CONTROL OF CARDIOVASCULAR FUNCTION DURING PROLONGED ANOXIA EXPOSURE IN THE FRESHWATER TURTLE (TRACHEMYS SCRIPTA)

by

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA
January 2007
ABSTRACT

Unlike the majority of vertebrates, the freshwater turtle (*Trachemys scripta*) can survive anoxia for hours at warm acclimation temperatures and weeks at cold acclimation temperatures. During anoxia exposure, the turtle heart continues its role in internal convection, but systemic cardiac output and systemic cardiac power output are massively reduced, primarily due to a decreased heart rate (f_H). Therefore, control of f_H is critical to cardiac energy management during anoxia.

This thesis investigated what extrinsic, autocrine/paracrine and intrinsic mechanisms control the cardiovascular system of anoxic freshwater turtles. Cardiac control was examined at the level of the whole animal, organ (isolated heart chambers), and cell (isolated cardiac myocytes). A 2 x 2 exposure design allowed comparisons between 21°C- and 5°C-acclimated turtles under normoxia and anoxia.

For warm-acclimated turtles, I discovered a re-setting of intrinsic f_H that accounts for up to 57% of the anoxic bradycardia, which, when combined with cholinergic cardiac inhibition (previously known), fully explains the depression of f_H with anoxia. Interestingly, prolongation of ventricular action potential duration (APD) by 47% with anoxia was proportional to the reduction in intrinsic f_H.

My thesis also revealed how cold acclimation prepared cardiac muscle for winter anoxic conditions. Cold temperature decreased intrinsic f_H, prolonged cardiac APDs and reduced the chronotropic sensitivity to extracellular anoxia and acidosis. Also, the decreased peak densities of ventricular I_Na and I_Ca and conductance of I_K, observed in 5°C-acclimated turtle hearts compared with 21°C-acclimated turtle hearts could serve to conserve the ATP cost of ion pumping.

When cold-acclimated turtles were exposed to anoxia, during which cholinergic cardiovascular control is blunted, my thesis discovered that a re-setting of intrinsic f_H accounts
for up to 66% of the anoxic bradycardia. However, contrary to 21°C, there was no prolongation of cardiac APDs. No evidence was found for either α-adrenergic or adenosinergic cardiac inhibition in cold-acclimated anoxic turtles, yet their reduction of in vivo cardiac activity correlated with alterations in myocardial high-energy phosphate metabolism, intracellular pH and free energy of ATP hydrolysis. These novel insights point to the importance of future studies on pacemaker currents and cardiac refractoriness.
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<table>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ADPf</td>
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<tr>
<td>APD</td>
<td>action potential duration</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>$f_h$</td>
<td>heart rate</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>inorganic phosphate</td>
</tr>
<tr>
<td>$P_{sys}$</td>
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</tr>
<tr>
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<td>ventral aortic blood pressure</td>
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<tr>
<td>$PCr$</td>
<td>phosphocreatine</td>
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<td>phosphodiester</td>
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<td>cardiac power output</td>
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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to those people who have helped, aided, advised and supported me in many ways throughout my work on this thesis.

First and foremost, I would like to express my thanks to Dr. Tony Farrell, my senior supervisor. Tony, I find it hard to believe that I spent almost 1/4th of my life completing two graduate degrees under your supervision! It doesn’t seem so long. What I remember from my first visit to SFU in 1999 was your extreme enthusiasm for science. This trait has not waned since then and is very contagious. Also, thank you very much for your extreme patience and also for the seemingly impossible feed-back times on the writings of this thesis.

I would like to thank Dr. Tobias Wang, Dr. Gōran Nilsson, Dr. Hans-Otto Pörtner and Dr. Matti Vornanen for welcoming me into their laboratories to pursue my Ph.D. research ideas. My travels to foreign labs to work with these wonderful scientists and learn new techniques would not have been possible without generous scholarships and grants from the Natural Sciences and Engineering Research Council of Canada, the Company of Biologists, the Society for Experimental Biology and the University of British Columbia.

Also, I must also express thanks to my committee members: Dr. Jones; Dr. Wang; and Dr. Williams.

I would also like to thank my parents (Jeanette and Robert) and my brothers (Robert and Mark) for their interest, guidance and support through the years.

Finally, thank you to my wife Agnieszka. Your presence always makes even the most stressful of days worth living!
CO-AUTHORSHIP STATEMENT

This thesis has been written in manuscript format according to the guidelines of the Faculty of Graduate Studies, University of British Columbia. The below listed, co-authored research articles comprise chapters II through VI and Appendix I. The vast majority of the research and all data analysis presented in these research articles were conducted by me. Listed co-authors either assisted with portions of the research, provided lab space and equipment and/or contributed towards design of experiments and interpretation of obtained results. All manuscripts were written solely by me in consultation with my senior supervisor (Dr. Tony Farrell) as well as the co-authors. Appendix I is included as it appears published in Science.


CHAPTER I:  
GENERAL INTRODUCTION AND THESIS OVERVIEW  

Anoxia Tolerance in Vertebrates

Vertebrates have evolved efficient respiratory and circulatory systems to ensure adequate oxygen delivery to body tissues. This is because the creation of metabolic energy, adenosine triphosphate (ATP), is most efficient when carbohydrate, lipid or amino acid fuels are completely catabolized to CO₂ and H₂O in the presence of oxygen (Hochachka and Somero, 2002). For example, the complete oxidation of 1 mol glucose yields 29 mol ATP (Brand, 2003). However, in the absence of oxygen (anoxia), the sole route for ATP production, anaerobic glycolysis, yields only $1/14^{\text{th}}$ the amount of ATP compared to oxidative phosphorylation (i.e., 2 mol of ATP per 1 mol glucose converted to pyruvate). Consequently, the vast majority of vertebrate species are dependent upon molecular oxygen for survival and are usually unable to successfully cope with periods of oxygen deprivation longer than a few minutes.

Death from anoxia is primarily ascribed to the disruption of cellular processes in critical tissues with a high ATP demand like the brain and heart, which arises from a mis-matching of anaerobic ATP production to a continued ATP demand. With oxygen deprivation, anoxia-intolerant species attempt to maintain normoxic cellular ATP levels through the rapid depletion of phosphogen reserves (Lutz and Storey, 1997) and the up-regulation of the anaerobic ATP supply pathway glycolysis, a phenomenon termed the Pasteur effect (Storey, 1985). However, phosphogens are almost instantaneously depleted (Lutz and Storey, 1997) and finite glycogen stores are also rapidly diminished with augmented glycolytic flux, which to match aerobic ATP production rates would need to be up-regulated 14-fold (Storey, 1985). Therefore, ATP production soon falls passively with oxygen deprivation. Consequently, ATP-dependent ion-motive pumps such as the Na⁺/K⁺-ATPase fail, resulting in disruption of cellular resting membrane potentials and loss of the ionic integrity of cellular membranes (Boutilier, 2001). The
subsequent drift of intracellular and extracellular ions towards their thermodynamic equilibrium eventually depolarizes the cellular membrane and leads to the uncontrolled influx of Ca\(^{2+}\) through voltage-gated Ca\(^{2+}\) channels (Hochachka, 1986). The increased concentration of intracellular Ca\(^{2+}\) activates Ca\(^{2+}\)-dependent phospholipases and proteases that perpetuate the rate of membrane depolarization, leading to cellular swelling and ultimately cell death (i.e., necrosis).

In addition, apoptotic cell death can occur with oxygen deprivation (Hochachka et al., 1996; Graham et al., 2004). Briefly, for mammalian cardiac myocytes, the accumulation of hypoxia-inducible factor-1 with hypoxia induces transcription of the death-promoting BNIP3 gene. At neutral pH, BNIP3 is inactive. However, with acidosis, BNIP3 is translocated from the cytosolic compartment into the mitochondria membrane where it stimulates opening of the mitochondrial permeability transition pore. This leads to the release of apoptosis inducing factor, cytochrome c and calcium, which stimulate proteases and DNases involved in cell death.

In contrast to the majority of vertebrate species, a select number of ectothermic vertebrates, specifically a few species of hagfish, cyprinid fish and freshwater turtle, exhibit a profound ability to endure prolonged periods (days to months) of severe hypoxia or even anoxia exposure. This is due to the natural history of these animals. Briefly, hagfishes use a feeding mechanism that precludes gill ventilation, besides which, their dead prey may be located in a hypoxic environment (Hansen and Sidell, 1983). Common carp inhabit ponds that are eutrophic and become diurnally hypoxic, especially in summer months when aquatic plant respiration is high and the oxygen content of water is already low (Garey and Rahn, 1970). Freshwater turtles and crucian carp can overwinter under ice in ponds for periods of up to several months. As a result, they become progressively hypoxic since they cannot access the water surface to either breathe air (turtles) or the water itself becomes anoxic (Vornanen and Paajanen, 2004). Subsequently, these organisms employ a plethora of physiological strategies and biochemical mechanisms that presumably allow for a matching of ATP synthesis and ATP demand such that
the ionic integrity of cellular membranes is preserved beyond the minutes of survival of anoxia-intolerant vertebrates (Boutilier, 2001). Further, these anoxia-tolerant vertebrates have developed creative waste management strategies to successfully cope with the acidosis that accompanies anaerobic metabolism (Jackson, 2000; Nilsson, 2001). A synopsis of these strategies follows.

**Balancing ATP Supply and Demand during Prolonged Anoxia Exposure**

Theoretically, anoxia-tolerant vertebrates could balance ATP supply and demand during prolonged anoxia exposure by up-regulating glycolytic ATP production to match ATP supply to a maintained demand (the Pasteur effect), reducing energy demand to a level that can be supported by the reduced ATP available from anaerobic metabolism (hypometabolism), or through a combination of both of these strategies (Lutz and Nilsson, 1997). There are clear trade-offs with both strategies. Up-regulating glycolysis allows for continued activity during anoxia. However, like for anoxia-intolerant vertebrates, fermentable fuel stores become limiting. Therefore, these stores need to be substantial for long-term anoxia survival. Also, up-regulating glycolysis will lead to a rapid accumulation of potentially harmful anaerobic end-products and acidosis (Lutz, 1989). In contrast, a benefit of the hypometabolic strategy is that it greatly slows fermentable fuel depletion and waste accumulation, both of which can be critical for long-term anoxia survival. However, hypometabolism may cause the brain to shut down to a comatose-like state and consequently impair the animal’s ability to respond to external stimuli (Lutz and Nilsson, 1997).

Regardless of the survival strategy, exposure or acclimation of anoxia-tolerant vertebrates to cold temperature serves to enhance anoxia survival time. Decreased temperature reduces kinetic energy of molecules. Therefore, in the absence of compensatory measures, exposure of ectothermic vertebrates to decreased temperature will result in decreased rates of
chemical reactions, physiological processes and ultimately, whole-body metabolic rate and overall ATP demand. Thus, for the up-regulation of glycolysis anoxia-survival strategy, cold temperature exposure translates to extended availability of fermentable fuel stores, whereas for the hypometabolic anoxia-survival strategy, cold temperature exposure aids in reducing metabolic rate. The significant role of cold temperature in facilitating anoxia survival may be best exemplified by the freshwater turtle (*Chrysemys picta bellii*), which can recover from an anoxic submergence greater than 91 days at 3°C (Ultsch and Jackson, 1982), but can only survive 24 h of anoxic exposure at 20°C (Herbert and Jackson, 1985a). The importance of low temperature in promoting anoxic survival is further demonstrated by the active selection of colder ambient temperatures by ectothermic vertebrates deprived of oxygen when in a thermal gradient (Tattersall and Boutilier, 1997).

*Up-regulation of Glycolysis in the Crucian Carp*

The strategy of up-regulating glycolytic ATP production to meet somewhat reduced ATP demands is best exemplified in the brain of the anoxia-tolerant crucian carp (*Carassius carassius*). Crucian carp survive the months of North European winters in small, shallow lakes and ponds that become completely anoxic due to thick ice coverage that inhibits both photosynthesis and oxygen diffusion from the air (Holopainen and Hyvärinen 1985). Amazingly, crucian carp remain active during anoxia exposure and continue to swim, albeit at a reduced level compared to normoxia (Nilsson et al., 1993). Consequently, their brain must remain functional to coordinate locomotion (Lutz and Nilsson, 1997; Nilsson, 2001). Accordingly, there is no evidence of reduced protein synthesis (Smith et al., 1996) or channel arrest, an energy conserving strategy in which the number of functional ion channels and/or the probability that ion channels are open is reduced such that the cost of ion pumping to maintain membrane integrity is diminished, in the brain crucian carp during anoxia exposure (Johansson...
and Nilsson, 1995). Although, some aspects of central nervous system function are reversibly reduced during anoxia in *Carassius*. For example, auditory nerve activity is suppressed in the closely-related anoxic goldfish (*Carassius auratus*; Suzue et al., 1987) and anoxic crucian carp become blind (Johansson et al., 1997). Further, microcalorimetric studies of crucian carp brain slices show that there is at least a 30% - 40% reduction in ATP turnover during anoxia, a finding indicative of metabolic depression (Johansson et al., 1995). However, despite this reduction, an approximate 8- to 10-fold increase in glycolytic rate is still needed to maintain ATP supply. Similarly, the reduction of body heat production to 1/3rd of the normoxic level in *Carassius* during anoxia (Van Waversveld et al., 1989) is not large enough to avoid activation of the Pasteur effect.

Indeed, ATP supply in the brain of crucian carp is maintained through a sustained increase in glycolytic rate (Johansson et al., 1995). Specifically, the increase in glycolysis arises from an up-regulation of key glycolytic enzymes and increase in fructose 2,6-bisphosphate, a potent activator of glycolysis (Storey, 1987). Simultaneously, brain blood flow is increased and sustained at an elevated level to deliver fermentable fuel and remove wastes (Nilsson et al., 1994).

Briefly, regulation of glycolysis occurs primarily through the allosteric control of three rate-limiting steps in the glycolytic enzyme cascade (Ramaiah, 1974). The principle rate-limiting step is the conversion of fructose 6-phophate to fructose 1,6-bisphosphate catalyzed by phosphofructokinase (PFK-1) (Ramaiah, 1974; Storey, 1985). Under normoxia, ATP and citrate inhibit PFK-1 such that glycolysis is coordinated with the citric acid cycle. In contrast, the activators of PFK-1, namely fructose 6-phosphate, adenosine monophosphate, adenosine diphosphate and inorganic phosphate, accumulate under anaerobic conditions. Furthermore, PFK-1 activity is also stimulated by fructose 2,6-bisphosphate, which is formed from fructose 6-phosphate by the action of the enzyme phosphofructokinase-2 when glucose is rapidly
metabolized (Storey, 1985). The activity of PFK-1 is coupled to the regulation of the two additional rate-limiting steps in glycolysis. These are the conversion of glucose to glucose 6-phosphate by hexokinase (HK) and the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase (PK) (Ramaiah, 1974). Specifically, a decrease in the concentration of fructose 6-phosphate, resulting from the activation of PFK-1 leads to a decrease in the concentration of glucose 6-phosphate because of the high activity level of glucose 6-phosphate isomerase and ultimately a promotion of HK activity. Increased concentration of fructose 1,6-bisphosphate, also resulting from the activation of PFK-1, activates PK through feed-forward activation.

**Down-regulation of Glycolysis and Metabolism in the Freshwater Turtle**

Freshwater turtles, like the crucian carp can overwinter under ice in ponds for periods of up to several months. As a result, they become progressively hypoxic since they cannot access the water surface to breath. However, in contrast to the crucian carp, freshwater turtles of the genera *Chrysemys* and *Trachemys* exhibit a prolonged anoxia-survival strategy of down-regulating ATP demand and ATP production in concert such that ATP supply and demand is matched. With the onset of anoxia exposure, the turtle first attempts to compensate for the reduction in ATP generation by increasing brain blood flow (by 171% – 242%; Hylland et al., 1994; Hylland et al., 1996) and up-regulating glycolysis, as evidenced by the quick depletion of brain, heart and skeletal muscle glycogen stores (Daw et al., 1967; Wasser et al., 1991; Lutz and Nilsson, 1993). However, with protracted periods of anoxia (i.e. hours to days), a biochemical reorganization occurs and leads to a coordinated metabolic depression and a suppression of the Pasteur effect. For example, turtle brain blood flow returns to pre-anoxic levels within 1-2 h of hypoxic exposure (Hylland et al., 1994; Hylland et al., 1996), and the initially activated pyruvate kinase activity in turtle muscle is inhibited (Kelly and Storey, 1988). Ultimately, whole-body metabolic rate of warm-acclimated turtles (20°C – 24°C) is depressed to 15% - 18% of the
normoxic metabolic rate during prolonged anoxia exposure (Jackson, 1968; Herbert and Jackson, 1985b). For cold-acclimated turtles (3°C), the decreases in metabolic rate is even greater, reaching values less than 10% of the normoxic metabolic rate at 12 weeks of anoxia exposure (Herbert and Jackson, 1985b).

Metabolic rate reduction in the anoxia-tolerant turtle during prolonged anoxia exposure occurs through the suppression of ion pumping and protein turnover, the key ATP consuming processes of a cell (Hochachka, 1986; Hochachka et al., 1996; Hochachka and Somero, 2002). Na\(^+\), K\(^+\) and Ca\(^{2+}\) channel activity is down-regulated during anoxia in the turtle brain and liver (Chih et al., 1989a; Pérez-Pinón et al., 1992; Buck et al., 1993; Bickler and Buck, 1998). Suppressed protein synthesis, or translational arrest, occurs in turtle heart (Bailey and Driedzic, 1996), hepatocytes (Land et al., 1993) and brain (Fraser et al., 2001). Although, some stress proteins (Chang et al., 2000) and glycolytic enzymes (Hochachka et al., 1996) can be up-regulated during anoxia. Additionally, increased levels of inhibitory neurotransmitters (Nilsson and Lutz, 1991) further contribute to energy conservation in the anoxic turtle by reducing the electrical activity and firing frequency of brain cells (Chih et al., 1989b; Sick et al., 1993), a phenomenon termed spike arrest.

The Pasteur effect, like metabolism, is inhibited by a variety of means during prolonged anoxia in the turtle. For instance, the activities of PFK-1, glycogen phosphorylase and pyruvate kinase are reduced through post-translational phosphorylation (i.e., covalent modification) in the anoxic turtle (Kelly and Storey, 1988; Brooks and Storey, 1988, Brooks and Storey, 1989ab; Mehrani and Storey, 1995). Further, since the association of enzymes with the particulate fraction of the cell (i.e. membrane fractions, glycogen particles or structural portions of the cell) increases the efficiency of enzymatic pathways (Storey, 1985), the proportion of glycolytic enzymes, namely pyruvate kinase and lactate dehydrogenase, associating with the particulate, versus soluble fractions of the cell is significantly reduced following a long-term (20 h) anoxic
exposure compared to a short (5 h) anoxic exposure in the turtle (Duncan and Storey, 1992). Finally, the activity of glycolytic enzymes can also be depressed by the fall in pH that accompanies prolonged anoxia (Storey, 1996).

**Glycogen Stores**

Both the crucian carp and freshwater turtle sustain prolonged glycolysis during anoxia exposure with large liver glycogen stores. Prior to the winter months, the crucian carp increases the fractional weight of its liver from 2% to 14%, and 30% of the liver mass is glycogen (Holopainen and Hyvärinen 1985; Hyvärinen et al. 1985). The amount of glycogen stored by the turtle is somewhat less, accounting for 15% of the mass of the liver (Clark and Miller, 1973). Further, large glycogen reserves are also found in the brain and heart of crucian carp (Schmidt and Wegener, 1988; Lutz and Nilsson, 1994; Vornanen, 1994; Vornanen and Paajanen, 2004; Vornanen and Paajanen, 2006) and in the brain, heart and skeletal muscles of turtles (Daw et al., 1967; Wasser et al., 1991; Lutz and Nilsson, 1993; Warren et al., 2006). However, it is the liver glycogen stores that are used to fuel anaerobic metabolism during prolonged anoxia. In fact, it has been shown that at 8°C, a temperature at which crucian carp survives approximately 2 weeks of anoxia, the only factor that eventually limits anoxic survival of the crucian carp is the total exhaustion of glycogen stores (Nilsson, 1990). Similarly, an exhaustion of tissue glycogen reserves, particularly those of the liver, coincides with the anoxia survival limit (≈ 44 d) of the red-eared slider turtle (*Trachemys scripta*; Warren et al., 2006).

The mobilization of metabolic fuel from the glycogen stores is facilitated through changes in enzyme activities that favor glycogenolysis. Briefly, increased levels of circulating catecholamines (Wasser and Jackson, 1991; Keiver et al., 1992) stimulate hepatic glycogenolysis and the release of glucose into the blood via adrenergic receptors in the liver (Hems and Whitton, 1980; Keiver and Hochachka, 1991; Horton et al., 1996). Hormonal stimulation results
in an activation of protein kinase A, which catalyzes the phosphorylation and activation of phosphorylase kinase, as well as the phosphorylation and inactivation of glycogen synthase. Phosphorylase kinase in turn catalyzes the phosphorylation and activation of glycogen phosphorylase, leading to glycogen degradation.

**Waste Management during Prolonged Anoxia Exposure**

In addition to being able to balance ATP supply and demand during anoxia, anoxia-tolerant vertebrates have developed creative waste management strategies to successfully cope with the acidosis that accompanies anaerobic metabolism. For instance, hagfish have a high blood volume (~15% of body mass; Forster et al., 1989), which is 5-times higher than teleosts (Olson, 1992) and may be important in buffering metabolic wastes. In addition, hagfish cardiac cells have a peculiarly thick glycocalyx, which may be important in protecting the extracellular calcium supply to cardiac myocytes from the effects of extracellular acidosis (Poupa et al., 1984).

Fish of the genus *Carassius* combat the decreased pH accompanying anaerobic metabolism by converting lactate produced by glycolysis into ethanol and CO₂, which are easily excreted into the ambient water (Shoubridge and Hochachka, 1980; Nilsson, 2001). The conversion of lactate to ethanol and CO₂ restores the NAD⁺/NADH ratio, but a disadvantage is the loss of chemical potential energy to the environment (van Waarde, 1991). Nonetheless, the benefit gained (i.e. survival) from the conversion of lactate to ethanol and CO₂ during periods of prolonged anoxia outweighs the associated cost.

Freshwater turtles utilize their bones and shell to reduce the harmful effects of H⁺ accumulation during prolonged anoxia exposure (Jackson et al., 1996; Jackson, 1997, Jackson, 2000; Jackson; 2002). Specifically, calcium, magnesium and sodium carbonates are released
from the shell into the extracellular fluid in exchange for lactate to supplement extracellular buffering of H\(^+\) (Jackson, 2004).

**Cardiovascular Status of Anoxia-tolerant Vertebrates during Prolonged Anoxia Exposure**

The heart is a unique tissue because its metabolic activity, cardiac output, is dependent on the whole-body demand for blood flow, which reflects whole-animal metabolic rate (Jackson, 2000). Thus, examination of the working heart provides perspective on whole-animal metabolic rate. For instance, under resting conditions, cardiac performance will be at some control level to support routine metabolic needs of the organism. However, if metabolic needs increase, for example, during exercise, cardiac performance will also increase to maintain sufficient oxygen delivery to the metabolically active tissues. Similarly, if metabolic rate is reduced, cardiac performance will decrease accordingly. For anoxia-tolerant species during prolonged anoxia exposure, the heart continues its role in internal convection despite the absence of oxygen. Specifically, continued cardiac activity is needed to transport metabolites and anaerobic waste products between tissues. Thus, like with normoxia, the level of cardiac performance with anoxia exposure will be set by whole-body demand for blood flow. Therefore, anoxia-tolerant vertebrates with differing anoxia-survival strategies would be expected to exhibit different cardiovascular responses to prolonged anoxia exposure.

Additionally, investigation of working hearts provides an excellent solution to the difficult challenge of studying the simple principle that anoxic cardiac failure can be traced to inadequate matching of ATP supply to ATP demand. Cardiac power output (\(PO\); estimated from the product of cardiac output and central arterial pressure and an indirect measure of cardiac ATP demand) is directly related to cardiac ATP supply up to some maximal level during both normoxia (ATP supply estimated from myocardial \(O_2\) consumption) and anoxia (ATP supply estimated from lactate production rates) (Reeves, 1963; Arthur et al. 1997; Farrell et al. 1985;
Graham and Farrell 1990). Therefore, under both aerobic and anaerobic conditions, a stable \( PO \) indicates a matching of ATP supply and demand. Conversely, a decreasing \( PO \) suggests that ATP supply and demand are not balanced.

