiitu and Vornanen, 2003). Similarly, SR Ca^{2+}-ATPase activity can accumulate Ca^{2+} (Aho and Vornanen, 1998), but at a slower rate than in rainbow trout (Vornanen et al., 2002a). In the crucian carp atria, the SR plays a more prominent role as SR inhibitors negatively affect contraction (Tiitu and Vornanen, 2001).

Similar to the anoxic turtle, crucian carp cardiac electrophysiology is largely unaffected by severe hypoxia and anoxia. AP shape for cardiomyocytes isolated from warm-acclimated carp is only marginally affected by cyanide, an inhibitor of mitochondrial cytochrome-*c* oxidase and aerobic ATP production (Vornanen and Tuomennoro, 1999; Fig. 7; Table 1). In atrial cardiomyocytes, cyanide poisoning depolarizes Vm, but does not affect AP shape, whereas in ventricular cardiomyocytes, AP duration is lengthened by ~15% in addition to Vm depolarization. Moreover, whole-cell conductance, single-channel conductance and open probability of ventricular I_{K1} are unaffected after four weeks of severe hypoxia exposure (<0.4 mg O_2 l^{-1}) at 4 °C (Paajanen and Vornanen, 2003; Table 1). Thus, channel arrest does not appear to apply to crucian carp I_{K1}. Even so, sarcolemmal Na^+/K^+-ATPase activity is reduced by one third within 4 days of anoxia exposure at 4 °C as well as with the onset of hypoxic conditions in the natural environment (Aho and Vornanen, 1997). This change likely conserves ATP, but is at odds with the channel arrest hypothesis because it is not accompanied by a concomitant reduction of the major K^+ current. In mammals, Na^+/K^+-ATPase activity is regulated by intracellular Na^+ load (Despa et al., 2002). Therefore, the possibility exists that the decreased Na^+/K^+-ATPase activity in the anoxic crucian carp heart is linked to a decreased I_{Na}.

Seasonal studies of cardiac L-type Ca^{2+} channel abundance and I_{Ca} density provide further evidence against anoxic channel arrest as an ATP conserving mechanism in the anoxic crucian carp heart. The number of ventricular dihydropyridine receptors (the subunit of the L-type Ca^{2+} cardiac channel that triggers channel opening) and the density of I_{Ca} do not change with the seasonal decrease in water oxygen content in the natural environment (Vornanen and Paajanen, 2004). Therefore, cardiac down-regulation of L-type Ca^{2+} channels is not triggered by seasonal anoxia.

ATP-sensitive K^+ channels ($I_{K,ATP}$) are thought to exert cardioprotective effects during periods of oxygen deprivation in anoxia-sensitive mammals (Grover and Garlid, 2000), being kept closed by high intracellular ATP concentrations during normoxia, but opened when ATP concentration decreases during hypoxia exposure. With $I_{K,ATP}$ channels open, K^+ efflux increases, shortening AP duration and preventing depolarization of the membrane. Sarcolemmal and mitochondrial $I_{K,ATP}$ channels enhanced hypoxia tolerance of warm-acclimated (21 °C) goldfish cardiomyocytes (Cameron et al., 2003; Chen et al., 2005), but $I_{K,ATP}$ is small even under complete metabolic inhibition of both cold- and warm-acclimated crucian carp cardiomyocytes (Paajanen and Vornanen, 2002). Thus, $I_{K,ATP}$ channels may not be essential for cardiac anoxia survival in the crucian carp.

5. Cold-temperature: preparing the heart for anoxia exposure

Temperature alters the kinetic energy of molecules. Consequently, not only the rate of chemical reactions, but also physiological processes and, ultimately, whole-body metabolic rate, are slowed in ectotherms by decreased ambient temperature. Some ectotherms respond by compensating to continue an active lifestyle. Cardiac compensatory changes include increased relative ventricular mass (Graham and Farrell, 1989), increased myofibrillar ATPase activity and decreased refractory period (Aho and Vornanen, 1999), proliferation of the SR (Bowler and Tirri, 1990), modulation of Ca^{2+} cycling (Shiels and Farrell, 1997; Shiels et al., 2000; Shiels et al., 2002a,b), increased I_{Na} (Haverinen and Vornanen, 2004), and alterations in K^+ conductances that shorten AP duration (Vornanen et al., 2002b; Paajanen and Vornanen, 2004).

For the freshwater turtle and crucian carp, priming physiological processes to conserve fuel take priority and make normal compensatory changes maladaptive during the preparation for winter anoxia (Hochachka, 1986). Thus, these anoxia-tolerant vertebrates exhibit inverse thermal compensation, which means that physiological processes not only decrease with cold temperature, but in addition, are actively down-regulated, especially energy consuming processes, to further minimize ATP consumption (Herbert and Jackson, 1985b; Jackson, 2000). Here, we briefly synthesize information from *in vivo*, *in situ* and *in vitro* studies of acute and chronic cold exposure and illustrate how cardiac muscle of turtle and carp heart prepares for a low energy supply during winter anoxic conditions via inverse thermal compensation. We end by discussing recent data that suggests modification of cardiac electrophysiology is involved in this preconditioning.

5.1. Effects of low temperature on the turtle heart

Cold-acclimation substantially reduces *in vivo* turtle cardiac activity: f_H, systemic cardiac output (Q_{sys}) and PO_{sys} decrease 5- to 15-fold following acclimation to 5 °C from 21 °C–22 °C (Herbert and Jackson, 1985b; Hicks and Farrell, 2000a; Stecyk et al., 2004a, Stecyk et al., 2007a;

see Fig. 2). With f_H as low as 3 min^{-1}, Q_{sys} reduced to 4 ml min^{-1} kg^{-1} and PO_{sys} a mere 0.044 mW g^{-1}, the corresponding Q_{10} values are 3.5 for f_H, 3.6 for Q_{sys} and 8.8 for PO_{sys}. Clearly, inverse compensation actively depresses cardiac activity and decreases ATP demand for mechanical work by almost 10-fold.

However, autonomic cardiac control is not involved in this radical downregulation. Autonomic control is absent in cold-acclimated, normoxic turtles (Hicks and Farrell, 2000b). Rather, physiological adjustments intrinsic to the cardiac tissues, including those associated with E–C coupling, play a prominent role in depressing turtle cardiac activity with cold acclimation. This has been indirectly evidenced by acutely-cooled turtle heart preparations having a higher spontaneous f_H (~8 min^{-1}) than the *in vivo* intrinsic f_H (~3 min^{-1}) of cold-acclimated turtles (Farrell et al., 1994; Hicks and Farrell, 2000a) as well as faster rates of contraction and relaxation than preparations obtained from cold-acclimated turtles (Overgaard et al., 2005).

Direct evidence towards intrinsic cellular modifications contributing to cardiac depression with cold acclimation is in the form of substantial reductions in the density of sarcolemmal ion currents in the turtle heart following cold-acclimation (Stecyk et al., 2007b). Compared to 21 °C-acclimated turtle ventricular cardiomyocytes, acclimation to 5 °C reduces peak I_{Na} density by 7.3-fold and peak I_{Ca} density by 13-fold (Table 1), changes that would likely produce the slow the rate of contraction observed in other studies (Overgaard et al., 2005). Similarly, inward I_{K1} density is reduced by 26% and the inward slope conductance of inward rectifier K^+ channels reduced by almost 50% (Table 1). These changes in current densities substantially modify the shape of the ventricular AP, including a 4.2-fold increase in AP duration (see Fig. 5). Also, ventricular Vm is depolarized by ~29 mV (consistent with the reduced I_{K1}), and AP upstroke rate decreased by 4.7-fold (consistent with the reduced I_{Na}). Comparable changes in Vm and AP shape are similarly evident in the turtle atria with cold-acclimation (see Fig. 5), but corresponding measurements of ion current densities are unavailable. Importantly for energy conservation, the density of ventricular Na^+/K^+-ATPase is lowered with cold-acclimation (Overgaard et al., 2005).

To determine whether the electrophysiological changes are a result of active down-regulation required experiments that measured current densities for warm- and cold-acclimated cells after ventricular myocytes were acutely switched to a common temperature of 11 °C (Stecyk et al., 2007b). The results of such experiments show the reduction in peak I_{Na} is predominantly a passive cold temperature effect, whereas the density of functional L-type Ca^{2+} channels is actively down-regulated with cold acclimation. The overall I_{K1} conductance also decreases with cold acclimation, but the density of inward rectifier K^+ channels is up-regulated to partially compensate for the negative direct effect of cold temperature on I_{K1}. As noted above, passive cooling effects, rather than temperature acclimation, predominate in reducing spontaneous f_H (Stecyk et al., 2007b).