Similar to the above discussion on anoxia-survival strategies, it appears that anoxia-tolerant vertebrates utilize one of two potential strategies to match \( PO \) to the less efficient generation of ATP from anaerobic glycolysis during prolonged anoxia exposure (Farrell and Stecyk, 2006). Firstly, some anoxia-tolerant vertebrates appear to have evolved a routine \( PO \) that falls within the maximum \( PO \) that can be supported by cardiac glycolytic potential (estimated to be \(-0.7 \text{ mW g}^{-1}\) for hearts of ectothermic vertebrates at 15°C; Farrell and Stecyk, 2006). For instance, routine \( PO \) under normoxia for the Atlantic hagfish (\textit{Myxine glutinosa}) and Pacific hagfish (\textit{Eptatretus cirrhatus}) of 0.1 and 0.4 mW g\(^{-1}\), respectively, are well below that found in elasmobranches, teleosts, and \( 0.7 \text{ mW g}^{-1}\) (Farrell, 1991; Forster et al., 1991; Farrell and Jones 1992). Indeed, hagfish cardiac performance during severe-hypoxia exposure, as assessed by perfused heart preparations, is comparable to during normoxia (Axelsson et al., 1990; Forster et al., 1992). Specifically, heart rate \( (f_{\text{H}}) \) does not change appreciably during severe hypoxia in either \textit{Eptatretus} or \textit{Myxine}. However, the hypoxic durations were relatively short (15 min at 10°C and 30 min at 17°C), and \textit{in vivo} cardiac capabilities of hagfish during prolonged anoxia exposure are unknown.

Similarly, crucian carp, which have an estimated \( PO \) of \( 0.46 \text{ mW g}^{-1} \) under normoxia at 8°C, retain normal cardiac performance for at least 5 days of anoxia (Stecyk et al., 2004; Farrell and Stecyk, 2006; Appendix I). Specifically, after an initial adjustment period, cardiac output, \( f_{\text{H}} \), stroke volume, and \( PO \) all return to pre-anoxic levels throughout days 2 to 5 of anoxia, while ventral aortic blood pressure and peripheral resistance decrease significantly by 30% and 40%, respectively. Presumably, the waste management strategy of crucian carp of converting lactate
to ethanol (Nilsson, 2001) necessitates an active circulatory system (Stecyk et al., 2004; Farrell and Stecyk, 2006). However, this hypothesis remains to be confirmed.

The second option is to temporarily and substantially decrease cardiac metabolism so that \( PO \) is below maximum cardiac glycolytic potential during prolonged anoxia (Farrell and Stecyk, 2006). This strategy is exemplified by the common carp (\textit{Cyprinus carpio}) and freshwater turtles. Common carp tolerate hours of severe hypoxia (<2 kPa), but remain motionless on the bottom of aquaria and ventilate very shallowly at a normal rate (Stecyk and Farrell 2002). Temperature plays a central role in their survival time in severely hypoxic water: it is \(~3\) h at 15°C vs \(~24\) h at 5°C (Stecyk and Farrell 2002). Correspondingly, routine \( PO \) is similarly temperature-dependent in normoxic common carp (0.88, 0.71 and 0.29 mW g\(^{-1}\) at acclimation temperatures of 15, 10 and 5°C, respectively; Stecyk and Farrell 2006). With severe hypoxia exposure, \( PO \) of common carp is quickly and substantially reduced, with the relative reductions in \( PO \) similar at all three acclimation temperatures (76%, 87% and 72%). The reductions result in minimum \( PO \) values of \( 0.21 \) mW g\(^{-1}\) within 30 min at 15°C, \( 0.09 \) mW g\(^{-1}\) within 45 min at 10°C and \( 0.08 \) mW g\(^{-1}\) by the 11th hour at 5°C that are well below the theoretical anoxic capability of \( 0.7 \) mW g\(^{-1}\). However, the maximal reductions in \( PO \) are only maintained temporarily by common carp before they begin to increase towards normoxic levels, a change that signals the end of the severe-hypoxia survival period (Stecyk and Farrell 2002). The 3.2 to 7.7-fold reductions in \( PO \) exhibited by the anoxic common carp heart fall short of the 14-fold reduction needed to preclude an activation of a Pasteur effect. This suggests that the anoxic common carp heart depletes metabolic fuel at a faster rate than in normoxia and accumulates harmful anaerobic waste products at a greater rate than if there was no Pasteur effect. Ultimately, this likely reduces survival time.

Freshwater turtles tolerate anoxia exposure longer and reduce \( PO \) to a greater degree than common carp. Under laboratory conditions, freshwater turtles have survived anoxia for 6 h at
22°C, 12 h at 20°C, 3 days at 12°C, 10 days at 10°C, 22 days at 5°C, and 91-168 days at 3°C (Ultsch and Jackson 1982; Jackson and Ultsch 1982; Herbert and Jackson 1985ab; Hicks and Farrell 2000ab). Although, species differences in anoxia tolerance do exist (Ultsch, 1985; Ultsch and Jackson 1995, Warren et al., 2006). In conjunction with the large reduction in whole-animal metabolic rate that accompanies anoxia exposure (Jackson, 1968; Herbert and Jackson, 1985b), systemic cardiac power output ($PO_{sys}$) of the turtle decreases exponentially by 6.6-fold to a minimum of 0.10 mW g⁻¹ at warm acclimation temperatures and by 20-fold from 0.044 mW g⁻¹ to 0.002 mW g⁻¹ at cold acclimation temperatures (Hicks and Wang, 1998; Hicks and Farrell, 2000a). The large reduction in $PO_{sys}$ with anoxia results from a minor arterial hypotension (~30% decrease in arterial blood pressure) and a large decrease in systemic cardiac output ($Q_{sys}$; up 4.5-fold and 12.5-fold in warm- and cold-acclimated turtles, respectively). The decrease in $Q_{sys}$ is primarily affected by marked bradycardia (Hicks and Wang, 1998; Hicks and Farrell, 2000a). Specifically, $f_1$ decreases 2.5-fold from ~25 min⁻¹ to ~10 min⁻¹ within 1 h of the commencement of anoxia exposure at 21°C - 25°C and by 5-fold from ~5 min⁻¹ to less than 1 min⁻¹ within 48 h of the commencement of anoxia exposure at 5°C. The 6.6-fold reduction in $PO_{sys}$ at warm acclimation temperatures places the ATP demand of the heart well below its capability for anaerobic ATP supply (Reeves, 1963; Farrell et al., 1994; Arthur et al., 1997). Nevertheless, these reductions are insufficient to prevent a Pasteur effect. For 5°C-acclimated turtles, the 20-fold reduction of $PO_{sys}$ indicates that a Pasteur effect is not needed in the heart during prolonged anoxia exposure.

**Autonomic Cardiovascular Control in Anoxia-tolerant Vertebrates during Prolonged Anoxia Exposure**

The circulatory system of vertebrates is primarily regulated by the autonomic nervous system, as well as by hormonal and local factors. The autonomic nervous system consists of a
parasympathetic vagal inhibitory component and a sympathetic adrenergic excitatory component and is of paramount importance for regulation of blood pressure, cardiac contractility and distribution of blood flows between various vascular beds (Nilsson, 1983; Morris and Nilsson, 1994). Interestingly, previous studies that have investigated autonomic cardiovascular control of anoxia-tolerant vertebrates during anoxia exposure through injection of pharmacological agonists and antagonists specific to cell surface receptors have revealed that the importance of autonomic cardiovascular control during anoxia exposure varies among anoxia-tolerant vertebrates.

As described above, the hagfish heart does not exhibit a major cardiac down-regulation during severe-hypoxia exposure (Axelsson et al., 1990; Forster et al., 1992). This is not unexpected because the hagfish heart is aneural, and thus lacks cardiac vagal innervation (Nilsson, 1983). Under normoxia, cardiac β-adrenergic stimulation does appear to be important to maintain cardiac performance (Axelsson et al., 1990; Johnsson and Axelsson, 1996), presumably from endogenous catecholamines. However, the role of β-adrenergic modulation of the hagfish heart during prolonged anoxia exposure has not been investigated.

Unlike hagfish, autonomic cardiovascular control is present during prolonged anoxia exposure in crucian carp, despite the similar cardiac anoxia-survival strategy between the two species. Specifically, injections of atropine (cholinergic antagonist) increase and propranolol (β-adrenergic antagonist) decrease $f_H$ during anoxia at 8°C, as is the case during normoxia (Stecyk et al. 2004; Appendix I). Further, tonic α-adrenergic vasoconstriction is present in crucian carp under normoxia and with prolonged anoxia exposure (Stecyk et al., 2004; Appendix I). Pharmacological α-adrenergic blockade with phentolamine decreases ventral aortic blood pressure and total peripheral resistance under both normoxia and anoxia. Thus, crucian carp retain cholinergic inhibitory and adrenergic excitatory cardiac control as well as excitatory adrenergic vascular control during anoxia. This phenomenon is consistent with the brain of
crucian carp remaining functional during prolonged anoxia exposure (Lutz and Nilsson, 1997; Nilsson, 2001). It remains to be determined if autonomic cardiovascular control persists beyond 5 d of anoxia and at colder acclimation temperatures.

In common carp, autonomic cardiovascular control is important in mediating the large changes in cardiovascular status that accompany prolonged severe-hypoxia exposure at both warm- (15°C) and cold-acclimation (5°C) temperatures (Stecyk and Farrell, 2006). Intra-arterial injections of α- and β-adrenergic and cholinergic antagonists clearly reveal that an inhibitory cholinergic cardiac tonus, an α-adrenergic mediated peripheral vasoconstriction and a large stimulatory cardiac β-adrenergic tone are present. Specifically, a vagal mediated bradycardia reduces cardiac output and creates an arterial hypotension, which is partially attenuated by an α-adrenergic mediated increase in peripheral resistance (Stecyk and Farrell, 2006). The underlying stimulatory cardiac β-adrenergic tone may possibly protect the heart from attendant acidic conditions during prolonged, severe hypoxia as it does in rainbow trout (*Oncorhynchus mykiss*) (Farrell et al., 1983; Farrell and Milligan, 1986, Farrell et al., 1986; Hanson et al., 2006).

In warm-acclimated freshwater turtles, autonomic control of the heart is retained during prolonged anoxia exposure. Injections of atropine increase (Hicks and Wang, 1998; Hicks and Farrell 2000b) and nadolol (β-adrenergic antagonist; Hicks and Farrell 2000b) decrease cardiac activity. In fact, at 22°C - 25°C, ~30% of the reduction in $f_H$ occurring with anoxia can be ascribed to cholinergic cardiac inhibition (Hicks and Wang, 1998; Hicks and Farrell, 2000b). However, in 5°C-acclimated freshwater turtles, vagal inhibition does not account for the marked anoxic bradycardia (Hicks and Farrell 2000b). Atropine injection does not result in the increase in $f_H$ that would be expected based on observations under normoxia, and under anoxia for warm-acclimated specimens. Similarly, β-adrenergic cardiac control is attenuated with cold anoxia in the turtle (Hicks and Farrell, 2000b), which may be related to the fact that ventricular β-adrenoceptor density is almost halved by cold, anoxia exposure (Hicks and Farrell, 2000b). The
basis of the massive reduction in cardiac function of cold-acclimated freshwater turtles during prolonged anoxia remains unknown. Similarly, the mechanism(s) underlying the unaccounted portion of cardiac depression of warm-acclimated turtles during anoxia is unknown. Further, autonomic control of the peripheral circulation in freshwater turtles during anoxia has not been investigated.

**Thesis Objectives**

The main objective of this thesis was to investigate what extrinsic, autocrine/paracrine and intrinsic mechanisms contribute to cardiovascular control in 5°C-acclimated, anoxia exposed freshwater turtles and account for the large depression in cardiovascular status. These investigations spanned various levels of biological organization (i.e., whole-animal, tissue-level, and cellular-level).

A secondary objective of this thesis was to examine the effect of temperature acclimation in modulating these cardiovascular controls during anoxia in the freshwater turtle. Therefore, for each of the studies of this thesis, a 2 x 2 exposure design compared 21°C- and 5°C- acclimated, normoxia- and anoxia-exposed turtles.

**Study Species**

The red-eared slider (*Trachemys scripta*) freshwater turtle was selected as the study species. Although this species is not the champion of anoxia-tolerance in turtles [anoxia survival time at 3°C is approximately 44 days in *T. scripta* (Ultsch, 1985; Warren et al., 2006) compared to 3 to 6 months in the western painted turtle *Chrysemys picta* (Jackson and Ultsch, 1982; Ultsch and Jackson, 1982; Herbert and Jackson, 1985ab)], its anoxia-survival time greatly surpasses that of most vertebrates. Further, the majority of previous research investigating cardiovascular control in freshwater turtles, specifically the *in vivo* studies examining autonomic cardiovascular
control, have utilized *T. scripta* (i.e., Hicks and Wang, 1998; Hicks and Farrell, 2000ab; Overgaard et al., 2002). Therefore, I believed *T. scripta* to be the most suitable species to utilize as a model organism to investigate anoxic cardiovascular control in the turtle. Specifically, I reasoned that investigations using *T. scripta* would: 1) allow for the best comparison of my research findings with previous research; 2) serve to best advance our understanding of cardiovascular control during anoxia exposure of an anoxia-tolerant vertebrate; and 3) allow for the acquisition of in depth knowledge for a single species. In comparison, equivalent study with the painted turtle would have first required repetition of studies previously conducted with *T. scripta*.

**Cardiovascular Control Mechanisms Investigated**

Of the many cardiovascular control mechanisms that exist in vertebrates, only 5 are examined in this thesis (Table 1.1). These mechanisms were selected on the merits of: 1) filling information gaps in the literature on anoxic cardiovascular control in the turtle; 2) presumed likeliness to modulate anoxic cardiovascular status; and 3) further investigating findings of previous studies of this thesis. Extrinsic control was defined as any control mechanism originating outside or external to the tissues of the cardiovascular system. Thus, extrinsic control includes neural and humoral controls. Conversely, intrinsic control was defined as any control mechanism relating to the essential nature of the tissues of the cardiovascular system. Thus, the role of cellular energy status and electrophysiological properties of cardiac myocytes in modulating cardiac activity were considered to be intrinsic types of cardiovascular control. Within these definitions, adenosingergic control could be considered to be either extrinsic (due to the possibility of paracrine activity) or intrinsic (due to its autocrine activity). Detailed rationales for each cardiovascular control mechanisms investigated are presented in the State of Knowledge: Past and Present section as well as in the individual chapters (II – VI).
### Table 1.1 Overview of cardiovascular control mechanisms investigated in this thesis.

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Cardiovascular Control Mechanism Investigated</th>
<th>Type of Cardiovascular Control</th>
<th>Level of Biological Organization</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>α-Adrenergic</td>
<td>Extrinsic (Neural/Humoral)</td>
<td>Whole-animal</td>
</tr>
<tr>
<td>III</td>
<td>Adenosinergic</td>
<td>Extrinsic Paracrine/ Autocrine Intrinsic</td>
<td>Whole-animal</td>
</tr>
<tr>
<td>IV</td>
<td>Cellular energy state (determined by NMR spectroscopy)</td>
<td>Intrinsic</td>
<td>Cellular-level (Whole-animal)</td>
</tr>
<tr>
<td>V</td>
<td>Extracellular factors</td>
<td>Extrinsic (Humoral)</td>
<td>Tissue-level</td>
</tr>
<tr>
<td>VI</td>
<td>Electrophysiological</td>
<td>Intrinsic</td>
<td>Cellular-level</td>
</tr>
</tbody>
</table>

### Methodological Considerations

Several different techniques were used in this thesis to investigate cardiovascular control mechanisms in the turtle. A brief description of these techniques as well as their associated assumptions and limitations follows. More detailed description is given in the Materials and Methods and/or Discussion sections of the individual studies.

All procedures were in accordance with Animal Care Guidelines of the university or research institution at which they were conducted. These were: Aarhus University, Aarhus, Denmark for chapter II; Simon Fraser University, Burnaby, Canada and the University of Oslo, Oslo, Norway for chapter III; the Alfred-Wegener-Institute for Marine and Polar Research, Bremerhaven, Germany for chapter IV; Simon Fraser University, Burnaby, Canada for chapter V; the University of Joensuu, Joensuu, Finland and the University of British Columbia, Vancouver, Canada for chapter VI.
Temperature and Anoxia Acclimation of Turtles

Turtles acclimated to and 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 acclimated to and 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 (Hicks and Farrell, 2000a). The 5°C turtles were acclimated during winter months and were fasted during this period.

For prolonged anoxia exposure, turtles were exposed to anoxia for 2.85 - 6 h at 21°C or for 11 - 14 d at 5°C, depending on the study. The anoxia exposure times were chosen to be consistent with previous investigations of turtle cardiac activity and its control during prolonged anoxia (Hicks and Wang, 1998; Hicks and Farrell, 2000ab). Further, previous study has shown turtle cardiac activity to be relatively stable at these times. The cardiovascular adjustments to anoxia exposure occur within the first 1 h of exposure for warm-acclimated turtles and within 48 h of exposure for cold-acclimated turtles (Hicks and Farrell, 2000a).

The required anoxic conditions were achieved by first individually placing turtles into an enclosed, water-containing plastic chamber that still allowed access to air for 24 hours. Then, the plastic chamber was completely filled with water, continuously bubbled with N₂ (water \( P_{O_2} < 0.3 \text{ kPa} \)) and the turtle denied air access by means of a mesh that was suspended below the surface of the water. The assumption throughout all studies that comprise this thesis is that both 21°C- and 5°C-acclimated turtles were anoxemic.

In vivo Cardiovascular Measurements and Pharmaceutical Injections

For chapters II and III, *in vivo* cardiovascular status was measured. This was accomplished using a surgically-implanted ultrasonic flow probe(s) to measure blood flow
through major systemic arteries and an arterial catheter that was subsequently connected to a
pressure transducer to record systemic blood pressure ($P_{sys}$) (pressure calibration was performed
against a static water column). Detailed surgical procedures for placement of catheter and flow
probe(s) are given in the Materials and Methods sections of chapters II and III.

The primary advantage of measuring \textit{in vivo} cardiovascular status is that the work output
of the heart, $PO_{sys}$, can easily be assessed by multiplying $Q_{sys}$ and $P_{sys}$. As discussed above,$PO_{sys}$ can be expressed as ATP demand, which in turn can be related to rates of glycolysis. An
obvious disadvantage is that the surgical procedures may stress the animal. However, in all
instances, turtles were allowed to recover from surgery for either 48 h at 21°C or 72 h at 5°C.
Previous study has shown that for warm-acclimated turtles, cardiovascular status stabilizes
within 48 h (Hicks and Farrell, 2000a).

Cardiovascular control was assessed through intra-arterial injections of phenylephrine (an
$\alpha$-adrenergic agonist; chapter II), phentolamine (an $\alpha$-adrenergic antagonist; chapter II) and
aminophylline (a non-specific adenosine receptor antagonist; chapter III). The doses injected
were selected to be within the range previously shown to be of sufficient potency in other
vertebrates, including turtles (phenylephrine and phentolamine: Overgaard et al., 2002;
aminophylline: Mohrman and Heller, 1984; Long and Anthonisen, 1994; Sundin and Nilsson
1996; Sundin et al., 1999; Söderström et al., 1999; Stensløkken et al., 2004; Stecyk and Farrell,
2006).

\textbf{The Microsphere Technique}

For chapter II, the coloured microsphere technique was used to quantify systemic blood
flow distribution of the turtle during normoxia, prolonged anoxia, and following $\alpha$-adrenergic
stimulation and blockade. This technique required the implantation of a catheter into the left
atria of the turtle in addition to the systemic catheter and flow probes.
With the microsphere technique, regional blood flow is assumed to be proportional to the number of microspheres trapped in the tissue of interest (Heyman et al., 1977; Bassingthwaigte et al., 1990). However, accurate use of coloured microspheres requires that several criteria be satisfied. Foremost, all microspheres must be trapped in the capillary beds during their first passage through the circulation. Secondly, injected microspheres must be adequately mixed with blood at the injection site to provide a homogenous solution such that the same concentration of microspheres prevails at all arterial sites. Finally, an efficient microsphere isolation and purification protocol is needed such that absorbance intensities correlate directly with the number of microspheres. Complete trapping of microspheres during normoxia and anoxia was confirmed by the lack of microspheres in arterial blood samples withdrawn 20 min after injections. Good mixing of microspheres was assured by the slow injection of microspheres into the atrium, such that atrial and ventricular contraction created a homogenous distribution of spheres in the blood. Further, under normoxia, microspheres were injected during breath-hold, when pulmonary blood flow and Left-to-Right shunting is low to avoid the shunting of microspheres into the pulmonary circulations (e.g. White et al., 1989; Hicks, 1994; Wang and Hicks, 1996). Pulmonary blood flow is markedly reduced during anoxia exposure (Hicks and Wang, 1998). Finally, tissue type did not influence the efficiency of microsphere recovery.

In vivo Nuclear Magnetic Resonance Spectroscopy and Imaging

For chapter IV, nuclear magnetic resonance (NMR) spectroscopy and imaging were utilized to continuously measure in vivo cardiac energetic state and cardiac activity during prolonged anoxia. The main advantage of NMR is that it is non-destructive and non-invasive. Consequently, metabolic processes can be followed as they take place within a living organism. Specifically, $^{31}$P-NMR allows for direct observation of freely mobile phosphorous containing metabolites in the cytosol, as compared to the total amounts obtained with the traditional
technique of freeze-clamping. Also, since the areas of $^{31}$P-NMR signals are proportional to the concentration of metabolites that gives rise to them, chronological changes of metabolite concentrations can be observed.

The main disadvantage of the technique is that it is insensitive. Only components present in millimolar quantities are clearly resolvable in the NMR spectrum. Also, with in vivo NMR, the possibility exists that other tissues rather than the tissue of interest contribute to the acquired spectra. To ensure that $^{31}$P-NMR measurements were acquired from turtle cardiac tissue, MR imaging was used to confirm the correct position of the turtle within the NMR magnet.

**In vitro Spontaneously Contracting Atrial Preparation**

For chapter V, a spontaneously contracting right-atrial preparation was used to investigate the extracellular effects of anoxia, acidosis, hyperkalemia, hypercalcemia and adrenaline on the spontaneous $f_t$ of normoxia- and anoxia-acclimated turtles at 21°C and 5°C. This preparation involved excising the right atrium, attaching it to a force-displacement transducer and fixed-arm, and suspending the preparation in a water-jacketed organ bath containing the starting saline solution that approximated the in vivo extracellular conditions. Sequential exposures to saline solutions were designed to mimic, in a step-wise manner, the shift from a normoxic to anoxic extracellular condition (for normoxia-acclimated preparations) or the reverse (for anoxia-acclimated preparations).

Results from this study can be extrapolated to the in vivo situation because: 1) spontaneously contracting right-atrial preparations contracted at rates comparable to the in vivo intrinsic $f_t$; 2) control preparations maintained a stable $f_t$ for the duration of the experimental protocol; 3) compositional changes in saline solutions were physiologically relevant; and 4) saline solution exposure time was sufficient to reach a new steady state for $f_t$. 
Myocardial Action Potential Recordings and Whole-cell Electrophysiology

Chapter VI presents novel recordings of cardiac action potentials (APs) from intact cardiac tissue and of ventricular sarcolemmal voltage-gated Na\(^+\), inward rectifier K\(^+\), and delayed rectifier K\(^+\) channel currents from enzymatically-isolated myocytes for the turtle. Further, novel measurements of ventricular L-type Ca\(^{2+}\) channels (\(I_{Ca}\)) 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 (\(Trachemys scripta scripta\)) (Galli et al., 2006b). At the outset of chapter VI, the whole-cell patch-clamp technique had not previously been utilized to examine electrophysiological properties of individual turtle cardiac myocytes. Therefore, the myocyte isolation protocol and intracellular and extracellular solutions were modified from established whole-cell patch-clamp methodologies for teleost fish to be relevant for the turtle (Vornanen, 1997; Shiels et al., 2000; Vornanen et al., 2002; Paajanen and Vornanen, 2003; Haverinen and Vornanen, 2004; Haverinen and Vornanen, 2006; Shiels et al., 2006). Measurements of APs and ventricular myocyte sarcolemmal ionic currents were made on \textit{in vitro} tissues/cells under conditions similar to those of the animal from which they were derived.