Similarly, not all of the changes in AP shape associated with cold-acclimation were reproducible by acute cold exposure, indicating active regulation (Stecyk et al., 2007b). Notably, the depolarization of Vm that occurs with cold-acclimation does not occur with an acute exposure of 21 °C-acclimated hearts to 5 °C. However, 5 °C-acclimated hearts acutely exposed to 21 °C have a Vm identical to that of 21 °C-acclimated hearts, suggesting that Vm depolarization with cold acclimation may be more quickly reversed (or compensated) than initiated.

Cold acclimation also appears to precondition the heart to better tolerate anoxia exposure and the accompanying extracellular changes. Spontaneous f_H of 5 °C-acclimated turtle hearts only slows when exposed to a combination of anoxia, acidosis and hyperkalemia, but these extracellular changes individually as well as collectively do slow the spontaneous f_H of warm-acclimated hearts (Reeves, 1963; Yee and Jackson, 1984; Jackson, 1987; Wasser et al., 1990a,b; Wasser et al., 1992; Farrell et al., 1994; Wasser et al., 1997; Stecyk and Farrell, 2007). The underlying mechanisms of this preconditioning effect remain to be discovered.

5.2. Effects of low temperature on the crucian carp heart

Laboratory-based cold acclimation and acute cold exposure studies, as well as those with seasonally-acclimatized fish captured from their natural ponds, have revealed that cold temperature induces numerous physiological changes in the crucian carp, many of which serve to prepare the heart for winter anoxia. For instance, in autumn, cardiac glycogen stores increase and are only depleted once anoxia ensues (Vornanen, 1994a; Vornanen and Paajanen, 2004). Also, the myosin heavy chain composition in crucian carp heart varies seasonally. In the winter, only the slow myosin heavy chain isoform is present, whereas both a fast and slow myosin heavy chain isoform are expressed during summer months; a finding that can be replicated with acclimation studies in the laboratory (Vornanen, 1994b). With a lower myosin-ATPase activity, the slow myosin heavy chain isoform is believed to produce force more economically than the fast summer counterpart, perhaps improving energetic economy of contraction and contributing to anoxia tolerance. Similarly beneficial may be the lower total ATPase activity of the carp heart in winter than summer (Aho and Vornanen, 1997).

In vivo f_H of laboratory acclimated, normoxic carp is temperature-dependent: ~65 min^{-1} at 22 °C, ~35 min^{-1} at 15 °C, ~17 min^{-1} at 8 °C, and ~15 min^{-1} at 5 °C (Vornanen and Tuomennoro, 1999; Matikainen and Vornanen, 1992; Stecyk et al., 2004b). For wild-captured fish, the f_H of ~65 min^{-1} in summer falls to ~10 min^{-1} in winter (Vornanen, 1994b). The relative importance of inhibitory cholinergic and stimulatory adrenergic tones on warm- and cold-acclimated carp heart is unknown, although, unlike cold-acclimated turtles, autonomic cardiac control is present in 8 °C-acclimated carp (Stecyk et al., 2004b). Nonetheless, part of the cold-induced bradycardia is intrinsic. At temperatures above 10 °C, spontaneous f_H of 5 °C-acclimated heart preparations is significantly lower than 15 °C-acclimated heats, indicating a modification of electrophysiological processes with cold-acclimation, but no difference in f_H exists at 4 °C (Matikainen and Vornanen, 1992).

Cardiac contraction kinetics are also modified by cold temperature. Consistent with a reduction in myofibrillar ATPase activity and isoform swapping, the velocity of cardiac contraction slows and contraction duration increases with cold acclimation (Matikainen and Vornanen, 1992; Vornanen, 1994a; Tiitu and Vornanen, 2001). Also, the refractory period of atrial and ventricular muscle is lengthened (Matikainen and Vornanen, 1992; Tiitu and Vornanen, 2001) and the rate of SR Ca^{2+} uptake is decreased (Aho and Vornanen, 1998), without alteration of the number of SR Ca^{2+} release channels in the ventricle (Tiitu and Vornanen, 2003). Further, the minor contribution of SR Ca^{2+} to atrial contraction at warm temperature is decreased in the cold (Tiitu and Vornanen, 2001).

Clearly, cold acclimation affects cardiac electrophysiology. Cold acclimation from 18 °C to 4 °C prolongs AP duration of crucian carp ventricular muscle by more than twofold from 1.3 to 2.8 s (Paajanen and Vornanen, 2004), which may explain the increase in refractory period (Vornanen, 1996). Cold acclimation (4 °C) also reduces the density of I_{Na} in ventricular myocytes to one-fifth and the I_{Ca} to one-sixth of that in warm-acclimated (18 °C) fish (Haverinen and Vornanen, 2004; Vornanen and Paajanen, 2004; Table 1). Further, the recovery of I_{Na} from inactivation becomes 44% slower with cold acclimation. I_{Na} is actively suppressed in cold-acclimated crucian carp in contrast to the predominant passive temperature effects in decreasing I_{Na} for turtles, but active down-regulation of I_{Ca} is similar in carp and turtle. Consequently, although 8 °C crucian carp are able to maintain routine *PO* during anoxia, it would seem that cold-acclimation plays a central role in pre-conditioning cardiac tissues and reducing its energy requirement as well as provisioning it with glycogen stores. Nevertheless, sarcolemmal Na^+/K^+-ATPase activity

does not differ between warm- and cold-acclimated or acclimatized carp, but instead decreases with the onset of hypoxia/anoxia (Aho and Vornanen, 1997; Paajanen and Vornanen, 2003).

6. Managing pH: another key to anoxia survival

Beyond balancing ATP supply and demand, anoxia-tolerant vertebrates must cope with the accompanying accumulation of lactate and H^+. This is because in all vertebrates studied thus far, acidosis reduces contractile force and can induce fatal ventricular arrhythmias, which dramatically decrease the ability of the heart to pump blood. In this section, we briefly summarize the strategies utilized by the turtle and crucian carp to combat a fall in pH during anoxia exposure. We then discuss cardiac cellular mechanisms employed to defend against acidosis and explore if differences exist between anoxia-intolerant and anoxia-tolerant hearts.

6.1. Anoxic acidosis in the turtle and carp

Turtles and carp both employ unique mechanisms to combat the extracellular acidosis (pH_e) that occurs during prolonged anoxia. Turtles release Ca^{2+}, Mg^{2+} and Na^+ carbonates from their bones and shell and also sequester lactate and H^+ into their bones and shell to buffer the accumulating wastes (Jackson et al., 1996; Jackson 1997, 2000, 2002, 2004). Carp convert the lactate into ethanol and CO_2 and release them to the surrounding water (Shoubridge and Hochachka, 1980; Nilsson, 2001). However, although the conversion of lactate to ethanol restores the NAD^+/NADH ratio, chemical potential energy is forever lost to the environment (van Waarde, 1991). Nonetheless, the survival benefit of preventing acidosis is of greater importance since acidosis debilitates cardiac function.

Despite these strategies, both turtles and crucian carp experience acidosis during anoxia, at least initially. Cold-acclimated anoxic turtles exhibit an almost linear decrease in plasma pH from a normoxic value of ~8.0 while plasma lactate concentration steadily increases up to 200 mM (Ultsch and Jackson, 1982). A similar, but more rapid pH change occurs with anoxia exposure for warm-acclimated turtles, with a pH of 6.7–7.0 representing the lower lethal limit for both warm- and cold-acclimated turtles (Herbert and Jackson, 1985a,b). In crucian carp, the onset of anoxia (at 15 °C) involves lactate accumulation in the plasma and a decrease of plasma pH from the normoxic level of ~7.7 (van Waarde, 1991). However, within 1.5 h of anoxic exposure and coincident with the commencement of ethanol production, both plasma pH (near 7.4) and plasma lactate (at ~8–10 mM) stabilize and are maintained for up to 26 h (van Waarde, 1991). At 8 °C, crucian carp, blood pH does not decrease below ~7.4 after a 7 days anoxia exposure (J. A. W. Stecyk, A. K. Dymowska and G. E. Nilsson, unpublished observation). Thus, an intriguing possibility is that the crucian carp heart can maintain routine cardiac performance during anoxia in part because extracellular acidosis is not severe.