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 (Ruppersberg, 2000), and leads to deterioration (i.e., rundown) of currents over time (Kostyuk, 1984). However, the magnitude and degree of repeatability of \(I_{Ca}\) that I report is comparable to that recently reported for cardiac myocytes of yellow bellied turtles with the perforated-patch technique (Galli et al., 2006b), a technique that overcomes this concern of whole-cell patch-clamp. Another limitation is the square voltage-clamp pulse protocols utilized do not emulate the change in membrane potential that occurs
during a physiological AP. Therefore, square pulse studies have only limited application to the
\textit{in vitro} condition.
References


CHAPTER II:
α-ADRENERGIC REGULATION OF SYSTEMIC PERIPHERAL RESISTANCE AND BLOOD FLOW DISTRIBUTION IN THE TURTE (TRACHEMYS SCRIPTA) DURING ANOXIC SUBMERGENCE AT 5°C AND 21°C

Introduction

Freshwater turtles, particularly those of the genera Chrysemys and Trachemys, exhibit a remarkable ability to survive prolonged periods of anoxia (Johlin and Moreland, 1933; Jackson and Ultsch, 1982; Ultsch and Jackson, 1982; Herbert and Jackson, 1985ab; Hicks and Farrell, 2000a). At 3°C, these turtles recover physiological functions after twelve weeks of anoxia and survive for up to five months of anoxia (Ultsch and Jackson, 1982; Herbert and Jackson, 1985b). This is achieved by metabolic depression, where biochemical and metabolic adaptations balance anaerobic energy production to the reduced demand, and a large capacity of the shell to buffer the ensuing metabolic acidosis and elevated lactate levels (Jackson and Schmidt-Nielsen, 1966; Jackson, 1968, 2000, 2002; Herbert and Jackson, 1985b; Storey, 1996; Lutz and Storey, 1997).

The cardiovascular system continues to operate during anoxia to transport metabolites between tissues, but because of the low metabolic demand of the body and direct effects of reduced oxygen on the myocardium, both heart rate (f_H) and systemic blood flow (Q_sys) are greatly depressed (Herbert and Jackson 1985b; Hicks and Wang, 1998; Hicks and Farrell, 2000a). While systemic blood pressure (P_sys) also decreases during anoxia, the reduction in Q_sys is considerably larger, indicating a substantial (three to five-fold) increase in systemic peripheral resistance (R_sys) (Hicks and Farrell, 2000a). The basis of this augmented R_sys, which maintains the systemic circulation in a state of hypotension during anoxia, remains unidentified (Hicks and Farrell, 2000b).

In most vertebrates, R_sys is predominantly controlled by α-adrenergic innervation of the resistance vessels, and α-adrenergic mediated peripheral vasoconstriction occurs during hypoxia in many species (Lillo, 1979; Fritsche and Nilsson, 1989; Axelsson and Fritsche, 1991; Stecyk
and Farrell, 2006). Likewise, systemic α-adrenergic tone mediates the increased peripheral vasoconstriction occurring during diving in mammals and birds (Butler and Jones, 1971; Butler, 1982; Lacombe and Jones, 1991; Signore and Jones, 1995). In freshwater turtles, adrenergic fibres innervate the systemic circulation (Berger and Burnstock, 1979), and α-adrenergic stimulation increases $R_{\text{sys}}$ in normoxic animals (Comeau and Hicks, 1994; Hicks and Farrell, 2000b; Overgaard et al., 2002). Anoxic exposure of turtles is associated with high concentrations of circulating catecholamines and it is possible that they increase $R_{\text{sys}}$ through arteriolar α-adrenergic stimulation (Wasser and Jackson, 1991; Keiver and Hochachka, 1991; Keiver et al., 1992). However, β-adrenergic regulation of the cardiovascular system seems depressed during anoxia as the stimulatory effects of adrenaline on $f_{\text{H}}$ and $P_{\text{sys}}$ are blunted during anoxia at both cold and warm temperatures and this is correlated with a reduced density of β-adrenergic receptors on the heart (Hicks and Wang, 1998; Hicks and Farrell, 2000b). The α-adrenergic control of vasomotor tone during anoxia has not been directly investigated in turtles. Consequently, it remains unknown whether the high levels of circulating catecholamines saturate the α-adrenergic receptors, so that exogenous application does not affect $R_{\text{sys}}$, or whether α-adrenergic control of vasomotor tone is suppressed during anoxia. Thus, our first objective was to test the hypothesis that increased α-adrenergic tone accounts for the augmented $R_{\text{sys}}$ during anoxia in the turtle (Trachemys scripta). We examined the α-adrenergic regulation of $Q_{\text{sys}}$, $P_{\text{sys}}$ and $R_{\text{sys}}$ with injections of α-adrenergic agonists and antagonists in normoxic and anoxic turtles acclimated to 5°C or 21°C.

The increase in $R_{\text{sys}}$ with hypoxia in vertebrate species is usually accompanied by a redistribution of systemic blood flow that is reflective of differences in metabolic needs among tissues, with critical systems, such as the brain and the heart, receiving a high priority to prevent damage from anoxia. For example, a high priority to cerebral blood flow has been observed in fish that are tolerant to oxygen shortage during both anoxia and severe hypoxia (Nilsson et al.,
1994; Yoshikawa et al., 1995; Söderström et al., 1999). Similarly, during hypoxia and underwater diving, endotherms redistribute blood flow towards cerebral, myocardial and adrenal vascular beds, while blood flow to visceral organs is reduced by a selective vasoconstriction, which, in many cases, is mediated by \(\alpha\)-adrenergic control (Johansen, 1964; Elsner et al., 1966; Chalmers et al., 1967; Krasney, 1971; Butler and Jones, 1971; Jones et al., 1979; Zapol et al., 1979). Consistent with these blood flow patterns, blood flow to various visceral organs is reduced during short-term anoxia in anaesthetized turtles, while brain blood flow is largely maintained (Davies, 1989, 1991; Bickler, 1992; Hylland et al., 1994, 1996).

The redistribution of blood flow during short-term anoxia in anaesthetized turtles (Davies, 1989) indicates that the different vascular beds respond differently to hypoxia. However, the anaesthetized turtle does not exhibit the otherwise well-documented depression of cardiac activity during anoxia, which may reflect the complex effects of anaesthetics on the heart and local blood flow regulation (e.g. Smith and Wollman, 1972; Marcus et al., 1976). Therefore, it is uncertain whether these findings can be applied to unanaesthetized animals and to a prolonged period of anoxia that can occur naturally. Thus, our second objective was to identify which critical tissues receive a high priority of blood flow during prolonged anoxia when cardiovascular function is depressed. Relative systemic blood flow distribution and absolute tissue blood flows were determined during normoxia and anoxia by the injection of coloured microspheres while simultaneously measuring absolute blood flows in the major arteries. Microspheres were also injected following \(\alpha\)-adrenergic stimulation and blockade during anoxia to determine \(\alpha\)-adrenergic control of blood flow redistribution.
Materials and methods

Experimental animals

Twenty-six red-eared sliders (*Trachemys scripta*, Gray) with body masses ranging between 0.45 and 1.9 kg (1.20 ± 0.07 kg, mean ± S.E.M.) were used in this study. Turtles were obtained from Lemberger Inc. (Oshkosh, WI, USA) and airfreighted to Aarhus University (Denmark), where they were maintained for several months before experimentation. The turtles studied at 21°C were held in large fibreglass aquaria under a 14 h: 10 h L:D photoperiod, had free access to dry basking platforms under infrared lamps to allow behavioural thermoregulation and were fed dead fish several times a week. Food was withheld during the experimentation period. The turtles studied at 5°C were kept in a large flow-through polypropylene tank at 5°C with access to air for six weeks prior to instrumentation. All 5°C turtles were fasted during this period. Experiments were performed between February and April 2001 and all procedures were in accordance with the laws of animal care and experimentation in Denmark.

Surgical procedures

Turtles were intubated with soft rubber tubing for artificial ventilation with isoflurane (4% in room air prepared by a Halothane vaporizer, Dräger, Lubeck, Germany) at a rate of 8-15 breaths min⁻¹ and a tidal volume of 10-20 ml kg⁻¹ using a Harvard Apparatus Ventilator (HI 665, Harvard Apparatus Inc., Holliston, MA, USA). Once a surgical plain of anaesthesia was achieved, as determined by the lack of a pedal withdrawal reflex, the isoflurane level was reduced to either 0.5% or 1% and maintained at this level throughout the operation, which lasted approximately 40 min.

For placement of catheters and flow probes, the heart and central vascular blood vessels were accessed by excision of a 3 x 4 cm piece of the plastron using a bone saw. An occlusive
catheter (PE-50 containing saline with 100 IU ml⁻¹ heparin) was advanced from the left thyroid artery into the right subclavian artery originating from the right aortic arch. For blood flow measurements, 1.0 - 1.5 cm sections of major systemic blood vessels were freed from the surrounding connective tissue for placement of ultrasonic blood flow probes (sizes 2 – 3 mm; Transonic Systems Inc., Ithaca, NY, USA). Turtles exposed to anoxia were instrumented with flow probes around the left aortic arch (LAo), the right aortic arch (RAo), and a single probe around both the left subclavian and left carotid arteries (Lsubcar). The use of one flow probe for monitoring blood flow through two vessels has previously been validated in turtles (Wang and Hicks, 1996, see also Akagi et al., 1987). In addition, the left atrium of these animals was cannulated for the injection of coloured microspheres into the systemic circulation. A PE-90 catheter, flared at the end to prevent withdrawal, was inserted through a 0.3 cm incision of the atrial wall into the lumen and fastened to the atrial wall by surgical silk (4-0). The pericardium was subsequently closed with two or three sutures (4-0 surgical silk). Control normoxic turtles were instrumented with a single flow probe around the LAo, with $Q_{sys}$ determined with the equation $2.8 \times Q_{LAO}$ as previously verified (Comeau and Hicks, 1994; Wang and Hicks, 1996). Acoustic gel was infused between the blood vessels and flow probes to enhance the signal and the excised piece of the plastron was resealed in its original position using surgical tape and fast-drying epoxy resin.

After completion of the operation, turtles were ventilated with room air until they resumed spontaneous ventilation. Turtles were then allowed to recover in individual water-filled aquaria (40 cm x 30 cm x 30 cm), covered with black plastic to minimize visual disturbance, for either 48 h at 21°C or 72 h at 5°C.
Experimental protocol

All experiments were performed on unrestrained turtles that were free to move within the aquaria. Prior to any experimental manipulation, arterial blood samples were obtained through the arterial cannula for the measurement of hematocrit and arterial pH. Turtles studied during anoxia were denied air access while the aquarium water was continuously bubbled with N\(_2\) (water \(P_{O2}\) less than 0.3 kPa) and were exposed to anoxia for either 6 h at 21°C or 12 days at 5°C. Normoxic turtles had free access to room air throughout the experimentation period.

Injections of \(\alpha\)-adrenergic agonists and antagonists were used to examine the \(\alpha\)-adrenergic regulation of \(R_{sys}\). Anoxic turtles were treated sequentially with the \(\alpha\)-adrenergic agonist phenylephrine (5 \(\mu\)g kg\(^{-1}\) and subsequently 50 \(\mu\)g kg\(^{-1}\)), the \(\alpha\)-adrenergic antagonist phentolamine (3 mg kg\(^{-1}\)), and, finally, phenylephrine (50 \(\mu\)g kg\(^{-1}\)). After injections of phenylephrine, cardiovascular variables were allowed to return to baseline values before continuing the protocol. Following phentolamine injection, cardiovascular function was allowed to stabilize before subsequent drug injections. Control normoxic turtles were only treated with phentolamine (3 mg kg\(^{-1}\)). All chemicals, purchased from Sigma-Aldrich, Denmark, were dissolved in physiological turtle saline (in mmol l\(^{-1}\): NaCl, 105; KCl, 2.5; CaCl\(_2\), 1.3; MgSO\(_4\), 1; NaHCO\(_3\), 15; NaH\(_2\)PO\(_4\), 1; pH 7.8) and injected as a single 0.5 – 1.0 ml bolus through the arterial cannula, which was subsequently flushed with saline. The total volume injected never exceeded 2 ml and control saline injections of 2 ml did not cause haemodynamic changes.

Coloured polystyrene microspheres (25 \(\mu\)m in diameter) (Dye Track, Triton Technologies, San Diego, CA, USA) were used to measure regional blood flow distribution. Microspheres, suspended in the manufacturer supplied saline, which contained 0.05% Tween 80 to prevent agglomeration and 0.01% Thimerosal to act as a bacteriostat, to a final concentration of 4.0 \(\times\) 10\(^5\) spheres ml\(^{-1}\) were injected into the anoxic exposed group of turtles in 1 ml aliquots through the left atrial cannula. This procedure was conducted during normoxia while the
animals were not ventilating their lungs (tangerine microspheres), during anoxia (orange microspheres), during the maximal haemodynamic response to the first 50 μg kg$^{-1}$ phenylephrine injection (lemon microspheres) and, finally, after subsequent injection of phentolamine (3 mg kg$^{-1}$; canary microspheres) when cardiovascular function stabilized. A minimum of 30 min was allowed between microsphere injections and any subsequent experimental manipulation. To minimize conglomeration of the microspheres in the heart, the microsphere solution was sonicated for 1 min and vortexed for 30 s immediately before injection. After microsphere delivery, which lasted approximately 1 min, the syringe and cannula were flushed twice (0.75 ml each time) with physiological turtle saline and rinsed four times with acidified ethanol (0.2% volume:volume HCl, 37%; ethanol) and the liquid retained such that the number of spheres remaining in the syringe could be determined. A reference blood sample (0.3 – 0.5 ml) was taken from the arterial cannula 20 min after the normoxic and anoxic microsphere injections to assess whether the microspheres had indeed been trapped in the tissues.

At the completion of the protocol, turtles were euthanized with a vascular injection of pentobarbital (100 μg kg$^{-1}$). All animals were then dissected to separate organs and tissues, including the integument, red and white muscle, bones, esophagus, stomach, intestines (including pancreas), spleen, ventricle, atria, brain, liver (including gallbladder), kidneys, gonads, fat, connective tissue (included major blood vessels, bladder and thyroid gland) and eyes, which were cut into 7 – 15 g pieces and placed in individual polypropylene conical centrifuge tubes. The shell was subsampled, using seven representative samples ranging from 1.0 to 8.9 g (mean of 3.8 ± 0.2 g) and representing 6.9 ± 0.4% of total shell mass. Three samples were taken from the plastron, one from each of the anterior, medial and posterior sections, and four from the carapace, one from each side of the shell (costal scutes) and two from the vertebral scutes, one anterior and one posterior. Tissue samples were stored at room temperature for up to
8 weeks, allowing for unaided tissue degradation to occur, before chemical digestion and microsphere recovery from the tissues.

*Tissue digestion and microsphere recovery*

Immediately prior to tissue digestion, 3000 control spheres (blue, 25 μm diameter), suspended in saline containing 0.05% Tween 80 and 0.01% Thimerosal, were added to each sample to evaluate the efficiency and quality of the recovery process. Extraction of microspheres from reference blood samples and soft tissues was as follows:

1. 3-5-times tissue volume of “alkaline digesting reagent” (2 M KOH) was added to the polypropylene tubes and the tissue left to digest for 24 h. During digestion, the tubes were maintained at 60°C and intermittently vortexed and sonicated for 45 s. After sonication, the probe-tip was rinsed into the sample tube with acidified ethanol.

2. After digestion, tubes were filled to capacity with 60°C distilled water and the contents mixed by repeated inversion. Samples were then centrifuged at 1500 g for 15 min and the supernatant aspirated to a level safely above the visible pellet.

3. The pellet was re-suspended by sonication in 3-5-times of the original tissue volume in 15% Triton-X solution (volume:volume Triton-X:distilled water, 0.1 g sodium azide L⁻¹) heated to 60°C. The tubes were then centrifuged for 5 min at 1500 g and the supernatant aspirated without disturbing the pellet. If the pellet was large after the Triton-X wash, the protocol was repeated from step 1 until the pellet was no longer visible.

4. The non-visible pellet was re-suspended by sonication in 2-4-times the original tissue volume of acidified ethanol, and again centrifuged for 5 min at 1500 g and the supernatant safely aspirated. Finally, the acidified ethanol wash was repeated, and after decanting, the remaining supernatant was left to evaporate at room temperature.
Microsphere recovery from bone and shell samples followed the same protocol as described above, but included two additional steps. After step 3, the pellet was resuspended by sonication in approximately 5-times the original tissue volume of “bone digesting reagent” (0.12% EDTA, 5.38% HCl, 94% H$_2$O). Tubes were then maintained at 60°C for at least 12 hours with intermittent sonication, centrifuged for 5 min at 1500 g and aspirated to a safe level. The procedure was repeated if bone fragments remained following centrifugation. Once the bone or shell was completely dissolved, the remaining pellet was re-suspended by sonication in 15% Triton-X solution and the protocol described above was followed. (i.e. alkaline digestion if a large pellet remained or two acidified ethanol washes if the pellet was not visible).

Measurement of microsphere distribution and terminology

Following tissue digestion, 250 µl of 2-(2-ethoxyethoxy)ethylacetate was added to each tube and vortexed to extract the dye from the microspheres. The dye was allowed to extract for 20 min and then centrifuged at 1500 g for five minutes. 200 µl of the supernatant was then transferred to a microplate and the absorption of each sample was measured at five wavelengths with a microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, California, USA) referenced to 200 µl of 2-(2-ethoxyethoxy)ethylacetate. The five wavelengths used were those of maximal absorbance for each microsphere colour label, namely 390 nm (lemon), 440 nm (canary), 495 nm (orange), 525 nm (tangerine) and 672 nm (blue). Samples with absorbance readings greater than 1.3 AU were diluted and re-analyzed to ensure linearity between absorbance and dye concentration. Correction for spectral over-lap among the five colour labels and subsequent determination of the number of each colour of microsphere per sample was resolved through a matrix conversion computer program (Triton Technologies Inc., San Diego, CA, USA).
The total number of each colour of microsphere recovered per animal was determined as the sum of all microspheres recovered and those estimated to be trapped in the non-digested portion of the shell (the number of microspheres recovered from each of the representative shell samples did not differ statistically, so the amount of microspheres in the non-digested portion of the shell was assumed to be identical). Recovery of injected microspheres was expressed relative to the number of microspheres injected (i.e. $4.0 \times 10^5$ minus the number of spheres remaining in the syringe after injection). The fraction of systemic cardiac output ($%Q_{sys}$) directed to each tissue was calculated as the quotient of the number of microspheres recovered per tissue and total microspheres recovered from systemic tissues. Absolute blood flows ($\mu l\ min^{-1}\ g^{-1}$) to systemic tissues were calculated by multiplying $%Q_{sys}$ for each tissue by systemic cardiac output ($Q_{sys}$), as measured by the ultrasonic flow probes (see below).

**Calculation of haematological and cardiovascular variables**

Hematocrit was determined as the fractional erythrocyte volume in a capillary tube following 3 min of centrifugation at 10000 g. Arterial pH was measured using a Radiometer pH electrode (PS-1 204, Copenhagen, Denmark) maintained and calibrated at the acclimation temperature of the experimental animal in a BMS Mk3 electrode set-up. Electrode output was displayed on a Radiometer PHM 73 pH monitor. Systemic blood pressure ($P_{sys}$) was measured by attaching the arterial cannula to a disposable pressure transducer (Baxter Edward, model PX600, Irvine, CA, USA) calibrated daily against a static water column. The signal from the pressure transducer was amplified using an in-house built preamplifier. Flow probes were connected to two, dual-channel blood flow meters (T201, Transonic Systems Inc., Ithaca, NY, USA). All signals were continuously recorded with a Biopac MP100 computer assisted data acquisition system (Biopac Systems Inc., Goleta, CA) at 50 Hz and data recordings were
analysed offline using AcqKnowledge data analysis software (version 3.5.7; Biopac Systems Inc., Goleta, CA).

Systemic cardiac output ($Q_{sys}$) was calculated as $Q_{LAo} + Q_{RAo} + 2Q_{subcar}$ for the anoxic exposed turtles, whereas $Q_{sys}$ was estimated as $2.8 \times Q_{LAo}$ for the control normoxic turtles. Heart rate ($f_{H}$) was derived from the beat-to-beat interval of the $P_{sys}$ trace. Systemic stroke volume ($V_{sys}$) was calculated as $Q_{sys}/f_{H}$ and systemic resistance ($R_{sys}$) as $P_{sys}/Q_{sys}$ with the assumption that right atrial pressure is negligible. Systemic power output ($P_{Osys}$) was calculated as $Q_{sys} \times P_{sys} / Mv$, where $P_{sys}$ is measured in kPa and Mv is ventricular mass (g).

Data analysis and statistics

Normoxic control values for all experimental groups were recorded for 30-60 min immediately before drug injections or anoxic exposure. At 21°C, haemodynamic variables were recorded continuously throughout the 6 h anoxic period; Reported haemodynamic values from this time period were averaged from 5-min periods at each hour of anoxic exposure. At 5°C, haemodynamic variables were recorded for 30 min on days 3, 8, and 12 of the anoxic exposure and continuously throughout the period of drug and microsphere injections on day 12. At both temperatures, we report the maximal response following injections of the $\alpha$-adrenergic agonist phenylephrine, which normally occurred within 5 min and 15 min after injection at 21°C and 5°C, respectively, while new steady state values were attained 0.5 h and 1 h after injection of the $\alpha$-adrenergic antagonist phentolamine at 21°C and 5°C, respectively.

Mean values ± S.E.M are presented for all haematological and cardiovascular variables, $%Q_{sys}$ and absolute tissue blood flows at each sample time. Within group comparisons of cardiovascular variables were determined using a one-way repeated measures analysis of variance (ANOVA), and comparisons of cardiovascular variables between acclimation
temperatures were performed using a t-test. Similarly, comparisons of $%Q_{sys}$ and absolute tissue blood flow between acclimation temperatures were performed using t-tests. Changes in $%Q_{sys}$ between routine normoxic and routine anoxic conditions, as well as following $\alpha$-adrenergic stimulation and blockade, were determined using a two-way repeated measures analysis of variance (ANOVA) on arcsine-transformed $%Q_{sys}$ data, with tissue $%Q_{sys}$ and condition as the two factors. Changes in absolute blood flow to different tissues during normoxic control, anoxic control, and following the $\alpha$-adrenergic agonist and antagonist drug injections were assessed with a one-way repeated measures ANOVA unless the data were not normally distributed, in which case a Friedman repeated measures ANOVA on ranks was used. Where appropriate, multiple comparisons were performed using Student-Newman-Keuls tests and in all instances significance was accepted when $P < 0.05$.

Results

Accuracy of microsphere technique

Accurate use of microspheres requires that several criteria be satisfied. Foremost, it must be assumed that that all microspheres are trapped in the capillary beds during their first passage through the circulation. Complete trapping of microspheres during normoxia and anoxia was confirmed by the lack of microspheres in arterial blood samples withdrawn 20 min after injections.

Secondly, injected microspheres must be adequately mixed with blood at the injection site to provide a homogenous solution such that the same concentration of microspheres prevails at all arterial sites. We injected the microspheres into the left atrium, and while atrial and ventricular contraction are likely to have assured good mixing, the undivided ventricle of turtles allows for some of the injected microspheres to be shunted into the pulmonary circulations (White et al., 1989). During normoxia, we therefore, injected the microspheres during breath-
hold, when pulmonary blood flow and Left-to-Right shunting is low (e.g. White et al., 1989; Hicks, 1994; Wang and Hicks, 1996). During anoxia, at least at warm temperatures, pulmonary blood flow is greatly reduced due to hypoxic pulmonary vasoconstriction (Hicks and Wang, 1998; Crossley et al., 1998). These precautions seemed effective as the relative number of spheres recovered from the lungs was low (5.2 ± 1.1%; N = 44) and did not change with either injection time or acclimation temperature.

The coloured microsphere technique also relies on an efficient microsphere isolation and purification protocol such that measured absorbance intensities correlate directly with the number of microspheres. Indeed, analysis of reference microsphere samples in the present study revealed linear relationships (P < 0.001 in all cases) between microsphere number and measured absorbance for each of the five colour labels (blue, y = 0.0002x, r² = 0.93; tangerine, y = 0.0022x, r² = 0.98; orange, y = 0.0004x, r² = 0.96; canary, y = 0.0006x, r² = 0.98; lemon, y = 0.0003x, r² = 0.87). Furthermore, there was a linear relationship (P < 0.001 in all cases) between the number of microspheres determined through matrix conversion and the number of microspheres in each experimental sample for all five colour labels (blue, y = 0.9712x, r² = 0.93; tangerine, y = 0.9873x, r² = 0.98; orange, y = 1.6673x, r² = 0.96; canary, y = 1.2674x, r² = 0.98; lemon, y = 1.3199x, r² = 0.93). Further, tissue type did not influence the efficiency of microsphere recovery. The relative recovery of blue control spheres (73 ± 2% (n = 1215)), used to assess microsphere loss during the extraction procedure, did not differ between tissue types. Relative recovery of injected microspheres differed among the different microsphere colour labels. Tangerine coloured spheres (injected at normoxic control) had a recovery of 29 ± 5%, whereas the lemon coloured spheres (injected after phenylephrine during anoxia) had a recovery of 140 ± 2%. 74 ± 13% of the canary (injected after phentolamine during anoxia) and 103 ± 10% of the orange (routine anoxic injection) microspheres were recovered.
The accuracy of the microsphere technique for determination of $\%Q_{sys}$ can be quantified in the present study because $Q_{sys}$ was measured simultaneously with blood flow probes (Fig. 2.1). $\%Q_{sys}$ to tissues perfused by the left subclavian (left foreleg integument, bone and muscle) and carotid arteries (head and neck bones, integument, muscle, esophagus, trachea, thyroid, brain and eyes) compared to the relative blood flow in these vessels were linearly-related, but the microsphere technique underestimated $\%Q_{sys}$ by approximately 30%.