6.2. Cellular mechanism employed to defend against acidosis

A decline in pH_e results in a secondary decline in intracellular pH (pH_i) which has significant and deleterious effects on E–C coupling. Additionally, acidosis decreases the sensitivity of the regulatory sites of TnC for Ca^{2+} such that less force is generated for a given amount of intracellular Ca^{2+} (Williamson et al., 1976; Fabiato and Fabiato, 1978; Orchard and Kentish, 1990). The cellular ion flux pathways involved in mounting a defence against an acidotic challenge have been worked out in considerable detail for the mammalian heart, but little is known for ectothermic vertebrate hearts. Isolated myocytes of acidotic-tolerant ectothermic species have not been studied in this regard except for the fall in pH_i in response to hypercapnia in the toad (*Buffo arenarum*; Salas et al., 2006). Also, ^{31}P nuclear magnetic resonance studies with both whole turtles (Stecyk, 2007) and isolated hearts (Wasser et al., 1990b) have documented the pH_i changes during extracellular acidosis and anoxia. For *C. picta bellii*, the rate and magnitude of intracellular acidosis depends on whether it is of metabolic or hypercapnic origin, but the defence of pH_i is less in the anoxic versus normoxic state (Wasser et al., 1990b).

pH_i must be controlled during anoxia-induced acidosis to prevent loss of cardiac force. The primary acid extrusion mechanism in mammalian cardiomyocytes is the Na^+-H^+ exchanger (NHE; Harrison et al., 1992). The NHE (and also a Na^+-HCO_3^- symport) increases Na^+_i during persistent acidosis which, through reverse-mode NCX, brings about an increase in intracellular Ca^{2+}. This increases $[Ca^{2+}]_i$ to directly recover contractile force. The real impact of increased reverse-mode NCX is mediated by the SR, which becomes progressively loaded with Ca^{2+} via increased Ca^{2+} influx. This SR Ca^{2+} is released in subsequent contraction cycles, dramatically increasing contractile strength. Pharmacologically blocking NHE prevents the recovery of pH_i, the rise in Na^+_i, the rise in Ca^{2+}_i, and the recovery of contractile force in mammalian cardiomyocytes (Harrison et al., 1992).

The cellular ion flux pathways initiated in response to acidosis have not been mapped in non-mammalian vertebrates. The dominant role for the SR in increasing $[Ca^{2+}]_i$ for recovery of force during persistent acidosis in mammals (Allen and Orchard, 1983; Orchard, 1987; Mattiazzi et al., 2007) raises the possibility that the very limited role for SR in cellular Ca^{2+} cycling in ectotherms could be a protective mechanism crucial for acidosis tolerance. The reasoning is as follows: SR Ca^{2+} loading during acidosis can be problematic for mammals when normal pH (and/or O_2) is restored. The increased SR Ca^{2+} release leads to Ca^{2+} overload causing potentially fatal ventricular arrhythmias. In some cases, this "reperfusion injury" results in severe myocardial damage and myocardial cell death (Maxwell and Lip, 1997) and constitutes a greater challenge for cellular ion homeostasis than acidosis or anoxia itself. The lack of an active SR in the turtle (Galli et al., 2006a,b) may explain the apparent absence of reperfusion injury following ischemia in the isolated turtle heart (Wasser et al., 1992).

Reverse-mode NCX plays an important role during normal ectotherm E–C coupling (Vornanen et al., 2002a; Galli et al., 2006b) and so could be involved in restoring contractility during acidosis. In toad ventricular myocytes recovery of pH_i, but not contractility, was dependent on NHE, suggesting that unlike for mammals, NHE does not play a critical role in the recovery of cardiac force during acidosis (Salas et al., 2006). Instead, reverse-mode NCX was important for contractile force recovery by altering the shape and duration of the cardiac AP. In the 20 °C-acclimated turtle, pH_i regulation occurred primarily via a Na^+-HCO_3^- symport, with NHE playing a minor role, and with anoxia decreasing the rate of pH_i regulation (Shi et al., 1997). Acidosis similarly alters cardiac AP shape and duration in isolated anoxic turtle hearts, with the exact response dependent on acclimation temperature and cardiac chamber (Stecyk et al., 2007b; Table 1). Thus, the plasticity of the ectotherm cardiac AP, and the ion channels that underlie it, may form the basis for acidotic tolerance. Clearly, further and direct investigations into these pathways are warranted. For instance, it would be interesting to determine intracellular Na^+ (Na^+_i) for normoxic and anoxic cardiomyocytes of the turtle and crucian carp as high Na^+_i may limit H^+ removal via NHE. Birkedal and Shiels (2007) recently report high (13 mM) Na^+_i in the anoxia-sensitive rainbow trout heart, which may reduce acidotic tolerance.