**Blood flow ratios among the major systemic blood vessels**

Blood flows in all major systemic arteries, with the exception of the right carotid and right subclavian arteries, were successfully measured in 9 of the 14 anoxic exposed turtles instrumented with three ultrasonic flow probes. Assuming that these vessels receive the same flows as the left carotid and left subclavian arteries, we estimate that $Q_{L Ao}$, $Q_{R Ao}$, and $Q_{Subcar}$ account for 36.3 ± 0.01%, 35.9 ± 0.01 %, and 13.9 ± 0.01% ($N = 9$ in all cases), respectively, of $Q_{sys}$ in turtles. This proportional distribution was not affected by acclimation temperature, oxygen availability (Table 2.1) or injections of phenylephrine and phentolamine during anoxia (data not shown).

*Effect of acclimation temperature on normoxic haematological variables, $\alpha$-adrenergic control of cardiovascular function, and systemic blood flow distribution*

Normoxic hematocrit did not vary significantly between acclimation temperatures, but arterial pH was significantly greater in 5°C normoxic turtles than 21°C normoxic turtles (Table 2.2). Cold-acclimation resulted in large reductions in $f_H$ and $Q_{sys}$, while $R_{sys}$ increased significantly (Table 2.3). $Q_{sys}$, $f_H$ and $PO_{sys}$ were between five- and eleven-times lower at 5°C compared to 21°C, with respective $Q_{10}$ values of 2.6, 2.8, and 4.7. In contrast, $R_{sys}$ was two-fold
greater at 5°C than at 21°C. As a result, $P_{VA}$ was reduced by only 46% at 5°C despite a 4.7-fold decrease in $Q_{sys}$.

$\alpha$-Adrenergic regulation of cardiovascular function differed between acclimation temperatures in normoxic turtles (Table 2.3). At 21°C, injection of the $\alpha$-adrenergic antagonist phentolamine did not affect any of the measured haemodynamic variables, whereas injection at 5°C significantly reduced $R_{sys}$, which was manifested as a decrease in $P_{sys}$ and augmented $Q_{sys}$ through an increase in $V_{Ssys}$. Thus, systemic $\alpha$-adrenergic tone was inversely related with acclimation temperature in normoxic turtles.

Blood flow distribution also differed with acclimation temperature under normoxic conditions (Fig. 2.4A,C; Table 2.5), with cold turtles having a significantly higher %$Q_{sys}$ to the integument, but a lower %$Q_{sys}$ to the intestines. Absolute tissue flows were greater to muscle (4.8-fold), bone (2.6-fold), intestines (10.4-fold), liver (2.5-fold), gonads (2.4-fold) and fat (2.5-fold) in warm turtles.

_Cardiovascular function and systemic blood flow distribution during anoxia_

$Q_{sys}, f_H$ and $P_{sys}$ were significantly reduced with anoxia at both acclimation temperatures (Figs. 2.2 and 2.3; Table 2.4). At 21°C, 2.1-fold and 2.6-fold reductions in $f_H$ and $Q_{sys}$, respectively, occurred by the 6th hour of anoxia and the accompanying fall in $P_{sys}$ led to an almost four-fold reduction in $P_{Osys}$. Correspondingly, $R_{sys}$ increased 2.3-fold by the 6th hour of anoxic exposure. At 5°C, the proportional changes in cardiovascular status during anoxia were larger than those observed at 21°C. $f_H$, $Q_{sys}$ and $P_{Osys}$ were reduced by 1.3- to 3.4-fold by the 3rd day of anoxia, and these initial reductions were then followed by a slower, gradual decline such that by the 12th day of anoxia, $f_H$, $Q_{sys}$ and $P_{Osys}$ were maximally reduced by 4.7-fold, 4.3-fold...
and 6.7-fold, respectively. Similar to the response at 21°C, there was a corresponding increase in $R_{sys}$ (2.9-fold by the 12th day of anoxic exposure) at 5°C. However, absolute $R_{sys}$ was 2.3 times greater during 5°C anoxia than 21°C anoxia (Table 2.4).

Systemic blood flow distribution was altered with anoxic submergence. After 6 h of anoxia at 21°C, $\%Q_{sys}$ decreased significantly in the stomach (6.2-fold) and intestines (3.8-fold), and increased significantly in the muscle (1.3-fold) and shell (1.7-fold) (Fig. 2.4C,D; Table 2.5). Absolute tissue blood flow decreased significantly to the intestines (14.4-fold), stomach (11.8-fold), kidneys (10.7-fold) and muscle (1.9-fold) (Table 2.5). After a 12-day anoxic exposure at 5°C, $\%Q_{sys}$ decreased significantly in the kidneys (2.7-fold) and gonads (2.2-fold) and increased significantly in the liver (1.7-fold) and shell (1.2-fold) (Fig. 2.4A,B; Table 2.5). Absolute blood flow decreased to all systemic tissues during anoxic submergence at 5°C (Table 2.5), with the largest decreases occurring in the digestive and urogenital tissues and the smallest decreases occurring in the brain, heart and liver (Table 2.6).

**α-Adrenergic control of cardiovascular function and systemic blood flow distribution during anoxia**

Systemic α-adrenergic tone in anoxic turtles also differed with acclimation temperature, but in contrast to normoxic turtles, systemic α-adrenergic tone increased with acclimation temperature. With the exception of a significant, but minor increase in $P_{sys}$ after a low dose of phenylephrine (5 µg kg⁻¹), there were no significant effects of α-adrenergic stimulation at 21°C, although $R_{sys}$ did tend to increase (Table 2.4). In contrast to these small effects of α-adrenergic stimulation, injection of the α-adrenergic antagonist phentolamine elicited a three-fold decrease in $R_{sys}$ at 21°C and completely abolished the effects of subsequent injection of phenylephrine.
Thus, the large anoxia-induced increase in \( R_{\text{sys}} \) at 21°C was a result of an elevated \( \alpha \)-adrenergic tone.

In anoxic turtles at 5°C, there was a clear dose-dependent increase in \( P_{\text{sys}} \) and \( R_{\text{sys}} \) following injection of phenylephrine, but phentolamine did not significantly affect routine anoxic \( R_{\text{sys}} \) (Table 2.4). Thus, although \( \alpha \)-adrenergic receptors remained functional, as attested by the eliminated effects of phenylephrine following phentolamine (Table 2.4), the increase in \( R_{\text{sys}} \) during anoxia was not a result of an increased \( \alpha \)-adrenergic tone. Consequently, the anoxia-induced \( \alpha \)-adrenergic mediated systemic vasoconstriction was blunted at 5°C despite being central to the systemic vascular tone during normoxia at this temperature.

Due to large individual variation there were no significant changes in either relative or absolute tissue blood flows following \( \alpha \)-adrenergic manipulation in anoxic turtles at 21°C (Table 2.5). At 5°C, phenylephrine injection significantly increased \( \%Q_{\text{sys}} \) in muscle and liver, while \( \%Q_{\text{sys}} \) to the shell decreased. Similarly, absolute blood flow to the liver increased following \( \alpha \)-adrenergic stimulation. However, only the increased liver \( \%Q_{\text{sys}} \), which occurred with \( \alpha \)-stimulation, was restored to routine anoxic levels with phentolamine injection. Shell \( \%Q_{\text{sys}} \) increased significantly following \( \alpha \)-adrenergic blockade, but did not fully return to the pre-\( \alpha \)-adrenergic stimulation value.

**Discussion**

*Normoxic cardiovascular function and systemic blood flow distribution: Effects of temperature*

Turtles were allowed to recover for 48 to 72 h after surgery to reduce the effects of surgical stress. Control normoxic hematocrit and arterial pH, recorded prior to anoxic exposure, are within previously reported ranges (Jackson and Ultsch, 1982; Ultsch and Jackson, 1982;
Hicks and Farrell, 2000b) and indicate a successful post-operative recovery. Additionally, normoxic blood flows, $P_{sys}$, $PO_{sys}$ and $R_{sys}$ at 21°C are similar to previous studies on turtles at 20-25°C (Shelton and Burggren, 1976; Herbert and Jackson, 1985b; Hicks, 1994; Wang and Hicks, 1996; Hicks and Wang, 1998; Hicks and Farrell, 2000ab). At 5°C, our values for $f_h$ and $P_{sys}$ during normoxia are also similar to previously reported values (Herbert and Jackson, 1985b; Hicks and Farrell, 2000ab), but our $Q_{sys}$ at 5°C ($9 - 14 \text{ ml min}^{-1} \text{ kg}^{-1}$) is greater than the value of 4.0 ml min$^{-1}$ kg$^{-1}$ reported by Hicks and Farrell (2000ab). Consequently, normoxic $V_{sys}$ and $PO_{sys}$ at 5°C are approximately four-fold higher and $R_{sys}$ two-fold lower than reported by Hicks and Farrell (2000ab). These quantitative differences may reflect the different 5°C acclimation procedures used in the two studies, or seasonal variability in the response to anoxia (our study was performed in winter/spring, while the study by Hicks and Farrell (2000ab) was performed in the fall/winter).

The reduction in $Q_{sys}$ with reduced temperature during normoxia is consistent with other ectothermic vertebrates, including freshwater turtles (Farrell and Jones, 1992; Hicks and Farrell, 2000ab; Stecyk and Farrell, 2002, 2006), and likely mirrors the reduction in metabolic rate (Jackson and Schmidt-Nielsen, 1966; Jackson, 1968; Herbert and Jackson, 1985b). Although $P_{sys}$ was also reduced with decreased temperature, $R_{sys}$ was greatly elevated at 5°C. $\alpha$-Adrenergic regulation of systemic vasomotor tone seems important in this response since $\alpha$-adrenergic blockade with phentolamine injection at 5°C reduced $R_{sys}$ to the 21°C normoxic level, but was without effect on $R_{sys}$ at 21°C (Tables 2.3 and 2.4). The increased $\alpha$-adrenergic vasomotor tone in 5°C normoxic turtles supplements the suppression of cholinergic inhibition of the heart at low temperature, which likely offsets the negative effects of temperature on cardiac activity (Hicks and Farrell, 2000b), and may represent an important mechanism to regulate blood flow distribution between priority and less essential tissues. In fact, differences in absolute
blood flow and \( \%Q_{\text{sys}} \) existed between warm- and cold-acclimated turtles. Absolute blood flow was decreased to muscle, bone, intestines, liver, gonads and fat in 5°C turtles relative to 21°C turtles. Additionally, \( \%Q_{\text{sys}} \) was increased to the integument at 5°C, which may reflect the increased reliance on cutaneous gas exchange at this temperature (Herbert and Jackson, 1985b; Ultsch and Jackson, 1982). The decrease in \( Q_{\text{sys}} \) to the intestines at 5°C compared to 21°C may reflect that these animals had fasted during the 1.5-month acclimation period. However, verification of an \( \alpha \)-adrenergic involvement in these phenomena is still needed.

**Control of systemic peripheral resistance during anoxia**

As previously reported, anoxia was accompanied by large depressions in \( f_{\text{Hb}}, Q_{\text{sys}} \) and \( P_{\text{sys}} \) at both 5°C and 21°C (Figs. 2.2 and 2.3; Table 2.4). These cardiovascular changes closely resemble those previously described at warm and cold temperatures (Herbert and Jackson, 1985b; Hicks and Wang, 1998; Hicks and Farrell, 2000a), although our \( Q_{\text{sys}} \) was higher than that reported by Hicks and Farrell (2000ab) due to an elevated \( V_{\text{sys}} \). The marked increase in \( R_{\text{sys}} \) accompanying anoxia at both temperatures is also consistent with earlier studies (Hicks and Wang, 1998; Hicks and Farrell, 2000ab), but contrasts the normal vasodilatory effects of oxygen lack, decreased pH and increased levels of vasoactive metabolites that are present during anoxia. Thus, the increased \( R_{\text{sys}} \) may be due to activation of vascular \( \alpha \)-adrenergic receptors by the elevated levels of circulating catecholamines (Wasser and Jackson, 1991; Keiver and Hochachka, 1991; Keiver et al., 1992) and/or increased sympathetic nerve activity. Indeed, \( \alpha \)-adrenergic peripheral vasoconstriction during oxygen limitation is well documented in different vertebrates (Butler and Jones, 1971; Lillo, 1979; Butler, 1982; Fritsche and Nilsson, 1989; Axelsson and Fritsche, 1991; Lacombe and Jones, 1991; Signore and Jones, 1995; Stecyk and Farrell, 2006), and *Trachemys* certainly has \( \alpha \)-adrenergic receptor mediated control of \( R_{\text{sys}} \) (e.g.
Overgaard et al., 2002). However, autonomic regulation of cardiac activity during anoxia in turtles is dependent upon acclimation temperature. Autonomic control is more pronounced at warm acclimation temperatures, while the direct effects of oxygen lack and acidosis seems to account for most of the decreased cardiac performance during cold, anoxic submergence (Hicks and Wang, 1998; Hicks and Farrell, 2000b).

The present study reveals that the $\alpha$-adrenergic system remains functional during anoxia at both warm and cold acclimation temperatures, but that the $\alpha$-adrenergic contribution to the increased $R_{sys}$ varies with temperature. Specifically, a large $\alpha$-adrenergic tone accounting for the increased $R_{sys}$ during anoxia at 21°C was revealed by the lack of haemodynamic responses following injection of phenylephrine and the large (3-fold) reduction in $R_{sys}$ following $\alpha$-adrenergic blockade with phentolamine. In fact, phentolamine reduced $R_{sys}$ to the 21°C normoxic level (Tables 2.3 and 2.4). The small effect of phenylephrine injection on cardiovascular function may possibly reflect the high levels of circulating catecholamines (>56 nmol norepinephrine, Wasser and Jackson, 1991) fully saturating the systemic $\alpha$-adrenergic receptors during anoxia.

In contrast to the high $\alpha$-adrenergic tone on $R_{sys}$ during anoxia at 21°C, the large progressive increase in $R_{sys}$ accompanying anoxia at 5°C does not seem to be mediated by $\alpha$-adrenergic vasoactivity. While inhibition of the $\alpha$-adrenergic receptors with phentolamine eliminated the effects of the preceding $\alpha$-adrenergic stimulation with phenylephrine, $\alpha$-adrenergic blockade did not affect routine anoxic $R_{sys}$ at 5°C. This finding is peculiar because it demonstrates that $\alpha$-adrenergic vasoactivity remains operational during anoxia, but that the systemic $\alpha$-adrenergic tonus is low. Thus, the increased concentration of plasma catecholamines present in cold, anoxic turtles (approximately 25 nmol norepinephrine, Wasser and Jackson, 1991) seemingly does not elicit $\alpha$-adrenergic mediated systemic vasoconstriction, perhaps, because of increased receptor density, decreased receptor affinity, or reduced signal transduction.
efficacy. Nevertheless, the low α-adrenergic tone during anoxia at 5°C is consistent with an overall blunting of the autonomic regulation of the cardiovascular system during cold anoxic submergence, when only small cholinergic and β-adrenergic tones exist on the cardiovascular system (Hicks and Farrell, 2000b).

Given the low α-adrenergic tone on the systemic circulation during anoxia at 5°C, other regulatory mechanisms must be responsible for the increased $R_{sys}$. Hicks and Farrell (2000a) suggested that the hypotension associated with anoxia at 5°C could directly affect $R_{sys}$ if $P_{sys}$ failed to surpass the critical closing pressure of certain vessels. However, our results from normoxic turtles at 5°C argue against such a mechanism because injection of phentolamine caused a very severe hypotension (<1.0 kPa) while $R_{sys}$ remained low (Table 2.3). Nonetheless, vessel diameters may be reduced at low blood flows and pressures, leading to a higher resistance (Lipowsky et al., 1978). Similarly, blood vessel tension is increased with cold temperature and thus, may also contribute to the increased $R_{sys}$ (Friedman et al., 1968; Dinnar, 1981), which would be exacerbated by the increased blood viscosity as temperature and flow decrease (Langille and Crisp, 1980). Finally, the low α-adrenergic vasomotor tone during anoxia may represent increased non-adrenergic, non-cholinergic regulation of $R_{sys}$.

Changes in systemic blood flow distribution with anoxic exposure

A redistribution of blood flow towards oxygen-sensitive tissues such as the brain and heart is critical to survival and is a commonly used survival strategy among vertebrates when exposed to hypoxia (Johansen, 1964; Elsner et al., 1966; Chalmers et al., 1967; Krasney, 1971; Butler and Jones, 1971; Jones et al., 1979; Zapol et al., 1979; Boutilier et al., 1986; Davies, 1989; Davies, 1991; Bickler, 1992; Hylland et al., 1994; Nilsson et al., 1994; Yoshikawa et al., 1995; Hylland et al., 1996; Söderström et al., 1999). Here, we provide a quantitative description
of systemic blood flow distribution during the large depression in cardiac status occurring with anoxic submergence in the anoxia-tolerant freshwater turtle. We clearly show that perfusion is sacrificed in subsidiary tissues, while the cerebral and myocardial circulations, as well as other critical organs, receive a priority of blood flow. Specifically, anoxia led to substantial depressions in $\%Q_{\text{sys}}$ and absolute tissue flow to digestive and urogenital organs at both temperatures, while $\%Q_{\text{sys}}$ was increased or maintained to the shell (5°C and 21°C), muscle (21°C) and liver (5°C) (Fig. 2.4; Table 2.5). These findings are in agreement with the redistribution of blood flow away from the renal and splanchnic circulatory beds observed after 30 minutes of $N_2$ ventilation in anaesthetized turtles (Davies, 1989) and are consistent with the greatly reduced renal function in conscious anoxic turtles (Warburton and Jackson, 1995; Jackson et al., 1996). Furthermore, the small number of microspheres directed towards the lungs during anoxia at 5°C implies the presence of a similar pulmonary vasoconstriction and reduced Left-to-Right shunt with anoxic exposure at 5°C to that exhibited during anoxic submergence at warm temperatures (Hicks and Wang, 1998; Crossley et al., 1998).

The importance of tissues containing large glycogen stores, specifically the liver and skeletal muscle, in fostering anoxic survival is highlighted in the present study. During anoxia, turtles must meet their energy demands through anaerobic metabolism, with glucose as the primary substrate. Glucose is derived from catecholamine-mediated breakdown of hepatic and skeletal muscle glycogen stores (Daw et al., 1967; Penny, 1974; Keiver and Hochachka, 1991; Wasser et al., 1991; Keiver et al., 1992), thus, maintained blood flow to the liver and muscle during anoxia may facilitate glucose export to other organs. Indeed, 6 h of anoxia at 21°C resulted in an increased $\%Q_{\text{sys}}$ to muscle and the maintenance of liver absolute blood flow at control normoxic levels. Similarly, $\%Q_{\text{sys}}$ to the liver was increased during anoxia at 5°C, and the reduction in absolute flows to the liver and muscle were minimal compared with the overall
reduction in $Q_{sys}$ (4.3-fold, Table 2.4) and the reductions in absolute blood flow to the bulk of systemic tissues (Table 2.6).

Anaerobic energy metabolism potentially threatens anoxic survival because of the accumulation of lactate and the ensuing acidosis (Herbert and Jackson, 1985b). However, the shell of the turtle acts as a powerful buffer reserve and diminishes the acidosis and accumulation of lactate in body fluids (reviewed by Jackson, 2000, 2002). The increased demand of blood flow to the shell observed in the present study is consistent with the increased demand on the shell as a buffer reserve during anoxia. In fact, $\%Q_{sys}$ directed to the shell increased significantly during anoxia at both temperatures, such that after cardiac depression, 40%-50% of $Q_{sys}$ was directed towards the shell. Furthermore, the homogenous distribution of microspheres in the shell is consistent with the uniform lactate accumulation of the entire shell during anoxia (Jackson et al., 1996; Jackson, 1997).

Turtles in the present study did not display the increased $\%Q_{sys}$ or absolute blood flow to the brain or myocardial circulations documented during short-term hypoxic exposure in other vertebrates (Johansen, 1964; Elsner et al., 1966; Chalmers et al., 1967; Krasney, 1971; Butler and Jones, 1971; Jones et al., 1979; Zapol et al., 1979; Nilsson et al., 1994; Yoshikawa et al., 1995; Söderström et al., 1999), including anaesthetized freshwater turtles (Davies, 1989; Davies, 1991; Bickler, 1992; Hylland et al., 1994; Hylland et al., 1996). Nevertheless, the importance of the brain and heart for anoxic survival and their corresponding demand for blood flow is clearly signified in the present study. At 21°C, brain and myocardial $\%Q_{sys}$ and absolute blood flow were maintained at control normoxic levels following the 6 h exposure period despite a 2.6-fold decrease in $Q_{sys}$ (Fig. 2.2; Tables 2.4 and 2.5). Likewise, 5°C cerebral and myocardial $\%Q_{sys}$ were maintained at control normoxic levels after 12 days of anoxia, while absolute blood flows were reduced less than the overall reduction in $Q_{sys}$ (4.3-fold), as well as the reductions in
absolute blood flows to the bulk of the systemic tissues (Tables 2.4 and 2.6). These differences in cerebral and myocardial blood supply during anoxia may simply reflect the long duration of anoxia in our study, which resulted in a complete transition from aerobic to anaerobic metabolism. Typically, normal cellular functions are maintained at the onset of anoxia and organ ATP levels preserved through activation of glycolysis. Consequently, increased tissue blood flow may be required for increased glucose delivery and waste removal. However, once biochemical reorganization has occurred, and glycolytic inhibition (reviewed by Storey, 1996) and metabolic depression are established, an increase in blood flow is no longer required. In fact, at 20°C, brain blood flow of anaesthetized anoxic turtles returns to normoxic levels within 1-2 h of anoxia (Hylland et al., 1994; Hylland et al., 1996).

α-Adrenergic control of systemic blood flow distribution during anoxia

It is well established that α-adrenergic control mediates peripheral vasoconstriction and subsequent redistribution of blood flow among tissues during hypoxia or diving in many groups of vertebrates (Butler and Jones, 1971; Butler, 1982; Lacombe and Jones, 1991; Signore and Jones, 1995). In the present study, the use of microspheres was unable to resolve many major changes in blood flow distribution between tissues following injection of α-adrenergic agonists and antagonists, and this, to some extent, may reflect a limitation of the methodology. However, given that α-adrenergic stimulation did not increase $R_{sys}$ during anoxia at 21°C, and given that $R_{sys}$ is not α-adrenergically mediated during anoxia at 5°C, major changes in blood flow distribution may not be expected after α-adrenergic manipulation. Nevertheless, there seems to be an α-adrenergic mediated dilation of the liver and an α-adrenergic mediated constriction in the shell during anoxia at 5°C (Table 2.5). Conversely, the general lack of changes at 21°C may simply reflect a global response of all tissues to α-adrenergic manipulation, with the resistances
in all tissue beds changing simultaneously such that no overall redistribution occurs.
Differentiation of the two possibilities is outside the scope of the present study and thus caution
must be exercised in interpreting the observed changes in $%Q_{sys}$ and absolute blood flow as a
reflection of tissue specific $\alpha$-adrenergic regulation.

**Concluding remarks**

In summary, our study reveals that $\alpha$-adrenergic regulation of $R_{sys}$ in the freshwater turtle
during anoxic submergence is temperature-dependent. The increased $R_{sys}$ during anoxia at 21°C
can largely be ascribed to an increased $\alpha$-adrenergic tone, whereas an $\alpha$-adrenergic tone does not
seem to contribute to the marked increase in $R_{sys}$ accompanying anoxia at 5°C. The large $\alpha$-
adrenergic tone on $R_{sys}$ during anoxia at 21°C is consistent with the importance of autonomic
regulation of the cardiovascular system during anoxia at warm temperatures and the blunting of
this response with cold anoxic exposure is consistent with the suppression of autonomic control
during cold anoxic submergence. However, while the intrinsic effects of anoxia and acidosis are
predominantly responsible for the depression in cardiac activity during anoxic submergence at
5°C, the primary determinant of the increased $R_{sys}$ and regulated hypotension remain to be
identified.