Ectotherms may also employ humoral means of protecting E-C coupling during an acidotic insult. The positive inotropic effect of adrenaline can protect the ectotherm myocardium from the adverse effects of hypoxia and exercise-induced acidosis (Milligan et al., 1989; Keiver and Hochachka, 1991; Wasser and Jackson, 1991; Keiver et al., 1992a,b). Adrenaline also increases f_H and can counteract acidosis-induced negative inotropy and chronotropy (Gamperl et al., 1998; Gesser, 1985; Milligan and Farrell, 1986; Hanson et al., 2006; Stecyk and Farrell, 2007).

Anoxia tolerant vertebrates withstand prolonged anoxia in part because they regulate pH_e and pH_i, which helps minimise disastrous effects on cardiac function. For the anoxic crucian carp, preventing severe acidosis through ethanol production could be the real key for maintaining cardiac activity, and thus survival, provided the glycolytic flux rate can be maintained at 14x normal (or enough to keep up with ATP demands). For the freshwater turtle, the pH_e excursion is greater, but so is the depression of cardiac contractility. Even so, pH_e must be maintained above pH 6.7–7.0 for continued cardiac function and anoxic survival. Thus, future research into adaptive mechanisms of anoxic cardiac tissue function should perhaps consider acidosis as being more detrimental in the long-term than anoxia *per se*, especially since cellular investigations with anoxia-tolerant vertebrate hearts are extremely limited. Available evidence suggests that unlike for anoxia-sensitive mammalian hearts, NHE is not a predominant means of pH_i regulation during persistent acidosis. Rather, the plasticity of the cardiac AP, and the ion channels hold greater promise for the basis for acidosis tolerance. Obviously, much future research is needed before the difference in the tolerance of acidosis between anoxia-intolerant and anoxia-tolerant hearts is fully comprehended.

7. Lessons from hypoxia-tolerant mammals

Although the mammalian myocardium is highly sensitive to oxygen deprivation, there are particular conditions and instances where the mammalian heart exhibits hypoxia, and even anoxia, tolerance. This phenomenon can be long-term, as with neonatal hearts, hibernators, altitude acclimation and the "hibernating myocardium" (Singer, 1999; Depre and Vanter, 2007), or transitory, as with pre-conditioning (Ravingerova, 2007). The final question we pose is: Are the ingredients of an anoxia-tolerant myocyte universal throughout the animal kingdom? If the answer is yes, then animals such as the freshwater turtle and the crucian carp provide a developmentally stable model to study anoxia tolerance, and their study may lead to the development of novel therapies for hypoxia related diseases of the heart (Ostadal et al., 1999). Unfortunately, very few researchers have utilized a comparative approach to investigate cellular cardiac adaptations to oxygen deprivation. The following section demonstrates that many of the fundamental mechanisms affording protection during hypoxia and ischemia in mammalian hearts are strikingly similar to those found in ectothermic anoxic survival.