The overall redistribution of systemic blood flow and changes in absolute blood flows to
specific tissues during anoxia are consistent with tissue metabolism and/or their respective
importance for survival during anoxia. Following 6 h of anoxia at 21°C, $%Q_{sys}$ and absolute
blood flow were reduced to the digestive and urogenital tissues while $%Q_{sys}$ and absolute blood
flows to the cerebral and myocardial circulations were maintained at control normoxic levels.
Following 12 days of anoxia at 5°C, $%Q_{sys}$ was reduced to the urogenital tissues, but maintained
at control normoxic levels in the brain and heart. This indicates that the digestive and urogenital
tissues are of reduced importance, whereas the myocardial and cerebral circulations remain a priority. Similarly, the increased importance of liver and muscle glycogen stores in fueling anaerobic metabolism during anoxia was indicated by the increased $\%Q_{\text{sys}}$ to the muscle (21°C) and liver (5°C) and minimally reduced absolute blood flow to the liver at 5°C. Finally, the crucial and increased importance of the turtle shell as a buffer reserve during anoxic submergence (Jackson, 2000, 2002) was highlighted by the increased $\%Q_{\text{sys}}$ directed towards the shell with anoxia at both 5°C and 21°C.

**Acknowledgements**

This work was supported by a Natural Sciences and Engineering Research Council of Canada research grant to A.P.F., a Simon Fraser University Graduate Fellowship awarded to J.A.W.S, and the Danish Research Council (Rømer stipend to T. W.). Special thanks to Johnnie Andersson, Dorte Olsson, Jenny Krautter and Steve Wong for help with the turtle dissections and microsphere recovery and also Dr. George Iwama for the use of the microplate spectrophotometer.
References


Figure Legend

**Figure 2.1** Regression plot of percent total systemic cardiac output directed to tissues perfused by the left subclavian and carotid arteries as measured with ultrasonic flow probes and the microsphere recovery technique. Values presented are from 5°C and 21°C acclimated turtles (N = 7) during normoxic control, routine anoxia and following α-adrenergic stimulation and blockade during anoxia.

**Figure 2.2** Chronological changes of cardiovascular function in 21°C-acclimated turtles to 6 h of anoxic submergence. 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 = 8 unless otherwise indicated above the error bar.

**Figure 2.3** Chronological changes of cardiovascular function in 5°C-acclimated turtles to 12 days of anoxic submergence. 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.

**Figure 2.4** Systemic cardiac output distribution (%Q_{sys}) between tissues at A) routine normoxic control at 5°C, B) following 12 days of anoxic submergence at 5°C, C) routine normoxic control at 21°C and D) following 6 h of anoxic submergence at 21°C. Significant (P<0.05) tissue specific differences in %Q_{sys} between acclimation temperatures at routine normoxic control are signified with asterisks. Exploded slices signify significant differences (P<0.05) in tissue %Q_{sys} between routine normoxia and anoxia within each acclimation temperature. N = 6 at 5°C and 5 at 21°C.
Table 2.1 *Blood flows through select major systemic arches of 5°C- and 21°C-acclimated turtles during normoxic and anoxic exposure.*

<table>
<thead>
<tr>
<th>Acclimation Temperature (°C)</th>
<th>Condition</th>
<th>Variable</th>
<th>Blood Flow (ml min⁻¹ kg⁻¹)</th>
<th>%Qsys</th>
<th>Factor*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Normoxia</td>
<td>Qsys</td>
<td>11.3±1.4</td>
<td>34.8±0.9</td>
<td>2.88±0.07</td>
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<td></td>
<td></td>
<td>QLAo</td>
<td>3.9±0.4</td>
<td>36.3±4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>QRAo</td>
<td>4.0±0.7</td>
<td>36.3±4.2</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>QLsubcar</td>
<td>1.7±0.4</td>
<td>14.5±2.2</td>
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</tr>
<tr>
<td></td>
<td>Anoxia</td>
<td>Qsys</td>
<td>2.68±0.35</td>
<td>36.9±3.6</td>
<td>2.79±0.27</td>
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<tr>
<td></td>
<td></td>
<td>QLAo</td>
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<tr>
<td></td>
<td></td>
<td>QRAo</td>
<td>0.90±0.14</td>
<td>33.3±1.3</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>QLsubcar</td>
<td>0.41±0.09</td>
<td>14.9±1.5</td>
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</tr>
<tr>
<td>21</td>
<td>Normoxia</td>
<td>Qsys</td>
<td>46.3±2.8</td>
<td>38.6±3.5</td>
<td>2.67±0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QLAo</td>
<td>18.1±2.7</td>
<td>37.1±1.5</td>
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<td></td>
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<td>QRAo</td>
<td>17.2±1.2</td>
<td>12.1±1.7</td>
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<tr>
<td></td>
<td></td>
<td>QLsubcar</td>
<td>5.5±0.7</td>
<td>12.1±1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anoxia</td>
<td>Qsys</td>
<td>15.4±2.6</td>
<td>34.6±1.9</td>
<td>2.92±0.16</td>
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<tr>
<td></td>
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<td>QLAo</td>
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<td>36.4±3.7</td>
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<tr>
<td></td>
<td></td>
<td>QLsubcar</td>
<td>2.3±0.5</td>
<td>14.5±1.1</td>
<td></td>
</tr>
</tbody>
</table>

* Multiple of QLAo needed to equal Qsys

Values are means ± S.E.M. (N=4 at 5°C and 5 at 21°C)

Qsys, systemic blood flow; QLAo, left aortic arch blood flow; QRAo, right aortic arch blood flow;

QLsubcar, left subcalvian and left common carotid blood flow.
Table 2.2 Hematocrit and arterial pH of normoxic turtles.

<table>
<thead>
<tr>
<th>Acclimation Temperature (°C)</th>
<th>Hematocrit (%)</th>
<th>Arterial pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>30 ± 3</td>
<td>7.82 ± 0.05</td>
</tr>
<tr>
<td>21</td>
<td>21 ± 3</td>
<td>7.53 ± 0.08*</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.  
N = 4 in all instances  
An asterisk indicates significant difference between acclimation temperatures.
Table 2.3 Cardiovascular status of 5- and 21°C-acclimated normoxic turtles before and after intra-arterial injection of the α-adrenergic antagonist phentolamine.

<table>
<thead>
<tr>
<th>Acclimation Temperature (°C)</th>
<th>Drug Injection Conditions</th>
<th>Heart Rate (min⁻¹)</th>
<th>Systemic Stroke Volume (ml kg⁻¹)</th>
<th>Systemic Cardiac Output (ml min⁻¹ kg⁻¹)</th>
<th>Systemic Blood Pressure (kPa)</th>
<th>Systemic Power Output (mW g⁻¹)</th>
<th>Systemic Resistance (kPa ml⁻¹ min kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Routine Normoxic</td>
<td>4.1±0.4</td>
<td>2.31±0.37</td>
<td>9.2±1.3 *</td>
<td>1.52±0.11 *</td>
<td>0.126±0.025 *</td>
<td>0.18±0.03 *</td>
</tr>
<tr>
<td></td>
<td>Phentolamine (3 mg kg⁻¹)</td>
<td>4.3±0.5</td>
<td>3.21±0.57</td>
<td>12.8±1.8 *</td>
<td>0.98±0.06 *</td>
<td>0.108±0.015</td>
<td>0.08±0.01 *</td>
</tr>
<tr>
<td>21</td>
<td>Routine Normoxic</td>
<td>21.3±2.0</td>
<td>1.95±0.29 (5)</td>
<td>42.9±8.0 (5)</td>
<td>2.83±0.53</td>
<td>1.506±0.323 (5)</td>
<td>0.09±0.02 (5)</td>
</tr>
<tr>
<td></td>
<td>Phentolamine (3 mg kg⁻¹)</td>
<td>22.6±3.4</td>
<td>2.84±0.90 (4)</td>
<td>63.5±18.6 (4)</td>
<td>2.08±0.33</td>
<td>1.777±0.591 (4)</td>
<td>0.06±0.02 (4)</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. (N=6 unless otherwise indicated in parenthesis)

Significant differences (P<0.05) between acclimation temperature for routine normoxic cardiovascular status are indicated by asterisks.

Significant differences (P<0.05) between routine normoxic and phentolamine for each variable at each acclimation temperature are indicated by the letter a.
Table 2.4  Cardiovascular status of 5- and 21°C-acclimated anoxic turtles before and after intra-arterial injections of the α-adrenergic agonist phenylephrine and antagonist phentolamine during anoxic exposure.

<table>
<thead>
<tr>
<th>Acclimation Temperature (°C)</th>
<th>Drug Injection</th>
<th>Heart Rate (min⁻¹)</th>
<th>Systemic Stroke Volume (ml kg⁻¹)</th>
<th>Systemic Cardiac Output (ml min⁻¹ kg⁻¹)</th>
<th>Systemic Blood Pressure (kPa)</th>
<th>Systemic Power Output (mW g⁻¹)</th>
<th>Systemic Resistance (kPa ml⁻¹ min kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Routine Normoxic</td>
<td>4.96±0.38</td>
<td>2.79±0.51</td>
<td>13.62±2.34</td>
<td>1.81±0.16</td>
<td>0.22±0.052</td>
<td>1.14±0.01</td>
</tr>
<tr>
<td></td>
<td>Routine Anoxic</td>
<td>1.06±0.11</td>
<td>3.10±0.40</td>
<td>3.18±0.33</td>
<td>1.22±0.14</td>
<td>0.03±0.006</td>
<td>0.41±0.06</td>
</tr>
<tr>
<td></td>
<td>Phenylephrine (5 µg kg⁻¹)</td>
<td>1.13±0.06</td>
<td>2.42±0.33</td>
<td>2.71±0.36</td>
<td>1.75±0.18</td>
<td>0.04±0.007</td>
<td>0.74±0.16</td>
</tr>
<tr>
<td></td>
<td>Phenylephrine (50 µg kg⁻¹)</td>
<td>1.33±0.07</td>
<td>2.03±0.42</td>
<td>2.64±0.62</td>
<td>2.11±0.18</td>
<td>0.04±0.009</td>
<td>1.02±0.19</td>
</tr>
<tr>
<td></td>
<td>Phentolamine (3 mg kg⁻¹)</td>
<td>1.25±0.08</td>
<td>3.58±0.69</td>
<td>4.70±1.12</td>
<td>1.36±0.13</td>
<td>0.05±0.015</td>
<td>0.46±0.20</td>
</tr>
<tr>
<td></td>
<td>Phenylephrine (50 µg kg⁻¹)</td>
<td>1.20±0.07</td>
<td>3.84±0.75</td>
<td>4.67±1.07</td>
<td>1.37±0.10</td>
<td>0.05±0.014</td>
<td>0.40±0.13</td>
</tr>
<tr>
<td>21</td>
<td>Routine Normoxic</td>
<td>21.1±1.1 (8)</td>
<td>2.02±0.22 (8)</td>
<td>39.2±3.6 (8)</td>
<td>3.05±0.45 (7)</td>
<td>1.05±0.195 (7)</td>
<td>0.08±0.01 (7)</td>
</tr>
<tr>
<td></td>
<td>Routine Anoxic</td>
<td>10.1±0.6 (8)</td>
<td>1.49±0.19 (8)</td>
<td>14.8±1.8 (8)</td>
<td>2.19±0.12 (7)</td>
<td>0.27±0.048 (7)</td>
<td>0.18±0.03 (7)</td>
</tr>
<tr>
<td></td>
<td>Phenylephrine (5 µg kg⁻¹)</td>
<td>10.4±0.5 (8)</td>
<td>1.39±0.23 (8)</td>
<td>14.3±2.4 (8)</td>
<td>2.71±0.18 (7)</td>
<td>0.31±0.056 (7)</td>
<td>0.27±0.07 (7)</td>
</tr>
<tr>
<td></td>
<td>Phenylephrine (50 µg kg⁻¹)</td>
<td>10.5±0.4 (8)</td>
<td>1.45±0.25 (8)</td>
<td>15.0±2.3 (8)</td>
<td>2.41±0.19 (7)</td>
<td>0.30±0.060 (7)</td>
<td>0.21±0.04 (7)</td>
</tr>
<tr>
<td></td>
<td>Phentolamine (3 mg kg⁻¹)</td>
<td>11.9±0.5 (8)</td>
<td>2.07±0.20 (8)</td>
<td>24.4±2.5 (8)</td>
<td>1.24±0.13 (7)</td>
<td>0.25±0.037 (7)</td>
<td>0.06±0.01 (7)</td>
</tr>
<tr>
<td></td>
<td>Phenylephrine (50 µg kg⁻¹)</td>
<td>12.4±0.7 (8)</td>
<td>1.96±0.20 (8)</td>
<td>23.9±2.3 (8)</td>
<td>1.41±0.16 (7)</td>
<td>0.28±0.056 (7)</td>
<td>0.07±0.01 (7)</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. (N=6 at 5 °C and are indicated in parenthesis at 21°C)

Significant differences (P<0.05) between routine anoxic and drug injections for each variable at each acclimation temperature are indicated by dissimilar letters.
Table 2.5 Percent systemic cardiac output (\%Q_{sys}) and absolute blood flow (\mu l min^{-1} kg^{-1}) to the various tissues of 5- and 21°C-acclimated turtles during routine normoxic and anoxic conditions and following anoxic injections of phenylephrine and phentolamine.

<table>
<thead>
<tr>
<th>Acclimation Temperature</th>
<th>Tissue</th>
<th>%Q_{sys} (50 \mu g kg^{-1})</th>
<th>Tissue flow (\mu l min^{-1} kg^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>5°C</td>
<td></td>
<td>Routine</td>
<td>Phentolamine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Normoxia</td>
<td>Anoxia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Routine</td>
<td>Phentolamine</td>
</tr>
<tr>
<td>Integument</td>
<td>4.4±0.2</td>
<td>6.3±1.3 ( ^\dagger )</td>
<td>6.1±1.1</td>
</tr>
<tr>
<td>Muscle</td>
<td>19.7±0.9</td>
<td>11.1±1.5</td>
<td>12.3±1.4 ( ^a )</td>
</tr>
<tr>
<td>Bone</td>
<td>6.6±0.6</td>
<td>8.2±0.9</td>
<td>7.2±1.0</td>
</tr>
<tr>
<td>Stomach</td>
<td>2.3±0.4</td>
<td>2.6±1.3</td>
<td>1.2±0.3</td>
</tr>
<tr>
<td>Intestines</td>
<td>4.9±0.8</td>
<td>4.2±0.8 ( ^d )</td>
<td>3.6±1.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.3±0.1</td>
<td>1.6±0.9</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Ventricle</td>
<td>0.2±0.02</td>
<td>0.7±0.3</td>
<td>0.8±0.3</td>
</tr>
<tr>
<td>Atria</td>
<td>0.07±0.02</td>
<td>0.2±0.05</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>Brain</td>
<td>0.3±0.2</td>
<td>0.7±0.3</td>
<td>0.9±0.4</td>
</tr>
<tr>
<td>Liver</td>
<td>9.4±0.6</td>
<td>4.8±1.1</td>
<td>8.3±2.1 ( ^d )</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.9±0.3</td>
<td>6.0±3.2</td>
<td>2.7±1.7 ( ^b )</td>
</tr>
<tr>
<td>Gonads</td>
<td>8.7±2.6</td>
<td>6.9±2.5</td>
<td>2.6±0.4</td>
</tr>
<tr>
<td>Fat</td>
<td>2.9±0.6</td>
<td>1.8±0.4</td>
<td>1.9±0.7</td>
</tr>
<tr>
<td>Connective Tissue</td>
<td>1.9±0.2</td>
<td>1.7±0.4</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>Shell</td>
<td>35.2±2.1</td>
<td>41.8±7.5</td>
<td>50.2±4.9 ( ^** )</td>
</tr>
<tr>
<td>Eyes</td>
<td>0.07±0.02</td>
<td>1.4±0.5</td>
<td>0.6±0.3</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. (N = 6 at 5°C and 5 at 21°C)
Significant differences (P<0.05) in normoxic \%Q_{sys} and absolute blood flow between acclimation temperature are indicated with \( ^\dagger \).
Significant differences (P<0.05) in \%Q_{sys} for each tissue between routine normoxia and routine anoxia are indicated with \( ^\cdot \).
Significant differences (P<0.05) in \%Q_{sys} for each tissue between routine anoxia, phenylephrine and phentolamine injections are indicated with dissimilar letters.
Significant differences (P<0.05) in absolute blood flow for each tissue between the four conditions are indicated by dissimilar letters.
Table 2.6 *Reductions in tissue absolute flow during chronic anoxic submergence at 5°C.*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidneys</td>
<td>14.2</td>
</tr>
<tr>
<td>Stomach</td>
<td>13.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>10.2</td>
</tr>
<tr>
<td>Connective Tissue</td>
<td>8.4</td>
</tr>
<tr>
<td>Eyes</td>
<td>8.4</td>
</tr>
<tr>
<td>Intestines</td>
<td>5.4</td>
</tr>
<tr>
<td>Gonads</td>
<td>5.2</td>
</tr>
<tr>
<td>Bone</td>
<td>4.9</td>
</tr>
<tr>
<td>Integument</td>
<td>4.2</td>
</tr>
<tr>
<td>Fat</td>
<td>4.1</td>
</tr>
<tr>
<td>Shell</td>
<td>3.8</td>
</tr>
<tr>
<td>Muscle</td>
<td>3.6</td>
</tr>
<tr>
<td>Ventricle</td>
<td>3.5</td>
</tr>
<tr>
<td>Brain</td>
<td>3.2</td>
</tr>
<tr>
<td>Liver</td>
<td>2.5</td>
</tr>
<tr>
<td>Atria</td>
<td>2.3</td>
</tr>
</tbody>
</table>
Figure 2.1
Figure 2.2

- Systemic Resistance (kPa ml⁻¹ min kg⁻¹)
- Systemic Cardiac Output (ml min⁻¹ kg⁻¹)
- Systemic Blood Pressure (kPa)
- Systemic Power Output (mW g⁻¹)
- Systemic Stroke Volume (ml kg⁻¹)
- Heart Rate (min⁻¹)
Figure 2.4
CHAPTER III: ADENOSINERGIC CARDIOVASCULAR CONTROL IN ANOXIA-TOLERANT VERTEBRATES DURING PROLONGED OXYGEN DEPRIVATION

Introduction

The vast majority of vertebrate species die after only a few minutes when deprived of molecular oxygen (anoxia). Anoxic death in these anoxia-intolerant animals primarily occurs due to the disruption of energy supply to cellular processes in critical tissues with a very high ATP demand like the brain and heart. The reason for an energy crisis is that aerobic ATP production can yield 14-times more ATP per mole of glucose than anaerobic glycolysis, which becomes the sole route for ATP production during prolonged anoxia. However, some anoxia-tolerant vertebrates survive prolonged periods (hours to weeks) of severe hypoxia and anoxia, and the basis of anoxic survival lies in their ability to balance cellular ATP supply with ATP demand, as well as cope with the acidosis associated with sustained anaerobic metabolism (Shoubridge and Hochachka, 1980; Boutilier, 2001; Jackson, 2002; Farrell and Stecyk, 2006).

Theoretically, cellular ATP supply and demand can be balanced during anoxia by up-regulating glycolytic ATP production to match ATP supply to demand (the Pasteur effect) and by reducing energy demand to a level that can be supported by the reduced ATP availability (hypometabolism), or through a combination of both of these strategies (Lutz and Nilsson, 1997).

A signaling molecule capable of coordinating the matching of ATP supply with ATP demand during periods of metabolic stress such as oxygen deprivation is adenosine, a ubiquitous, endogenous nucleoside. Adenosine is primarily formed from the breakdown of cytosolic ATP and released into the extracellular space from energy-deficient cells when energy supply and demand are mismatched. Adenosine can mediate numerous physiological and metabolic effects that promote substrate availability and suppress metabolic demands via interaction with at least four (i.e., A₁, A₂A, A₂B and A₃) cell membrane receptors (Newby, 1984;
Newby et al., 1990; Belardinelli and Shryock, 1992; Mubagwa et al., 1996; Mubagwa and Flameng, 2001; Villarreal et al., 2003). This retaliatory role of adenosine is well documented for cardiac tissues of anoxia-intolerant mammalian species. Specifically, adenosine vasodilates the coronary artery, stimulates glucose uptake by myocardial cells, causes bradycardia, antagonizes the stimulatory cardiac effects of catecholamines, decreases the velocity of cardiac impulse conduction and decreases the force of cardiac contractions (Wyatt et al., 1989; Belardinelli and Shryock, 1992; Mubagwa et al., 1996). In the periphery, adenosine is equally important, regulating vascular tone through relaxations and contractions, depending on vessel type and basal tone (Tabrizchi and Bedi, 2001).

For anoxia-tolerant vertebrates, the role adenosinergic cardiovascular control plays during prolonged periods of oxygen deprivation is unclear, in part because previous findings appear to be contradictory. For example, in vitro studies on atrial tissue of the hypoxia-tolerant common carp (Cyprinus carpio; Cohen et al., 1981) and the anoxia-tolerant crucian carp (Carassius carassius; Vornanen and Tuomennoro, 1999) indicate that adenosine can have negative chronotropic and inotropic cardiac effects under normoxic conditions. However, results from in vivo studies show that adenosinergic cardiac control during oxygen deprivation does not exist. Intraperitoneal injection of the adenosinergic antagonist aminophylline into 22°C-acclimated crucian carp caused tachycardia in normoxic fish, but did not abolish the bradycardia associated with a brief (30 min) hypoxic (0.8 mg O₂ l⁻¹) exposure (Vornanen and Turmennoro, 1999). Likewise, aminophylline did not inhibit the hypoxic bradycardia exhibited by the hypoxia-tolerant epaulette shark (Hemiscyllium ocellatum) during 20 minutes of severe hypoxia (0.3 mg O₂ l⁻¹) at 25°C (Stensløkken et al., 2004). Although, absence of adenosinergic cardiovascular control may have been due to the relatively short hypoxic exposure times at warm temperatures because with prolonged hypoxia in common carp, an adenosinergic inhibition of cardiac activity was suggested (but not definitively demonstrated because adenosine antagonist
injection followed other antagonists injections) in 5°C-acclimated fish, but not in 10°C- or 15°C-acclimated animals (Stecyk and Farrell, 2006).

In view of this uncertainty, coupled with the fact that cardiovascular depression by anoxia-tolerant vertebrates during prolonged oxygen deprivation (Hicks and Farrell, 2000ab; Stecyk and Farrell, 2002; Stecyk et al., 2004ab; Stecyk and Farrell, 2006) mirrors the effects of adenosine on the mammalian cardiovascular system, the present study investigates adenosinergic regulation of the cardiovascular system during normoxia and prolonged anoxia in three vertebrate species. The freshwater turtle (*Trachemys scripta*), common carp and crucian carp are considered to exhibit different levels of anoxia tolerance, as well as different anoxic survival strategies and cardiovascular responses (Lutz and Nilsson, 1997; Hicks and Farrell, 2000a; Stecyk and Farrell, 2002, Stecyk et al., 2004b; Farrell and Stecyk, 2006; Stecyk and Farrell, 2006). Given the receptor- and tissue-specific physiological effects of adenosine (see reviews by Shryock and Belardinelli, 1997; Mubagwa and Flameng, 2001), we tested for the presence of adenosinergic cardiovascular control using an intra-arterial injection of the non-specific adenosine receptor antagonist aminophylline while continuously measuring cardiovascular status. Our null hypothesis was that adenosinergic mechanisms do not regulate cardiovascular status during prolonged oxygen deprivation in the freshwater turtle, common carp and crucian carp.