The enhanced hypoxia tolerance of mammalian neonates has long been known among experimental and clinical researchers. Survival times of neonatal guinea pigs breathing pure nitrogen is 7 min and an impressive 50 min for newborn rats, while their adult counterparts rarely exceed 3 min (Fazekas et al., 1941). With respect to morphology and Ca^{2+} handling, neonatal myocytes more closely resemble those from ectotherms rather than adult mammals. They have a spindle-like appearance with a large surface area to volume ratio (Lu, 1999) due to a width and depth (7–10 μm) that is half those found in adults, but very similar to those found in fish and reptiles (Vornanen, 1998; Perasalmi, 1997; Galli et al., 2006b). Furthermore, neonatal myocytes lack the characteristic adult T-tubular network (Lu, 1999), similar to fish and reptiles. Neonatal and ectothermic myocytes are therefore adapted for sarcolemmal Ca^{2+} entry, and are heavily reliant on the L-type Ca^{2+} channel and the NCX, while the SR plays a limited role in cardiac contraction (Vornanen et al., 2002a; Nakanishi and Jarmakani, 1984). But whether these anatomical and physiological similarities contribute to hypoxia tolerance in ectotherms and neonates is unknown. Ca^{2+} release from the SR is the main cause of Ca^{2+} overload in anoxic adult mammalian myocytes, and it is tempting to speculate that the absence of Ca^{2+} overload in ectotherms and neonates may contribute to their protection against anoxic injury.

For newborn mammals, the most powerful line of defense against hypoxic insult is their ability to lower metabolism. Major energy savings come from abandoning temperature regulation and falling into a hypothermic state (Singer, 1999). Similar to the freshwater turtle, newborns subjected to severe hypoxia (6% oxygen) respond with a reduction in oxygen consumption which can fall to 75% of normoxic values (Moore, 1959) and respond to severe hypoxia or ischemia (which more closely resembles the anoxic condition) with a profound bradycardia and decrease in cardiac output (James et al., 1972; Harris et al., 1982), the opposite of the responses in adult mammals. Furthermore, severe hypoxia (7% oxygen, 30 Torr) combined with acidosis (pH 7) leads to a reduction in the rate of sino-atrial node firing and the rate of slow diastolic depolarization (Stowe et al., 1985). Action potential duration and amplitude are also markedly reduced (Stowe et al., 1985).

Hypometabolism is not restricted to neonatal mammalians. A phenomenon termed "hibernating myocardium", lasting for days, months and even years, has been demonstrated clinically in patients that have previously undergone coronary bypass surgery and in various animal models (Bito et al., 2004; Depre and Vanter, 2007). Also, a reversible reduction in myocardial metabolism and contractile function following a brief period of ischemia serves to protect the heart from reperfusion injury and from future bouts of ischemia (ischemic preconditioning). Researchers have often assumed that the reduction in metabolism is entirely mechanical in origin (decreased contractile activity), but recent research has demonstrated non-contracting mammalian myocytes reduce metabolism (Casey and Arthur, 2000, Casey et al., 2002), suggesting myocardial hibernation down-regulates energy consuming processes other than contractile function. Indeed, reductions in proton leak, RNA and mRNA synthesis and protein synthesis have been measured in non-contracting myocytes exposed to severe hypoxia (Casey et al., 2002).

Other studies demonstrate electrical and cellular remodelling occurs in the hibernating myocardium. Decreased SERCA-2 activity and increased phospholamban protein expression have been found in human myocardial biopsies from patients with hibernating myocardium syndrome, but expression of NCX and ryanodine receptor remained unchanged (Nef et al., 2006). In isolated pig cardiac myocytes with hibernating myocardium syndrome, myocytes were enlarged, had a reduced and slowed contraction and a longer AP duration (Bito et al., 2004). A reduction in the global Ca^{2+} transient was also observed, and this was attributed to a reduction in the L-type Ca^{2+} channel current, while SR Ca^{2+} content remained stable (Bito et al., 2004).

In true hibernating mammals, hypometabolism and hypothermia are similarly critical survival strategies. Cardiac cellular remodelling is apparent in hibernators. The density of the SR per cell volume is greater in hibernators than in other adult mammals and increases further with hibernation, as does SR Ca^{2+} uptake rate (Skepper and Navaratnam, 1995; Belke et al., 1991), placing the SR central in the regulation of inotropic and arrhythmogenic effects. Additionally, the density of the SR Ca^{2+} release channel is increased and the SR Ca^{2+} content is higher (Liu et al., 1997; Dibb et al., 2004; Yatani et al., 2004). Taken together, these factors lead to an increase in the systolic Ca^{2+} transient (Dibb et al., 2004). In addition to alterations in SR function, myocytes isolated from hibernating woodchucks exhibit shorter AP durations than non-hibernating woodchucks (Kondo, 1987; Yatani et al., 2004), which is mainly due to a reduction in L-type Ca^{2+} channel current and faster inactivation kinetics (Yatani et al., 2004). In the same study, protein expression of SERCA-2 increased and phospholamban decreased in hibernating hearts (Yatani et al., 2004).

Although the cellular mechanisms underlying anoxia tolerance in the freshwater turtle and crucian carp remain largely unknown, studies from hypoxia/ischemia tolerant mammals provide a useful platform for designing future experiments. Furthermore, the striking similarities in survival strategies across the animal kingdom suggest fundamental cellular mechanisms have been conserved during the evolution of anoxia tolerance. Thus, investigation of these mechanisms in the freshwater turtle and crucian carp could find clinical relevance.

8. Concluding remarks

Hearts of freshwater turtles and crucian carp continue to function for prolonged time periods when completely deprived of oxygen. Such prolonged cardiac anoxia survival arises from an apparent ability to match cardiac ATP demand to the limited ATP supply from anaerobic glycolysis. In terms of mechanical work, turtles achieve this by substantially reducing *PO* during anoxia exposure to a level well below the maximum glycolytic potential. Conversely, crucian carp have evolved a normoxic *PO* that falls below the maximum glycolytic potential because of a low arterial blood pressure.

Despite these different cardiac anoxia survival strategies and contrary to the prevailing channel arrest hypothesis, current evidence suggests altered cardiac electrophysiology during prolonged anoxia exposure is neither a central nor an important means of cardiac energy conservation for either the anoxic turtle or crucian carp. For crucian carp, ventricular I_{K1} and I_{Ca} densities do not differ between normoxic and oxygen-deprived fish. For warm-acclimated turtles, ventricular I_{Na} density doubles, whereas densities of ventricular I_{Ca} and I_{K1} are unaffected. For cold-acclimated anoxic turtles, ventricular I_{Na} and I_{Ca} are unaffected by anoxia exposure, but ventricular I_{K1} is modestly reduced during anoxia, which may help to conserve ATP. Thus, rather than arresting individual currents, the anoxic turtle appears to conserve cardiac ATP during anoxia by reducing the frequency of APs (i.e., action potential arrest) and thereby the demand on cardiac ATPases. Available evidence indicates that a re-setting of intrinsic f_H to a reduced level at the level of the pacemaker cells is imperative to this strategy.

Unlike anoxia exposure *per se*, passive and acclimatory electrophysiological responses to cold temperature are imperative to preparing the turtle and carp heart for winter anoxia under ice. Cold temperature alters cardiac AP characteristics through substantial changes in the density of sarcolemmal ion currents and slows f_H. Such modifications presumably contribute to the depression of cardiac activity *in vivo*. Further, reductions in I_{Na}, I_{Ca} and I_{K1} should decrease ATP demand by reducing the energetic costs associated with ion pumping. The active down-regulation of some of these current densities beyond the acute effect of temperature in turtles and carp suggests that inhibition of these currents is imperative for energy conservation and prolongation of anoxia survival. Thus, the hypothesized "arrest" of channel currents with cold exposure appears to be a valid ATP conserving strategy for the hearts of anoxia-tolerant vertebrates. Still undiscovered, however, are the underlying mechanisms, which could include temperature-dependent changes in channel phosphorylation, transcription, translation, rate of protein degradation, or trafficking of channels to the sarcolemmal membrane.

Nevertheless, it is important to note that the effects of anoxia on APs and ion current densities summarized in the present review may differ from those *in vivo*. This is because turtle and carp ventricular I_{Na}, I_{Ca}, I_{K1} and I_{Kr} were characterized using square voltage clamp pulses which do not reflect the changes that occur with an AP. Moreover, extracellular recording solutions did not mimic the changes in ionic composition, pH and adrenaline that accompany prolonged anoxia. While defense strategies against pH_e are extremely important for continued function of the turtle and crucian carp hearts during anoxia, pH_e does decline progressively with anoxia in turtles and acidosis has been proposed as an ultimate limiting factor in turtles. Thus, future electrophysiological studies using physiologically relevant AP pulse-protocols as well as intracellular and extracellular conditions that better emulate extracellular and intracellular conditions of the anoxic turtle and crucian carp would be very insightful.

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