**Materials and methods**

**Experimental animals**

Twenty-three red-eared slider turtles (*Trachemys scripta*; body masses ranging between 0.56 and 1.01 kg; 0.80 ± 0.15 kg, mean ± S.D.), 13 common carp (*Cyprinus carpio*; 0.91 - 1.46 kg; 1.17 ± 0.18 kg, mean ± S.D.) and 12 crucian carp (*Carassius carassius*; 0.21 - 0.34 kg; 0.27
± 0.04 kg, mean ± S.D.) were used in this study. Turtles were obtained from Lemberger Inc. (Oshkosh, WI, USA) and airfreighted to Simon Fraser University. Turtles acclimated to 21°C were held indoors in glass aquaria under a 12 h:12 h L:D photoperiod, had free access to basking platforms and diving water and were fed several times a week with a mixture of commercial trout feed pellets, cat food, and fresh vegetables. The turtles acclimated to 5°C were kept in glass aquaria with access to air in a temperature-controlled room set to 5°C for 5 weeks prior to experimentation to allow adequate time for cold-acclimation (Hicks and Farrell, 2000a). The 5°C-acclimated turtles were fasted during this period. Two acclimation temperatures were used for turtles because of the temperature-dependence of anoxia tolerance in this species (maximum anoxia survival time is 24 h and 44 d at 21°C and 3°C, respectively; Ultsch, 1985; Warren et al., 2006). Common carp were captured using traps by a local fisherman and maintained indoors at Simon Fraser University. Fish were acclimated to 5°C for several weeks in a 2000 l flow-through fiberglass tank receiving aerated dechlorinated water, fed ad libitum with commercial trout food pellets (ProForm Aquaculture Feeds, Chilliwack, BC, Canada) and maintained under a 12 h:12 h L:D photoperiod. By acclimating common carp to 5°C we could extend the maximum hypoxia exposure period to 24 h (as compared with just 3 h at 15°C; Stecyk and Farrell, 2002). Crucian carp were captured in the Svartkulp pond, Oslo community, kept for at least 2 weeks in tanks continuously supplied with aerated and dechlorinated Oslo tap water (8°C), maintained under a 12 h:12 h L:D photoperiod and were fed daily with commercial carp food (Tetra Pond, Tetra, Melle, Germany). Crucian carp can tolerate at least 5 days of anoxia at 8°C (Stecyk et al, 2004b). All procedures were in accordance with Simon Fraser University Animal Care Guidelines and the Norwegian Animal Research Authority.
Surgical procedures

Cardiovascular status was evaluated by monitoring heart rate ($f_H$), cardiac output ($Q$) and ventral aortic pressure ($P_{VA}$) for fish, and $f_H$, systemic cardiac output ($Q_{sys}$) and systemic blood pressure ($P_{sys}$) for the turtle during normoxia, severe hypoxia (common carp only) and anoxia. Surgical procedures for placement of catheter and flow probe lasted approximately 30 - 45 min in each species and were as follows.

Surgical operations for 21°C-acclimated turtles were at room temperature and in a 7 - 10°C room for 5°C-acclimated turtles. Turtles were intubated with soft rubber tubing for artificial ventilation with isoflurane (4% in room air, prepared by a Halothane vaporizer, Dräger, Lubeck, Germany) at a rate of 8-15 breaths min⁻¹ and a tidal volume of 10-20 ml kg⁻¹ using a Harvard Apparatus Ventilator (HI 665, Harvard Apparatus Inc., Holliston, MA, USA). Once a surgical plain of anaesthesia was achieved, as determined by the lack of a pedal withdrawal reflex, the isoflurane level was reduced to and maintained between 0.5% and 1%. The heart and central vascular blood vessels were accessed by excision of a 2 x 2 cm piece of the plastron using a bone saw, and an occlusive catheter (PE-50 containing saline with 100 IU ml⁻¹ heparin) was advanced from the left thyroid artery into the right subclavian artery originating from the right aortic arch. For measurement of $Q_{sys}$, a 1.0 - 1.5 cm section of the left aortic arch (LAo) was freed from the surrounding connective tissue for placement of an ultrasonic blood flow probe (sizes 2 – 3 mm; Transonic Systems Inc., Ithaca, NY, USA). Acoustic gel was infused between the blood vessel and the flow probe to reduce signal noise and the excised piece of the plastron was resealed in its original position using surgical tape and fast-drying epoxy resin. Turtles then were ventilated with room air until they resumed spontaneous ventilation and placed in individual water-filled plastic chambers (30 cm x 20 cm x 10 cm), covered with black plastic.
to minimize visual disturbance to recover for either 48 h at 21°C, or 72 h at 5°C before being tested.

Common carp were anaesthetized with buffered tricaine methanesulphonate (MS-222) (0.2 g l⁻¹ MS-222 + 0.2 g l⁻¹ NaHCO₃) and crucian carp with benzocaine [50 g l⁻¹ dissolved in 96% ethanol and added to the water to a final concentration of 100 mg l⁻¹] until opercular movements ceased. The fish were placed on an operating table and the gills continuously irrigated with a chilled, recirculating anaesthetic solution (0.1 g l⁻¹ MS-222 + 0.1 g l⁻¹ NaHCO₃ for common carp; 40 mg l⁻¹ benzocaine for crucian carp). An ultrasonic flow probe (common carp, sizes 2–3 mm, Transonic Systems Inc.) or a Doppler flow probe (crucian carp, 20 MHz, 45°, Iowa Doppler Products) was positioned around the ventral aorta to measure \( Q \). It was not possible to use ultrasonic flow probes with crucian carp because of their small size. To measure \( P_{VA} \), the ventral aorta was non-occlusively cannulated (see Axellson, et al., 1992; Stecyk and Farrell, 2002; Stecyk et al., 2004b; Stecyk and Farrell, 2006 for details on the surgical procedure). Flow probe leads and the cannula were securely fastened with several skin sutures, including one to the base of the dorsal fin. Recovery of the fish began when gill irrigation was switched from the anaesthetic solution to aerated fresh water and with the onset of opercular movements, which usually occurred within 5 min, common carp were transferred to flow-through experimental holding tubes (previously described by Stecyk and Farrell, 2002) and crucian carp were transferred to submerged, open-ended, but mesh-covered cylindrical tubes. Both fish species were provided at least a 48 h post-operative recovery before being tested.

**Experimental protocols**

All experiments were performed on unrestrained, temperature-acclimated animals that were able to move within their experimental chambers. Normoxic turtles had free access to room air throughout the experimentation period. Anoxia turtles were denied air access by
completely filling the housing chamber with water that was continuously gassed with N₂ (water oxygen concentration was reduced to <0.1 mg O₂ l⁻¹) and suspending a metal mesh below the surface of the water. For common carp, severe hypoxia (water oxygen concentration always <0.3 mg O₂ l⁻¹ and usually <0.1 mg O₂ l⁻¹) was achieved by continuously gassing the water with 100% N₂ in an exchange column before it entered the flow-through holding tube. For crucian carp, anoxia was induced by continuously gassing the water in the tank housing the fish with N₂. Anoxic conditions (<0.01 mg O₂ l⁻¹; regularly monitored with an oxygen electrode) were reached within 3 h.

Adenosinergic cardiovascular control during normoxia and anoxia was assessed by injecting aminophylline [(theophylline)₂ ethylenediamine; Sigma-Aldrich, Oakville, ON, Canada], which after injection rapidly forms free theophylline, a potent, non-specific antagonist of adenosine receptors (Cotgreave and Caldwell, 1983; Snyder, 1985). Aminophylline was dissolved in physiological saline [in mmol l⁻¹, turtle: NaCl, 105; KCl, 2.5; CaCl₂, 1.3; MgSO₄, 1; NaHCO₃, 15; NaH₂PO₄, 1; pH 7.8; common carp: NaCl, 124.1; KCl, 3.1; CaCl₂, 2.5; MgSO₄, 0.9; N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic (TES) free acid, 3.6; TES Na salt, 6.4; pH 7.8 (Keen et al. 1993); crucian carp: NaCl, 140; KCl, 2.95; CaCl₂, 1; MgSO₄, 1] and injected as a single intra-arterial bolus at a concentration of 3 mg kg⁻¹ (turtle) or 1 mg kg⁻¹ (common and crucian carp) through the arterial cannula, which was subsequently flushed with heparinized (10 IU ml⁻¹) saline. These doses of aminophylline fall in the middle of the range of doses previously shown to be of sufficient antagonistic potency in fish, reptiles and mammals (0.02 to 20 mg/kg; Mohrman and Heller, 1984; Long and Anthonisen, 1994; Sundin and Nilsson 1996; Sundin et al., 1999; Söderström et al., 1999; Stenslökken et al., 2004; Stecyk and Farrell, 2006). The total volume injected never surpassed 1.5 ml kg⁻¹ and control saline injections of equal volume did not cause any statistically significant changes in cardiovascular status (see Figs. 3.1, 3.2, 3.3, 3.4). Control normoxic animals were injected at the conclusion of the post-operative recover period,
while anoxic (severely hypoxic for common carp) animals were injected following their respective anoxic exposures: 6 h for 21°C-acclimated turtles, 14 days for 5°C-acclimated turtles, 12.5 h for 5°C-acclimated common carp and 5 days for 8°C-acclimated crucian carp. These anoxia durations are known to result in new steady states for cardiovascular status for each species and temperature acclimation (see Hicks and Farrell, 2000a; Stecyk and Farrell 2002; Stecyk et al., 2004ab, Stecyk and Farrell, 2006).

Calculation of haematological and cardiovascular variables

Normoxic hematocrits of 19 ± 2% (21°C-acclimated turtles, N = 8), 28 ± 2% (5°C-acclimated turtles, N = 9), 32 ± 4% (common carp, N = 5) and 23 ± 2% (crucian carp, N = 7) measured at the conclusion of the post-operative recovery period, were determined as the fractional erythrocyte volume in a capillary tube following 3 min of centrifugation at 10,000 g. $P_{\text{sys}}$ and $P_{\text{VA}}$ were measured by attaching the arterial cannula to a pressure transducer [Baxter Edward, model PX600, Irvine, CA, USA (common carp and red-eared sliders); Gould Statham, model P23 Db, Gould-Statham, Oxnard, CA, USA (crucian carp)]. Pressure transducers were calibrated daily against a static water column, and the signals amplified with suitable preamplifiers [Senselab, Somedic Sales AB, Hörby, Sweden (common carp and red-eared sliders); University of Oslo in-house built preamplifier (crucian carp)]. The ultrasonic flow probe implanted in turtles and common carp, and Doppler flow probe implanted in crucian carp, were connected to a T201 Transonic blood flow meter (Transonic Systems, Inc., Ithaca, NY, USA) and a 545C-4 directional pulsed Doppler flow meter (Bioengineering, University of Iowa, IA, USA), respectively, for measurement of either $Q$ or $Q_{\text{sys}}$. For common and crucian carp, $Q$ is blood flow through the ventral aorta, while for the turtle, $Q_{\text{sys}}$ was estimated as $2.8 \times Q_{\text{LAo}}$ (as previously verified by Comeau and Hicks, 1994; Wang and Hicks, 1996; Stecyk et al., 2004a). $f_H$ was derived from the beat-to-beat interval from either the flow or pressure traces. Stroke
volume [cardiac stroke volume ($V_{SH}$) for common and crucian carp, systemic stoke volume ($V_{sys}$) for turtle] was calculated as $Q / f_{f1}$ (common and crucian carp) and $Q_{sys}/f_{f1}$ (turtle). Calculation of peripheral resistance [total peripheral resistance ($R$), i.e. the sum of branchial and systemic resistance for common and crucian carp, systemic resistance ($R_{sys}$) for the turtle] as $P_{VA}/Q$ (common and crucian carp) and $P_{sys}/Q_{sys}$ (turtle) assumed that central venous blood pressure was negligible (an assumption that overestimate the change in $R$ if venous blood pressure increased during hypoxia/anoxia). Cardiac power output [$PO$ for common and crucian carp, systemic cardiac power output ($PO_{sys}$) for turtle], was calculated as $Q \times P_{VA} / Mv$ (common and crucian carp) and $Q_{sys} \times P_{sys} / Mv$ (turtle), where $Q$ and $Q_{sys}$ are measured in ml s$^{-1}$, $P_{VA}$ and $P_{sys}$ are measured in kPa and $Mv$ is ventricular mass (g). Cardiovascular signal traces acquired from turtles and common carp were recorded at 50 Hz with an in-house programmed computer assisted data acquisition system (National Instruments, Austin, TX, USA) and data analysis was performed offline using in-house data analysis software. Cardiovascular signals from crucian carp were recorded to a computer with a Powerlab 4/20 data acquisition system (AD Instruments, Colorado Springs, CO, USA) and analyzed offline with the accompanying software.

**Data analysis and statistics**

Normoxic control values for all experimental animals were determined from the averages of continuous recordings of cardiovascular status for 30-60 minutes immediately prior to the commencement of either the exposure or the aminophylline injection. For 21°C-acclimated turtles and common carp, haemodynamic variables were recorded continuously throughout the severely hypoxia/anoxia exposure period, and at each hour of severe hypoxia/anoxia exposure an average for a 5-min period was calculated and reported here. For crucian carp, haemodynamic variables were recorded and averaged for a 30-min period on each day of the 5-day anoxia
exposure. For 5°C-acclimated turtles, haemodynamic variables were recorded and averaged for a 30-min period at the same time of day on days three, six, nine, twelve and fourteen of the 14-day anoxia exposure period. For all animals, cardiovascular status was recorded continuously following control saline and aminophylline injections; Reported haemodynamic values from these time periods were averaged (from 3- to 5-min periods) at 5 min and 10 min following injection, and then at 10-min intervals to a maximum of 30 min (control saline injections), 60 min (aminophylline injection in turtles), 50 min (aminophylline injection in common carp) and 40 min (aminophylline injection in crucian carp).

Mean values ± S.E.M are presented for all cardiovascular variables at each sample time. Paired t-tests or Wilcoxon signed rank tests (crucian carp percentage data) were used to determine statistical significance between control normoxic and anoxic/severely hypoxic cardiovascular status (Table 3.1). Statistically significant changes in cardiovascular variables following aminophylline injection were detected by utilizing a 2-way repeated measures analysis of variance (RM ANOVA) to compare the cardiovascular responses to the control saline and aminophylline injections at 5, 10, 20 and 30 min following injection. Multiple comparisons were performed using Student-Newman-Keuls tests, and for crucian carp percentage data, values were first arcsine transformed. Further, paired t-tests or Wilcoxon signed rank tests (crucian carp percentage data) between pre-aminophylline control values and the last recorded data point following aminophylline injection (i.e. the 40, 50 and 60 min data points for crucian carp, common carp and turtles, respectively) were used to test for statistically significant changes in cardiovascular status beyond the 30 min period tested with the two-way RM ANOVA. In all instances, \( P<0.05 \) is used as the level of significance.
Results

Normoxic and anoxic/severe hypoxic cardiovascular status

The control cardiovascular status for normoxic animals, as well as the cardiovascular responses to anoxia/severe hypoxia reported here (Table 3.1) are in accord with previous studies on red-eared slider turtles, common carp and crucian carp (Garey, 1970; Itazawa, 1970; Hicks and Farrell, 2000ab; Stecyk and Farrell, 2002; Stecyk et al., 2004ab; Stecyk and Farrell, 2006). Briefly, the response to 6 h (21°C) and 14 days (5°C) of anoxia in the red-eared slider included large reductions in $Q_{sys}$ (69% at 21°C and 70% at 5°C), $f_H$ (70% at 21°C and 63% at 5°C), $P_{sys}$ (42% at 21°C and 53% at 5°C) and $PO_{sys}$ (82% at 21°C and 88% at 5°C). Correspondingly, $R_{sys}$ increased significantly by 71% at 21°C and 65% at 5°C. Similarly, 5°C-acclimated common carp also exhibited significant reductions in cardiovascular status when deprived of oxygen, but not quite of the same magnitude as those displayed by cold-acclimated freshwater turtles. By 12.5 h of severe hypoxia, $Q$ was maximally depressed in common carp by 50%, $f_H$ by 42%, $P_{VA}$ by 24% and $PO$ by 64%, while $R$ significantly increased by 64%. In stark contrast to the turtles and common carp, crucian carp following 5 days of anoxia maintained all cardiovascular variables ($Q$, $f_H$, $V_{SH}$, $R$ and $PO$) at control normoxic levels with the exception of a modest arterial hypotension (18% reduction in $P_{VA}$). Given that the present data conform well to earlier data, we are confident of the results for aminophylline injections during normoxia and severe hypoxia/anoxia.

Cardiovascular responses to control saline injection

Control saline injections of 1.5 ml kg$^{-1}$ (i.e., a volume equivalent to the aminophylline injections) did not cause any statistically significant haemodynamic changes in control normoxic
or anoxic/severely hypoxic animals (Figs. 3.1, 3.2, 3.3, 3.4). These control injections were made 0.5 h before aminophylline injection.

**Cardiovascular responses to aminophylline injection**

**Normoxic animals**

Under normoxic conditions, aminophylline injection in both warm- and cold-acclimated turtles resulted in a short-lived tachycardia, while other cardiovascular variables remained unchanged (Figs. 3.1, 3.2). At 21°C, $f_H$ increased significantly by 25% from 26.6 min$^{-1}$ to a maximum of 33.4 min$^{-1}$ within 20 min of the aminophylline injection, but returned to pre-injection levels by 60 min (Fig. 3.1B). Similarly, at 5°C, $f_H$ increased significantly by 30% from 3.0 min$^{-1}$ to 3.9 min$^{-1}$ within 30 min of the aminophylline injection before returning to the pre-aminophylline value (Fig. 3.2B).

In contrast to normoxic turtles, aminophylline injection had no significant effect on the cardiovascular status of normoxic crucian carp and common carp (Figs. 3.3, 3.4).

**Anoxia- and hypoxia-exposed animals**

The species-specific cardiovascular responses to aminophylline injection observed under normoxia were significantly altered by the anoxia/hypoxia exposure. Specifically, the increase in $f_H$ observed in both warm- and cold-acclimated normoxic turtles following aminophylline injection was no longer present in either 21°C- or 5°C-acclimated anoxic turtles (Figs. 3.1B, 3.2B). Aminophylline injection did not have any statistically significant effects on any of the measured cardiovascular variables in anoxic turtles at either acclimation temperature (Fig. 3.1, 3.2). Similarly, aminophylline injection did not cause any statistically significant changes in the
cardiovascular status of anoxic crucian carp (Fig. 3.3). Thus, adenosinergic regulation of cardiovascular status, which is present in normoxic turtles, is suppressed during anoxia, whereas adenosinergic regulation of cardiovascular status is not present in either normoxic or anoxic crucian carp.

Severely hypoxic common carp contrast with the anoxic turtle and crucian carp. Aminophylline injection significantly altered cardiovascular status by increasing $Q$ maximally by 63%. In fact, $Q$ remained elevated for the entire 50-min, post-aminophylline recording period (Fig. 3.4C). This increase in $Q$ reflected a significant 30% increase in $f_H$ (Fig. 3.4B), but only the 5-minute $V_{SH}$ value was statistically significantly greater than the pre-aminophylline control (Fig. 3.4D). $P_{VA}$ remained unchanged in severely hypoxic common carp following aminophylline injection (Fig. 3.4A), while $R$ significantly decreased by 45%, indicating adenosinergic vasodilation during severe hypoxia (Fig. 3.4E). The prolonged $f_H$ response of severely hypoxic common carp to aminophylline injection also contrasts with the transient increases in $f_H$ of both warm- and cold-acclimated normoxic turtles.

**Discussion**

Our objective was to determine if adenosinergic cardiovascular control modulates cardiovascular function in the hypoxia/anoxia-tolerant vertebrates, the freshwater turtle, common carp and crucian carp, during prolonged oxygen deprivation. Given the retaliatory role adenosine plays in coordinating the matching of cardiac ATP supply and demand during periods of energy-limitation and its well-documented effects on the cardiovascular system of anoxia-sensitive species during oxygen deprivation (Newby, 1984; Newby et al., 1990; Belardinelli and Shryock, 1992; Mubagwa et al., 1996; Mubagwa and Flameng, 2001; Villarreal et al., 2003), we expected to reject our null hypothesis. However, in support of the null hypothesis, our results
revealed that adenosinergic cardiovascular control during prolonged oxygen deprivation was absent in both red-eared sliders and crucian carp, and present only in common carp. We propose that this species-specific finding may be explained by differences in anoxia tolerance and anoxic survival strategies displayed by our study species.

**Critique of Methods**

The present study tested for the presence of adenosinergic modulation of cardiovascular status with an intra-arterial injection of the non-specific adenosine receptor antagonist aminophylline while continuously measuring cardiovascular status. The aminophylline concentration should be of sufficient potency to ensure antagonism of adenosine receptors, but moderate enough to avoid non-specific physiological effects. Therefore, we selected doses (1 mg kg\(^{-1}\) for crucian carp and common carp and 3 mg kg\(^{-1}\) for turtle) well within the range of doses known to be of sufficient potency in fish, reptiles and mammals (0.02 to 20 mg/kg; Mohrman and Heller, 1984; Long and Anthonisen, 1994; Sundin and Nilsson, 1996; Sundin et al., 1999; Söderström et al., 1999; Stensløkken et al., 2004; Stecyk and Farrell, 2006). Given known blood volumes (of 3% - 5% for teleost fish and 7% for turtles; Smits and Kozubowski, 1985; Olson, 1992), our aminophylline doses correspond to initial blood concentrations of approximately of between 5 \times 10^{-5} \text{ and } 8 \times 10^{-5} \text{ M in the carp and } 1 \times 10^{-4} \text{ M in the turtle. These concentrations are within the range of theophylline (aminophylline dissociates to theophylline once injected) concentrations (10^{-7} to 10^{-3} \text{ M}) previously shown to have vasoactive effects in fish (Okafor and Oduluye, 1986; Small et al., 1990; Mustafa and Agnisola, 1998).}

Our experimental technique also assumes that a change in cardiovascular status as a result of aminophylline injection is attributable to adenosinergic modulation of cardiovascular function. However, aminophylline, in addition to binding adenosine receptors, can also
potentiate cAMP-induced actions such as β-adrenergic cardiovascular effects through its inhibition of phosphodiesterase enzymes that degrade cAMP (Manallack et al. 2005). Thus, the observed increases in cardiac activity following aminophylline injection could also be a consequence of this non-specific effect. Nevertheless, the variability of cardiovascular response to aminophylline across species and experimental conditions argues against a general non-specific effect of the injections. Further, it should be stressed that the present study did not attempt to distinguish either the specific roles of the different types of adenosine receptors, or if adenosinergic cardiovascular control involved interaction with other signaling molecules such as nitric oxide (eg., Mustafa and Agnisola, 1998; Pellegrino et al., 2005). Such objectives were outside the scope of the current study, but could be investigated in the future.

Another potential concern of our experimental technique is that the heparin included in the saline solutions could affect cardiovascular status (eg., Susic et al., 1982). However, the concentration of heparin was (10 IU ml$^{-1}$ of saline), and control heparinised saline injections did not cause significant cardiovascular changes in any study species under any experimental condition. Thus, we do not believe that the inclusion of heparin in our drug injection saline affected our findings.

Finally, the authors acknowledge that some minor differences exist between experimental protocols for each study species. Specifically, crucian carp were instrumented with Doppler flow probes due to their small size. Consequently, measurements of $Q$, $V_{SH}$, $R$ and $PO$ in crucian carp are expressed as a percentage of control, whereas actual values are presented for the turtle and common carp. Further, the length of time cardiovascular status was measured following aminophylline injection varied somewhat between species, ranging from 40 min for crucian carp to 60 min for turtles. However, we do not believe that this difference in recording time limited our ability to detect changes in cardiovascular status. The present study, like a previous study
with common carp (Stecyk and Farrell, 2006), clearly shows that when cardiovascular responses to aminophylline do occur, they do so within 15 to 30 min of injection. Thus, the occurrence of significant changes in cardiovascular status after 40 to 60 min seems highly unlikely. Lastly, it could be argued that the different anaesthesia techniques utilized for common carp and crucian carp compromises our comparison between these related species. However, previous studies on these two carp species show a stable cardiovascular status after 48 h of recovery (Stecyk and Farrell, 2002; Stecyk et al., 2004b) and they respond similarly to other autonomic cardiovascular antagonists despite different anaesthesia techniques (Stecyk et al., 2004b; Stecyk and Farrell, 2006). In the present study, fish were allowed minimally 48 h, and up to 72 h post-operative recovery.

*Adenosinergic cardiovascular control during normoxia and severe hypoxia/anoxia*

Our results revealed a clear adenosinergic, inhibitory cardiovascular control (bradycardia and peripheral vasodilation) during prolonged oxygen deprivation in 5°C-acclimated common carp and not in normoxia. These findings are consistent with a retaliatory role for adenosine and with our earlier, but inconclusive findings for 5°C-acclimated common carp (Stecyk and Farrell 2002). However, a retaliatory role for adenosine was not identified in anoxic turtles and crucian carp, although adenosinergic, inhibitory cardiovascular control was present in normoxic turtles, but not crucian carp. These findings indirectly suggest that the red-eared slider and crucian carp are able to balance cardiac ATP supply and demand during prolonged anoxia, but common carp at 5°C are not.

We propose that this species-specific difference in adenosinergic cardiovascular control may reflect differences in the anoxia tolerance and survival strategies among these three species. Specifically, common carp are considerably less anoxia-tolerant than the freshwater turtle or
crucian carp, surviving severe hypoxia for only short periods (survival time is limited to ~24 h at 5°C and is even less at warmer acclimation temperatures; Blazka, 1958; Stecyk and Farrell 2002). In contrast, crucian carp and freshwater turtles overwinter in ice-covered ponds that become anoxic for periods of up to several months (Herbert and Jackson, 1985; Vornanen and Paajanen, 2004). Moreover, while hypoxic carp down-regulate cardiac activity with reductions in $f_h$, $Q$ and $P_O$ through vagal and adenosinergic mediated mechanisms (Stecyk and Farrell 2002; Stecyk and Farrell 2006; present study), the reduction in $P_O$ falls short of a 14-fold reduction (only 2.8-fold in the present study) needed to preclude an activation of a Pasteur effect for the heart. Thus, hypoxic heart of common carp depletes metabolic fuel and accumulates harmful anaerobic wastes at rates faster than in normoxia, reducing survival time. Consequently, we suggest that cardiac energy supply and demand in 5°C common carp never reaches steady state during severe hypoxia and, therefore, the presence of an adenosinergic inhibition of $f_h$ and $Q$ plays a critical role in extending the anoxic survival time. The negative chronotropic and inotropic adenosinergic effects on cardiac activity in the common carp during severe hypoxia most likely involve increases in $K^+$ conductance through the inwardly rectifying $K^+$ channels (Belardinelli et al., 1995; Aho and Vornanen, 2002) and an inhibition of L-type $Ca^{2+}$ current (Qu et al., 1993), respectively. However, future studies are needed to confirm the exact mechanisms and adenosine receptor types involved in adenosinergic cardiac inhibition.

In contrast to the common carp, the results for aminophylline injections in anoxic red-eared sliders and crucian carp indirectly suggests that they both are able to balance cardiac ATP supply and ATP demand during prolonged anoxia and that a retaliatory role for adenosine is not needed. In addition, turtles and crucian carp have unique mechanisms to defend against acidosis, while the common carp does not. The shell and bones of turtles play a crucial role in buffering the potential catastrophic acidosis that would otherwise accompany sustained anaerobic metabolism (Jackson, 2002). In anoxic crucian carp, the acidosis is combated by a unique ability
to convert the lactate into ethanol and CO₂, which are excreted into the ambient water (Shoubridge and Hochachka, 1980).

Freshwater turtles survive prolonged anoxia by entering a hypometabolic state that allows them to reduce their energy demands, conserve fermentable fuel stores and limit the accumulation of harmful anaerobic end-products (Jackson, 1968; Lutz and Nilsson, 1997). In concert with the reduction in whole-animal metabolic rate and the subsequent decreased demand for blood flow, cardiac activity of anoxic turtles is profoundly suppressed (Herbert and Jackson, 1985; Hicks and Wang, 1998; Hicks and Farrell, 2000ab, Stecyk et al., 2004a; present study). At 21°C, there is an autonomic depression of cardiovascular status during anoxia, but autonomic cardiovascular control is blunted at 5°C (Hicks and Farrell, 2000b). While the exact mechanism(s) for cardiac depression during anoxia at 5°C remains unclear, intrinsic f₁ is re-set to a reduced level at both warm and cold acclimation temperatures (Stecyk and Farrell, 2007). Most importantly, cardiac work ($P_{\text{Osys}}$, i.e. ATP demand) is reduced by 4.6- to 6.6-fold (at 21°C) and by 20-fold (at 5°C) with prolonged anoxia (Hicks and Farrell, 2000a; Stecyk et al., 2004a). At both temperatures, this depression of cardiac work puts cardiac ATP demand well below its capability for anaerobic ATP supply. Furthermore, the depression of cardiac work is so profound at 5°C that an up-regulation of glycolysis is not even required to meet cardiac ATP demand (Hicks and Farrell, 2000a; Farrell and Stecyk, 2006). Thus, through a massive reduction in cardiac activity during prolonged anoxia, the turtle can likely balances its cardiac ATP supply and ATP demand, thus precluding the need for adenosinergic inhibition of cardiac activity.

Crucian carp utilize a different anoxic-survival strategy than the freshwater turtle (Lutz and Nilsson, 1997) and, unlike turtles, do not become comatose (Nilsson et al., 1993; Nilsson, 2001). In fact, anoxic crucian carp maintain their cardiovascular status near normoxic levels (Stecyk et al., 2004b; present study) and retain autonomic cardiovascular control (Stecyk et al.,
To do this, crucian carp must up-regulate glycolysis during anoxia, as exemplified in the brain, where key glycolytic enzymes are up-regulated, and store massive amounts of glycogen in the liver and heart prior to the winter months (Holopainen and Hyvärinen 1985; Hyvärinen et al. 1985; Vornanen and Paajanen, 2004). Also, there is no evidence of membrane channel arrest in either the brain (Lutz and Nilsson, 1997; Vornanen and Paajanen, 2006) or cardiac tissues of anoxic crucian carp (Paajanen and Vornanen, 2003; Vornanen and Paajanen, 2004). Thus, the crucian carp, like the freshwater turtle, is truly-anoxia tolerant and likely balances cardiac ATP supply and demand during anoxia, thus precluding the formation of adenosine and an adenosinergic inhibition of cardiac activity.

Interestingly, the apparent lack of adenosinergic cardiovascular control in the turtle and crucian carp reported here contrasts with the important role of adenosine in mediating metabolic protection in the brain of these species during short-term anoxia. Specifically, a small, temporary fall in ATP, ADP and AMP occurs in the brain of turtles as an immediate response to anoxia and corresponds to an increase in extracellular adenosine (Nilsson and Lutz, 1992), which facilitates the balancing of ATP supply and ATP demand in the turtle brain by decreasing neuronal excitability, limiting excitable neurotransmitter release and mediating ion channel arrest (Buck, 2004), as well as causing vasodilation and increased cerebral blood flow (Hylland et al., 1994). Similarly, adenosine suppresses metabolic rate (Nilsson, 1991) and increases cerebral blood flow (Nilsson et al., 1994) during short-term (a few hours) anoxia in the crucian carp. For prolonged anoxia exposures (i.e., at 6 h, 14 d and 5 d of anoxia in 21°C turtles, 5°C turtles and crucian carp, respectively), adenosinergic cardiovascular control was absent in the present study. Collectively, these results raise the possibility that transient disruptions in cardiac energetic status at the onset of anoxic exposure when large and rapid changes in cardiac activity occur both in turtles and crucian carp (Hicks and Farrell, 2000a; Stecyk et al., 2004ab) may invoke adenosinergic cardiovascular depression. However, previous studies utilizing nuclear
magnetic resonance spectroscopy to follow turtle cardiac energetic status *in vitro* turtle hearts reported that myocardial ATP levels are maintained during the first 4 h of an anoxia exposure, with $f_H$ either remaining stable (Wasser et al., 1990) or decreasing (Wasser et al., 1997).

Although, the value of this observation is compromised by the fact that these spontaneously beating heat preparations were not performing at *in vivo* levels of cardiac work and so ATP demand was reduced. Ideally, simultaneous measurements of *in vivo* cardiac energetic status and cardiac activity are needed to better comprehend the relationship between cardiac energetic status and cardiac activity in the turtle and crucian carp during prolonged anoxia exposure.

In view of the above discussion, our finding that adenosinergic inhibitory cardiovascular control was present only in normoxic red-eared sliders, regardless of their acclimation temperature, and not in the normoxic carp, seems to disagree with the view of adenosinergic cardiac inhibition being an important mechanism of cardiac control during energy-limited conditions. However, adenosinergic cardiovascular control during normoxia is possible because it is formed during normoxia, both intracellularly from ADP and ultimately the AMP formed during ATP hydrolysis, as well as from the transmethylation pathway, and extracellularly from extracellular AMP (see Villarreal et al., 2003; Buck 2004 for detailed descriptions of these pathways). In fact, intracellular adenosine formation is regulated through an AMP/adenosine cycle that allows for precise control of intracellular adenosine concentrations (Bontemps, 1983; Buck, 2004). Furthermore, the breath-hold diving behaviour of turtles may result in temporary hypoxic states and energy imbalance. The adenosinergic inhibition of $f_H$ during normoxia in both warm- and cold-acclimated turtles could be associated with such imbalances. Indeed, the cardiovascular adjustments associated with diving in the turtle closely resemble the known cardiovascular effects of adenosine. Specifically, normoxic turtles exhibit cardiorespiratory synchrony, where bradycardia and decreased $Q_{sys}$ occur during apnea and increases in $f_H$ and $Q_{sys}$ accompany breathing episodes (Wang and Hicks, 1996). Further, adenosine is a known
neuromodulatory respiratory depressant in mammalian species (Long and Anthonisen, 1994) and adenosinergic inhibition of respiration rate has been observed in elasmobranch (Stensløkken et al. 2004) and, potentially, teleost fish (Stecyk and Farrell, 2006). Perhaps, the tachycardia in normoxic turtles following aminophylline injection was associated with an increased respiration frequency that occurred as a result of the adenosinergic blockade. Of course, future studies investigating adenosinergic control of respiration and cardiorespiratory synchrony in the turtle are needed to test these ideas.

The absence of cardiovascular responses to aminophylline in the normoxic common carp contrasts with a previous inconclusive study on this species (Stecyk and Farrell, 2006), which reported a slight decrease in $P_{VA}$ in 5°C-acclimated common carp following aminophylline injection but no cardiac depression. Stecyk and Farrell (2006) injected aminophylline after sequential $\alpha$-adrenergic and cholinergic pharmalogical blockade, which resulted in an elevation of cardiac activity. Thus, the previously observed fall in $P_{VA}$ of common carp with aminophylline injection could have been a phenomena resulting from an interaction between the various injected pharmaceuticals.

Concluding remarks

In summary, we examined adenosinergic cardiovascular control in three vertebrate species tolerant of prolonged periods of severe hypoxia/anoxia, but with differences in their anoxic survival strategies and cardiovascular responses to prolonged anoxia. Our results revealed that adenosinergic cardiovascular control is important in regulating cardiovascular status in 5°C-acclimated hypoxic common carp, but not in anoxic freshwater turtles and crucian carp. We suggest these species-specific differences relate to differences in anoxia-tolerance among the three species. Future studies are needed to 1) fully understand the exact mechanisms
(i.e., adenosine receptor sub-types) underlying the adenosinergic cardiac inhibition in hypoxic common carp, and 2) test our suggestion that turtles and crucian carp balance cardiac ATP supply and ATP demand during prolonged anoxic exposure.

Acknowledgements

This work was supported by Natural Sciences and Engineering Research Council of Canada research grants to A. P. F. and J. A. W. S. and Research Council of Norway funding to G. E. N. Special thanks to Dr. Dave Jones for the use of the Halothane vaporizer and Harvard Apparatus Ventilator, Dr. William Milsom for the use of the bone saw and to Dr. M. Axelsson and Dr. J. Altimiras for writing the data acquisition and analysis programs for Labview.


Figure Legend

**Figure 3.1** Chronological changes of cardiovascular function in 21°C-acclimated normoxic and anoxia-exposed (6 h) turtles following control saline and aminophylline injections. Significant differences ($P<0.05$) from control values as determined with a 2-way RM ANOVA are indicated by asterisks. Values are means ± S.E.M.; $N=6$ (normoxic animals) and 5–6 (anoxic animals).

**Figure 3.2** Chronological changes of cardiovascular function in 5°C-acclimated normoxic and anoxia-exposed (14 d) turtles following control saline and aminophylline injections. Significant differences ($P<0.05$) from control values as determined with a 2-way RM ANOVA are indicated by asterisks. Values are means ± S.E.M.; $N=5$ for both normoxic and anoxic animals unless otherwise indicated below the error bar.

**Figure 3.3** Chronological changes of cardiovascular function in 8°C-acclimated normoxic and anoxia-exposed (5 d) crucian carp following control saline and aminophylline injections. Values are means ± S.E.M.; $N=5$ (normoxic animals), 7 unless otherwise indicated below the error bar ($f_{H}$ and $P_{VA}$ of anoxic animals) and 4 ($Q$, $V_{SH}$, $R$ and $PO$ of anoxic animals).

**Figure 3.4** Chronological changes of cardiovascular function in 5°C-acclimated normoxic and severely hypoxia-exposed (12.5 h) common carp following control saline and aminophylline injections. Significant differences ($P<0.05$) from control values as determined with a 2-way RM ANOVA are indicated by asterisks. Significant difference ($P<0.05$) of the 50 minute time point from control, pre-aminophylline values as determined with a paired t-test are indicated by double crosses. Values are means ± S.E.M.; $N=6$ (normoxic animals) and 5-6 (anoxic animals).
Table 3.1 Cardiovascular status of the red-eared slider, common carp and crucian carp during normoxia and prolonged oxygen deprivation.

<table>
<thead>
<tr>
<th>Species</th>
<th>Acclimation Temperature (°C)</th>
<th>Condition</th>
<th>Cardiac Output* (ml min⁻¹ kg⁻¹)</th>
<th>Heart Rate (min⁻¹)</th>
<th>Stroke Volume* (ml kg⁻¹)</th>
<th>Blood Pressure* (kPa)</th>
<th>Cardiac Power Output* (mW g⁻¹)</th>
<th>Peripheral Resistance* (kPa ml⁻¹ min kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red-eared slider</td>
<td>21</td>
<td>Normoxia</td>
<td>46.9±5.1</td>
<td>26.4±2.6</td>
<td>1.96±0.42</td>
<td>2.55±0.13</td>
<td>1.097±0.133</td>
<td>0.059±0.008</td>
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<td></td>
<td>Anoxia §</td>
<td></td>
<td>14.7±1.0†</td>
<td>7.9±0.9†</td>
<td>1.92±0.15</td>
<td>1.47±0.16†</td>
<td>0.194±0.018†</td>
<td>0.101±0.010†</td>
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<tr>
<td>Red-eared slider</td>
<td>5</td>
<td>Normoxia</td>
<td>8.6±1.5</td>
<td>3.5±0.1</td>
<td>2.46±0.39</td>
<td>1.69±0.10</td>
<td>0.160±0.036</td>
<td>0.204±0.047</td>
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<td></td>
<td>Anoxia §</td>
<td></td>
<td>2.6±0.4†</td>
<td>1.3±0.3†</td>
<td>2.40±0.65</td>
<td>0.79±0.12†</td>
<td>0.019±0.003†</td>
<td>0.336±0.069†</td>
</tr>
<tr>
<td>Common carp</td>
<td>5</td>
<td>Normoxia</td>
<td>4.4±0.6</td>
<td>7.1±0.7</td>
<td>0.64±0.07</td>
<td>3.26±0.30</td>
<td>0.335±0.077</td>
<td>0.809±0.121</td>
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<tr>
<td></td>
<td>Severe hypoxia §</td>
<td></td>
<td>2.2±0.5†</td>
<td>4.1±0.8†</td>
<td>0.61±0.13</td>
<td>2.48±0.16†</td>
<td>0.121±0.032†</td>
<td>1.370±0.227†</td>
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<td>Cardiac Output (%)Normoxia</td>
<td>Heart Rate (min⁻¹)</td>
<td>Stroke Volume (%)Normoxia</td>
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<td>Crucian carp</td>
<td>8</td>
<td>Normoxia</td>
<td>-</td>
<td>12.8±1.8 (7)</td>
<td>-</td>
<td>1.69±1.11 (7)</td>
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<td>Anoxia §</td>
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<td>145.3±12.8</td>
<td>15.7±1.1 (7)</td>
<td>84.5±14.4</td>
<td>1.39±1.50 (7)</td>
<td>132.9±10.4</td>
<td>65.3±8.1</td>
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* Refers to systemic cardiac output, systemic stroke volume, systemic blood pressure, systemic resistance, and systemic cardiac power output for the red-eared slider.

§ Anoxia and severe hypoxia values were taken at the conclusion of the anoxic/severe hypoxic exposure periods.

† indicates statistical significance between normoxia and anoxia/severe hypoxia for each group.

Values are means ± S.E.M. N=6 (21°C slider turtles), 5 (5°C slider turtles), 7 (common carp) and 4 (crucian carp) unless otherwise indicated (numbers in parenthesis).
Figure 3.2
Figure 3.3
Figure 3.4
CHAPTER IV:

³¹P-NMR AND MRI MEASUREMENTS OF IN VIVO ENERGETIC STATUS AND CARDIAC ACTIVITY DURING PROLONGED ANOXIA EXPOSURE IN THE TURTLE (TRACHEMYS SCRIPTA)

Introduction

Most vertebrates die within minutes when deprived of molecular oxygen (anoxia), in part because of cardiac failure. In contrast, freshwater turtles (genera Chrysemys and Trachemys) survive 12 – 24 h of anoxic submergence at a warm-acclimation temperature. At cold-acclimation temperatures (3°C - 5°C), anoxia tolerance is more spectacular, with Chrysemys recovering physiological functions following 5 months of anoxia (Johlin and Moreland, 1933; Jackson and Ultsch, 1982; Ultsch and Jackson, 1982; Herbert and Jackson, 1985ab) and Trachemys surviving for up to 44 d (Ultsch, 1985; Warren et al., 2006). Regardless of acclimation temperatures, the anoxic heart of the turtle continues its role in internal convection during prolonged anoxia exposure, but at a massively reduced rate (Herbert and Jackson, 1985b, Hicks and Wang, 1998; Hicks and Farrell, 2000ab, Stecyk et al., 2004a).

One key to prolonged anoxia survival is balancing cellular ATP supply to ATP demand such that cellular energetic state is protected (Boutilier, 2001). Indeed, previous in vitro studies on warm-acclimated turtle hearts report that myocardial ATP content can be maintained near control levels (Wasser et al., 1990b; Jackson et al., 1995a; Wasser et al., 1997), and cardiac cellular energy state decreased at a slower rate and maintained at a higher level than for cardiac tissue of anoxia-intolerant vertebrates with severe hypoxia/anoxia exposure (Hartmund and Gesser, 1996; Overgaard and Gesser, 2004). However, in vivo extracellular conditions and cardiac performance may differ considerably from in vitro experiments. For cold-acclimated turtles, terminal sampling revealed a 35 – 40% decrease for cardiac ATP content after 12 weeks of anoxia (Jackson et al., 1995b). Even so, the 20-fold reduction of systemic cardiac power output (i.e., ATP demand) and lack of adenosinergic cardiac inhibition with prolonged anoxia
exposure indirectly suggest that cardiac ATP supply and demand remain balanced in cold, anoxic turtles (Hicks and Farrell, 2000a; Farrell and Stecyk, 2006; Stecyk and Farrell, 2007).

Thus, the present goal was to continuously measure in vivo cardiac energetic state during prolonged anoxia in the turtle for the first time. We utilized in vivo $^{31}$P-nuclear magnetic resonance (NMR) spectroscopy to directly and repetitively measure cardiac high-energy phosphates and intracellular pH (pH$_i$) of unanaesthetized, warm- and cold-acclimated turtles (Trachemys scripta) during prolonged anoxia exposure (2.85 h at 21°C and 11 d at 5°C). We wished to establish the time course and stability of the changes in cellular ATP reported earlier by other methods. Additionally, we wanted to examine if changes in turtle cardiac high-energy phosphate metabolism, pH$_i$ and/or energetic state contribute directly to the known large depression of cardiac activity with prolonged anoxia (Herbert and Jackson, 1985b, Hicks and Wang, 1998; Hicks and Farrell, 2000ab, Stecyk et al., 2004a). For instance, increased inorganic phosphate (P$_i$) and intracellular acidosis, which have been suggested to be the primary causes of cardiac contractile failure when cellular energy is compromised in mammals (Godt and Nosek, 1989), can have negative inotropic effects on turtle cardiac tissue (Gesser and Jørgensen, 1982; Wasser et al., 1990a; Jensen and Gesser, 1999). Further, for mammals, it has been suggested that decreased cardiac function with hypoxia is due to a reduction in the amount of free energy released from ATP hydrolysis (dG/dξ), although previous findings are contradictory (Kammermeier et al., 1982; Kentish and Allen, 1986). Therefore, we also repeatedly monitored turtle heart rate ($f_h$) as well as aortic and pulmonary blood flows during prolonged anoxia exposure using flow-weighted magnetic resonance imaging (MRI) techniques, which have been used previously in fish (Bock et al., 2002; Lannig et al., 2004). We reasoned that if alterations in myocardial high-energy phosphate metabolism, pH$_i$ and/or dG/dξ dictate the depression of turtle cardiac activity with anoxia exposure, then changes in $f_h$ would parallel changes in cardiac bioenergetic status.
Materials and Methods

Experimental animals

Fourteen red-eared slider turtles (*Trachemys scripta*, Gray) obtained from Lemberger Inc. (Oshkosh, WI, USA) and with body masses ranging between 546 and 748 g (630 ± 76 g, mean ± S.D) were used in this study. Turtles acclimated to and 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 acclimated to and 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 (Hicks and Farrell, 2000a). The 5°C turtles were fasted during this period. All procedures were in accordance with the animal care guidelines of the Alfred-Wegener-Institute (AWI) for Marine and Polar Research.

Experimental protocol

Each experimental animal served as its own control because MR measurements were obtained at the acclimation temperature of the animal first during normoxia and then at regular intervals during a prolonged anoxia exposure. 24 h prior to MR measurements, turtles were placed in an enclosed, water-containing plastic chamber that still allowed access to air. To prevent motion artifacts in the MR images, the turtle was immobilized by fastening two Velcro straps glued to the bottom of the chamber around the shell of the turtle. This arrangement allowed for movement of appendages and the head, but not the body. For MR measurements during normoxia, the chamber containing the turtle was placed within the magnet [Bruker 47/40 Biospec DBX system with a 40 cm wide bore and actively shielded gradient coils (50 mT m^-1)] and the heart centred over a triple tuneable surface coil (^31P, ^13C, ^1H; 5 cm diameter) that was
used for $^{31}$P-NMR spectroscopy. An actively decoupled $^1$H cylindrical birdcage resonator (20 cm diameter) was used for the MR imaging experiments. The coil circuit and field homogeneity were optimized to the experimental set-up, and the location of the heart within the magnet confirmed via coronal, sagittal and transverse scout images collected using a gradient echo sequence [excitation pulse shape: hermite; pulse length: 2000 µs, $\phi = 22.5^\circ$; matrix size: 128 x 128; field-of-view (FOV): 12 cm$^2$; slick thickness: 3 mm; repetition time (TR): 100 ms; echo time (TE): 5 ms; resulting scan time: 25 s] (Fig. 4.1). Presented data for control normoxic $^{31}$P-NMR spectra and MR images were averaged from results of 4 or 5 sets of measurements (see below for MR spectroscopy and imaging measurement parameters) that were obtained over a period of 45 – 60 min.

For MR measurements under prolonged anoxia, the chamber containing the turtle was removed from the magnet, completely filled with the water of the appropriate temperature, continuously bubbled with N$_2$ (water $P_{O_2} < 0.3$ kPa) and once again centred in relation to the magnet, surface coil and resonator. For 21°C-acclimated turtles, $^{31}$P-NMR spectra and MR images were acquired regularly and almost continuously over a 2.85 h anoxia exposure period. For 5°C-acclimated turtles, $^{31}$P-NMR spectra and MR images were acquired regularly for the first ~18 h and then on days 3, 7 and 11 of an 11-day anoxia exposure period (presented data from days 3, 7 and 11 are averages from 4 or 5 sets of $^{31}$P-NMR spectra and MR images that were obtained over a period of 45 – 60 min). The anoxia exposure times were chosen to be consistent with previous investigations of turtle cardiac activity and its control during prolonged anoxia (Hicks and Wang, 1998; Hicks and Farrell, 2000ab, Stecyk et al., 2004, Stecyk and Farrell, 2007; Stecyk et al., 2006ab). When not in the magnet, 5°C-acclimated anoxic turtles were maintained in a cold room set to 5°C, and the housing chambers continuously bubbled with N$_2$. 
**31P-NMR spectroscopy**

*In vivo* 31P-NMR spectroscopy parameters were as follows. Sweep width: 4000 Hz; flip angle: 60° (pulse shape: bp32; pulse length 200 μs); TR: 1 s; scans: 512; total acquisition time: 8 min 32 s. 31P-NMR spectra were processed using TopSpin v1.0 software (BrukerBioSpin MRI GmbH, Ettlingen, Germany) and a macro (written by R.-M. Wittig, AWI) to yield integrals of all major peaks within the spectrum (Fig. 4.2), as these correlate with the amount of substance within the detection volume of the 31P-NMR coil.

**MR imaging**

Alternating with spectroscopy, flow-weighted MR imaging methods were applied to measure heart rate \( f_H \) as well as aortic and pulmonary blood flows. \( f_H \) was measured using a single slice fast gradient echo MRI sequence [Snapshot Flash (Haase, 1990)] with parameters: excitation pulse shape: hemrite; pulse length: 2000 μs; flip angle: 80°; FOV: 6 cm; slice thickness: 2 mm; matrix: 128 x 64; TR: 8.53 ms; TE: 3.1 ms, resulting scan time 545 ms; dummy scans: 117; repetitions: 32 for 21°C experiments, 64 for 5°C experiments; receiver gain (rg): 500. Blood flows in the left aorta and pulmonary artery were determined from a flow-weighted gradient echo MRI sequence similar to Bock et al. (2001) with parameters: excitation pulse shape: hemrite; pulse length: 2000 μs; flip angle: 80°; FOV: 6 cm; slice thickness: 2 mm; matrix 128 x 128; averages: 4, dummy scans: 59; rg: 1500.

**Data analysis and statistics**

All results are expressed as means ± S.E.M. Concentrations of ATP, phosphocreatine (PCr) and P, were expressed as a percentage of the total 31P-NMR signal (i.e., the sum of the 7 major peaks: phosphomonoester (PME), P, phosphodiester (PDE), PCr, γ-ATP, α-ATP and β-ATP; see Fig. 4.2) to control for: 1) possible differences in 31P-NMR signal intensities that can
occur from slight movement of the animal; and 2) minor differences in the position of the turtle in the magnet prior to and following commencement of anoxia (21°C and 5°C turtles) and between measurement days (5°C turtles). This approach assumes that no major phosphate export from turtle cardiac muscle occurs with anoxia exposure. Although no previous study has reported on the effect of anoxia on all phosphate compounds in the turtle heart, data from Jackson et al. (1995b) show that with anoxia, the sum of P, PCr, β-ATP, PDE and PME does not change in 20°C-acclimated turtle hearts, but is reduced slightly (~13%) in 3°C-acclimated hearts. Similarly, in the present study, no statistically significant changes in total 31P signal were observed during prolonged anoxia at 21°C, and a small, but insignificant 13% decrease occurred with anoxia exposure at 5°C (see Fig. 4.3), therefore validating our approach. Percentages of metabolite concentrations were transformed to μmol g⁻¹ quantities by setting control β-ATP values to 2.9 μmol g⁻¹ w.w.⁻¹ and 2.6 μmol g⁻¹ w.w.⁻¹ for warm- and cold-acclimated turtles, respectively (Jackson et al., 1995b), and using this value as a conversion factor to calculate the other metabolite concentrations. pH, was calculated from the chemical shift of P, relative to PCr using previously published formulas describing the relationship between pH and chemical shift difference for the turtle (Wasser et al., 1990b; Jackson et al., 1995b).

The free energy of ATP hydrolysis (dG/dξ) was estimated from the equation dG/dξ = ΔG°ATP + RT ln {([ADPf][P,])/[ATP]}, where ΔG°ATP (−30.5 kJ mol⁻¹) is the value of ATP hydrolysis under standard conditions of molarity, temperature, pH and [Mg²⁺], the gas constant (R) is 8.324 J mol⁻¹ K, and temperature (T) in Kelvin is 278.15 K (5°C) or 294.15 K (21°C) (Kammermeier, 1987). With this equation, more negative values of dG/dξ indicate greater free energy available from the hydrolysis of ATP to drive ATP-requiring reactions. Molar concentrations of metabolites were calculated assuming water content of turtle cardiac tissue to be 80% (Gesser and Jørgensen, 1982). Myocardial free ADP (ADPf) levels were calculated from the creatine kinase equilibrium {[ADPf] = ([ATP][Cr])/([PCr] x Keq)}, where Keq is the
equilibrium constant for the creatine kinase reaction (Lawson and Veech, 1979). The value of $K_{eq}$ was corrected for measured pH (Lawson and Veech, 1979). Cytostolic $[\text{Mg}^{2+}]$ was assumed to be 1 mM. The creatine content (Cr) was estimated as the difference between the total Cr content in the turtle heart and $^{31}$P-NMR measured PCr content. For 21°C-acclimated turtles, total Cr is 8.14 uM g$^{-1}$ (Overgaard and Gesser, 2004). Since no previous study has reported total Cr content of 5°C-acclimated turtle hearts and PCr concentration of 5°C-acclimated hearts (14.8 ± 0.8 μmol g$^{-1}$; see Fig. 4.7B) was greater than 8.14 uM g$^{-1}$, total creatine content of 5°C-acclimated turtle hearts was estimated using the ratio of Cr:PCr for 21°C-acclimated turtle hearts.

$f_H$ was calculated from the time interval between Snapshot Flash MR images that depicted blood flow through the central blood vessels. Relative changes in aortic and pulmonary blood flow were determined by manually selecting regions of interest (ROIs) in the flow weighted gradient echo MR images and comparing changes in the mean signal intensity of the ROIs (Fig. 4.4). Depending on the location of the heart and quality of image, blood flow was measured from either the left or right aortic arch as previous study has shown blood flow through these vessels to be equivalent independent of acclimation temperature or anoxia exposure (Stecyk et al., 2004). Likewise, blood flow was measured from either the right or left pulmonary artery under the assumption that blood flow is equivalent in these vessels. To better compare data among turtles, baseline corrections were applied to individual ROIs by subtracting signal intensity of a ROI placed in a region of the image where flow effects could be excluded.

Statistically significant changes in measured or calculated parameters between acclimation temperatures were determined using t-tests. Statistically significant changes in measured or calculated parameters over time with prolonged anoxia exposure were determined using a one-way repeated analysis of variance. Where appropriate, multiple comparisons were performed using Student-Newman-Keuls tests and in all instances significance was accepted when $P < 0.05$. 
Results

*fH and blood flow response to prolonged anoxia exposure*

Our MR imaging techniques proved suitable to measure *in vivo* \( f_H \) and blood flows through the central blood vessels of anoxia exposed turtles. The \( f_H \) and blood flow responses of both warm- and cold-acclimated turtles to prolonged anoxia exposure presented here match previously reports of anoxic cardiovascular status (Herbert and Jackson, 1985b, Hicks and Wang, 1998; Hicks and Farrell, 2000ab, Stecyk et al, 2004, Stecyk et al, 2006a). Specifically, at 21°C, anoxia exposure resulted in a decrease of \( f_H \) from 14.1 ± 1.7 to ~10 min\(^{-1}\) (Fig. 4.6A) and reductions of aortic and pulmonary blood flow of ~50% and ~85 - 90%, respectively (Fig. 4.5A). With prolonged anoxia exposure at 5°C, \( f_H \) was reduced from 4.2 ± 0.6 min\(^{-1}\) to ~1 min\(^{-1}\) (Fig. 4.7B) and aortic blood flow decreased by ~80 – 90% (Fig. 4.5B). A novel finding is the cessation of pulmonary blood flow with prolonged anoxia exposure at 5°C (Figs. 4.4B and 4.5B).

*Phosphorous metabolite, pH\(_i\) and dG/d\(\zeta\) during normoxia*

Our *in vivo* \(^{31}\)P-NMR spectroscopy of the turtle heart was able to distinguish the resonance peaks previously reported for isolated, perfused turtle hearts (Wasser et al, 1990b, Wasser et al., 1992; Jackson et al., 1995b) (Fig. 4.2). However, under normoxic conditions, the relative magnitudes of identifiable peaks varied with acclimation temperature (Fig. 4.2). From this observation we conclude that the content of the various phosphorous metabolites in the turtle heart changes with temperature acclimation. Specifically, using previously published (Jackson et al., 1995b) turtle cardiac ATP contents of 2.9 \( \mu \text{mol g}^{-1} \) (warm-acclimated) and 2.6 \( \mu \text{mol g}^{-1} \) (cold-acclimated) as reference values to quantify peak areas, we found that PCr (14.8 ± 0.8 \( \mu \text{mol g}^{-1} \)) and \( P_i \) (7.0 ± 1.2 \( \mu \text{mol g}^{-1} \)) content of 5°C-acclimated turtle hearts is 2.6x and 2.2x greater than in 21°C-acclimated turtle hearts (Figs. 4.6BC, 4.7BC). Similarly, PME (10.1 ± 0.8 \( \mu \text{mol g}^{-1} \)) and PDE (16.5 ± 0.9 \( \mu \text{mol g}^{-1} \)) content at 5°C is 4.6x and 4.5x greater than at 21°C,
respectively (data not shown). Normoxic pH, at 5°C (7.66 ± 0.06) is also greater than at 21°C (7.40 ± 0.10) (Figs. 4.6E, 4.7E). In contrast, estimated dG/dξ values reveal that the less energy is available from the hydrolysis of ATP for 5°C-acclimated turtle hearts (-52.3 ± 0.6 kJ mol⁻¹) than for 21°C-acclimated hearts (-56.5 ± 0.6 kJ mol⁻¹) (Figs. 4.6F, 4.7F).

Phosphorous metabolite, pH, and dG/dξ changes with prolonged anoxia exposure

Prolonged anoxia exposure modified turtle myocardial phosphorous metabolite content, pH, and dG/dξ. After 2.85 h of anoxia at 21°C, PCr was reduced by 69% from 5.8 ± 0.9 μmol g⁻¹ to 1.8 ± 0.3 μmol g⁻¹, Pᵢ increased by 140% from 3.2 ± 0.8 μmol g⁻¹ to 7.7 ± 0.6 μmol g⁻¹, pHᵢ reduced by 0.39 units from 7.40 ± 0.10 to 7.01 ± 0.04, and dG/dξ decreased by 5.8 kJ mol⁻¹ from -56.5 ± 1.3 kJ mol⁻¹ to -50.7 ± 0.9 kJ mol⁻¹ (Fig. 4.6). The minor (17%) decrease in ATP from 2.9 ± 0.3 μmol g⁻¹ to 2.4 ± 0.2 μmol g⁻¹ at 2.85 h of anoxia exposure was not statistically significant (Fig. 4.6D). Thus, during prolonged anoxia at 21°C, ATP levels are maintained at control normoxic levels via the creatine kinase equilibrium. Nevertheless, cardiac energetic status does not stabilize during anoxia despite the constant ATP content. dG/dξ continuously decreased in a linear fashion throughout anoxia exposure period (Fig. 4.6F).

The fᵢ response to prolonged anoxia exposure at 21°C did not temporally match changes in turtle cardiac phosphorous metabolite content, pHᵢ, or dG/dξ (Fig. 4.6). Specifically, whereas fᵢ stabilized within 0.5 h of the commencement of anoxia, PCr, Pᵢ, pHᵢ, and dG/dξ all appeared to change linearly and did not reach levels statistically similar to 2.85 h values until 0.90 -1.33 h of exposure. Indeed, when fᵢ is plotted as a function of PCr, Pᵢ, ATP pHᵢ or dG/dξ, no prominent correlations exist (Fig. 4.8). Therefore, the changes in fᵢ with 21°C-acclimated turtles during prolonged anoxia are not dictated by alterations in myocardial high-energy phosphate metabolism, pHᵢ, or dG/dξ.
Prolonged anoxia exposure of 5°C-acclimated turtles resulted in quantitatively similar changes of PCr and P, greater reductions of ATP and pH, and a smaller reduction of dG/dξ than 21°C-acclimated turtles. After 11 d of anoxia at 5°C, PCr was reduced by 63% from 14.8 ± 0.8 μmol g⁻¹ to 5.5 ± 1.3 μmol g⁻¹, P, increased by 114% from 7.0 ± 1.2 μmol g⁻¹ to 15.0 ± 1.0 μmol g⁻¹, ATP decreased by 50% from 2.6 ± 0.3 μmol g⁻¹ to 1.3 ± 0.1 μmol g⁻¹, pH, reduced by 0.54 units from 7.66 ± 0.06 to 7.12 ± 0.04, and dG/dξ decreased by 4.0 kJ mol⁻¹ from -52.3 ± 0.6 kJ mol⁻¹ to -48.3 ± 0.9 kJ mol⁻¹ (Fig. 4.7). However, in contrast to the continuous, linear changes over time for PCr, P, ATP, pH, and dG/dξ with prolonged anoxia at 21°C, patterns of change over time for PCr, P, ATP, pH, and dG/dξ were all asymptotic with prolonged anoxia at 5°C (Figs. 4.6 and 4.7). This finding suggests that turtle myocardial high-energy phosphate metabolism and energetic state is maintained at a new steady-state with prolonged cold anoxia exposure.

Unlike the absence of temporal correlations between f₄ and anoxia-induced alterations in myocardial high-energy phosphate metabolism, pH, or dG/dξ at 21°C, the bradycardia exhibited by 5°C-acclimated anoxia exposed turtles closely reflected changes of myocardial PCr, P, ATP, pH, and dG/dξ. Plotting 5°C anoxic f₄ against changes in PCr, P, ATP, pH, or dG/dξ (Fig. 4.9) revealed that commencing at 1.7 h of anoxia exposure and continuing through to day 11 of anoxia exposure, f₄ is tightly matched with the changes in PCr, P, pH, ATP and dG/dξ. Indeed, linear regression analysis revealed that these relationships were statistically significant (P<0.001) with r² values of 0.86 for PCr, 0.90 for P, 0.56 for ATP, 0.87 for pH, and 0.77 for dG/dξ.

**Discussion**

The present study is the first to utilize in vivo ³¹P-NMR spectroscopy and MR imaging to repeatedly measure key components of cardiac bioenergetics and cardiac performance of turtles during prolonged anoxia exposure. Our objectives were to: 1) determine the time-dependent
changes in energetic status of the turtle heart during prolonged anoxia exposure; 2) investigate if alterations of myocardial high-energy phosphate metabolism, pH, and/or dG/dξ correlate with the known depression of cardiac activity displayed by turtles with prolonged anoxia exposure (Herbert and Jackson, 1985b; Hicks and Wang, 1998; Hicks and Farrell, 2000ab, Stecyk et al., 2004); and 3) determine if differences existed between the effects of anoxia in warm- and cold-acclimated turtles. Our novel measurements at 21°C revealed that although myocardial ATP remains above 80% of control normoxia levels via the creatine kinase equilibrium reaction with prolonged anoxia exposure, dG/dξ continuously decreased. However, the bradycardia that accompanies anoxia in 21°C-acclimated turtles does not correlate with the alterations in myocardial high-energy phosphate metabolism, dG/dξ or a reduced pH, In contrast, the anoxic bradycardia exhibited by 5°C-acclimated turtles with 11 d of anoxia exposure did significantly correlate with changes in myocardial high-energy phosphate metabolism, pH, and dG/dξ, which all reached a new steady state with prolonged anoxia.

Cardiac activity and energetic status during normoxia

For normoxic turtles, our in vivo 31P-NMR and MRI measurements of cardiac energetic status and contractile performance are comparable with previously published findings using in vitro 31P-NMR spectroscopy of isolated perfused turtle heart preparations and 31P-NMR spectroscopy of tissue samples, as well as traditional in vivo measurement of cardiovascular variables (Malan et al., 1976; Wasser et al., 1990ab; Wasser et al., 1992; Jackson et al., 1995ab; Hartmund and Gesser, 1996; Wasser et al., 1997; Hicks and Wang, 1998; Hicks and Farrell, 2000ab; Overgaard and Gesser, 2004; Stecyk et al., 2004; Stecyk et al., 2006a). The similarity of past and present results indicates that MRI can be used to obtain reliable measurements of turtle cardiac activity for both warm- and cold-acclimated animals and gives support to our findings on the effect of prolonged anoxia exposure on key bioenergetic components of turtle cardiac tissue.
Nevertheless, some notable differences in cardiac phosphorous metabolites and cardiac activity do exist between the present study and past investigations. Specifically, control normoxic \( f_H \) at 21°C reported here (\( \sim 14 \text{ min}^{-1} \)) is approximately half the rate previously reported for warm-acclimated normoxic turtles (Herbert and Jackson, 1985a; Hicks and Wang, 1998; Hicks and Farrell, 2000ab, Stecyk et al., 2004; Stecyk et al., 2006a). This discrepancy could be the result of our acquisition frequency for Snapshot Flash images being too slow to capture actual \( f_H \), or more likely due to turtles exhibiting a vagal inhibition in response to the noise produced by the NMR magnet during measurement. Regardless, anoxic \( f_H \) of 21°C-acclimated turtles was identical to previous reports (Fig. 4.6A) (Herbert and Jackson, 1985a; Hicks and Wang, 1998; Hicks and Farrell, 2000ab, Stecyk et al., 2004; Stecyk et al., 2006a). For \(^{31}\text{P}-\text{NMR} \) measurements, our control normoxic PCR for both 21°C- and 5°C-acclimated turtles (Figs. 4.6B and 7B) are 1.2x - 3.5x greater than previous reports (Jackson et al., 1995b; Hartmund and Gesser, 1996; Overgaard and Gesser, 2004). Conversely, our control normoxic PME and PDE for both 21°C- and 5°C-acclimated turtles are approximately 1/3\(^{rd}\) to 1/7\(^{th}\) less than previously published values (Jackson et al., 1995b). Finally, it should be noted that control normoxic pH, for 5°C-acclimated turtles (7.66 ± 0.06) was 0.28 units higher than previously reported (Jackson et al., 1995b), but is in exact accordance with pH, predicted from the data of Malan et al. (1976). The underlying reasons for these differences are unclear at present, but they could simply reflect differences in experimental technique or study species.

An interesting finding for normoxic turtles was the greater abundance of PCR and P, at 5°C than 21°C (Figs. 4.6B and 4.7B). The increased PCR with cold acclimation translates to an increase in energy reserve, and thus, could be viewed as a preparatory response of turtle cardiac physiology for winter anoxic conditions. However, we are at present unable to explain the concomitant increase in P, with cold acclimation to almost the 21°C anoxic level (Figs. 4.6C and 4.7C).
Cardiac activity and energetic status during prolonged anoxia exposure

Prolonged anoxia exposure of both 21°C- and 5°C-acclimated turtles resulted in the expected decreases in $f_{H}$ and blood flows (Figs. 4.5, 4.6, 4.7; Hicks and Wang, 1998; Hicks and Farrell, 2000a; Stecyk et al., 2004). The depression of cardiac activity was accompanied by significant decreases in myocardial PCr, pH, and dG/d$\xi$ and a concurrent increase in P$, although the magnitude and pattern of change (i.e., linear at 21°C vs. asymptotic at 5°C) for these parameters varied with acclimation temperature (Figs. 4.6BCE and 4.7BCE). Similarly, the response of cardiac ATP to anoxia exposure varied between 21°C- and 5°C-acclimated turtles. Specifically, at 21°C, ATP was maintained at control normoxic levels throughout anoxia exposure, whereas at 5°C, ATP decreased by 50% before stabilizing at this level by the 3$^{rd}$ day of anoxia exposure (Figs. 4.6D and 4.7D).

The present in vivo findings of cardiac high-energy phosphorous metabolites for 21°C-acclimated turtles are consistent with previous $^{31}$P-NMR studies that investigated the effects of anoxia as well as combined anoxia with acidosis on of turtle cardiac high-energy phosphorous metabolites in vitro (Wasser et al., 1990b; Jackson et al., 1995b; Wasser et al., 1997). Therefore, it is clear that cardiac ATP supply during anoxia exposure at warm temperatures is maintained from PCr via the creatine kinase equilibrium, as it is in the turtle brain during diving (Wemmer et al., 1982). The maintenance of ATP at control normoxic levels with prolonged, warm anoxia confirms previous indirect evidence of a balanced cardiac ATP supply and demand during prolonged warm anoxia exposure (Stecyk et al., 2006a).

The 0.39 unit decrease of pH, with anoxia at 21°C in the present study (Fig. 4.6E) is considerably larger than previous reports of a relatively stable or slightly depressed pH, (i.e., 0.08 – 0.15 unit reduction) with anoxia or combined anoxia with acidosis exposures of similar duration to that utilized in the present study (Wasser et al., 1990b; Jackson et al., 1995b; Wasser
et al., 1997). This discrepancy may be due to the lower levels of cardiac performance with isolated, perfused turtle heart preparations compared to the in vivo condition. Alternatively, these equivocal results may be due to species-specific differences between studies and reflective of the important difference in anoxia-tolerance between Trachemys scripta and Chrysemys picta (Ultsch, 1985).

Similar to 21°C-acclimated turtle hearts, the decreased PCr and increased P, at the onset of anoxia exposure at 5°C indicates that the creatine kinase equilibrium plays an important role in supplying ATP during this time period (Fig. 4.7BC). However, an important distinction between warm- and cold-acclimated turtles revealed in the present study is the different pattern of change of bioenergetic parameters to prolonged anoxia (Figs. 4.6 and 4.7). Specifically, the asymptotic-like changes of PCr, Pi, ATP, pH, and dG/dz at 5°C (Fig. 4.7) indicates that cold-acclimated turtles, unlike 21°C-acclimated turtles, are able to reorganize cardiac energetic status with prolonged anoxia to a decreased, but steady state. Such a feat requires that energy consuming processes also be reduced with prolonged anoxia exposure. Clearly, anoxic bradycardia would serve to limit energy use by reducing the frequency of recruitment of ion channels and thus the load on ATP-dependent ion pumps (the mechanisms underlying the anoxic bradycardia are discussed below). Further, cold-acclimation as well as prolonged anoxia exposure results in modifications of turtle cardiac electrophysiology that presumably reduces energy demands (Stecyk et al., 2006b). Also, the down-regulation of ventricular β-adrenergic receptor density with cold anoxia (Hicks and Farrell, 2000b) and insensitivity of the cold, anoxic turtle heart to adrenergic stimulation (Hicks and Farrell, 2000b; Stecyk and Farrell, 2007) in face of increased levels of circulating catecholamines during anoxia (Wasser and Jackson, 1991) may be energy conserving mechanisms. This idea may be best exemplified by the fact that the use of inotrope agents to increase cardiac performance and counteract cardiac failure in the human heart actually results in increased, rather than decreased heart failure mortality due to augmented
cardiac energetic costs (Ingwall and Weiss, 2004). Finally, increased efficiency of cardiac contraction under oxygen limited conditions would also serve to conserve energy during prolonged anoxia (Overgaard and Gesser, 2004).

*Correlation of turtle cardiac bioenergetic components and $f_H$*

Although the bradycardia and associated depression of cardiac activity exhibited by anoxic turtles is well documented and quantified (Herbert and Jackson, 1985b; Hicks and Wang, 1998; Hicks and Farrell, 2000a), the mechanisms underlying the decreased $f_H$ were not, until recently thoroughly investigated. Briefly, our current understanding is that for warm-acclimated turtles, a simultaneous cholinergic, vagal cardiac inhibition and $\beta$-adrenergic cardiac stimulation combine to reset $f_H$ during prolonged anoxia with cholinergic inhibition accounting for $\sim 30\%$ of the anoxic bradycardia (Hicks and Wang, 1998; Hicks and Farrell, 2000b). Additionally, intrinsic $f_H$ is re-set to a reduced level with prolonged anoxia, in association with a prolongation of ventricular action potential duration, and could contribute a further $57\%$ of the anoxic bradycardia (Stecyk and Farrell, 2007; Stecyk et al., 2006b). Therefore, for warm-acclimated turtles, $\sim 90\%$ of anoxic bradycardia can be mechanistically accounted for. $\alpha$-adrenergic (Stecyk et al., 2004) and adenosinergic cardiac inhibition of cardiac activity (Stecyk et al., 2006a) do not contribute to anoxic bradycardia, but negative chronotropic effects of extracellular anoxia, acidosis and hyperkalemia and positive chronotropic effects of hypercalcemia and adrenaline may be additional modulators of anoxic $f_H$ *in vivo* (Stecyk and Farrell, 2007).

For cold-acclimated turtles, autonomic cardiovascular control is blunted during prolonged anoxia and does not account for the large anoxic bradycardia (Hicks and Farrell, 2000b; Stecyk et al., 2004). Adenosinergic cardiac inhibition during prolonged, cold anoxia is similarly not involved (Stecyk et al., 2006a). However, cold acclimation pre-conditions the cardiac electrophysiological status of turtles for winter anoxia (Stecyk et al., 2006b), and similar
to 21°C-acclimated turtles, intrinsic $f_H$ is re-set to a reduced level at 5°C that may contribute up to 66% of the anoxic bradycardia (Stecyk and Farrell, 2007). But, unlike at 21°C, the re-setting of intrinsic $f_H$ with anoxia at 5°C does not appear to result from a prolongation of the cardiac cycle (Stecyk et al., 2006b). Therefore, other important mechanisms must contribute to the remaining, unaccounted proportion of the anoxic bradycardia for cold-acclimated turtles.

The present findings strongly suggest that for 5°C-, but not 21°C-acclimated turtles, alterations in high-energy phosphate metabolism, pH, and/or $dG/d\xi$ may play an important role in facilitating the depression of cardiac activity with prolonged anoxia. The anoxic bradycardia of 5°C-acclimated turtles closely correlated with changes in PCr, P$_i$, pH, and $dG/d\xi$ (Fig. 4.9), whereas for 21°C-acclimated turtles, $f_H$ was stable despite changing PCr and P$_i$ levels, pH, and $dG/d\xi$ (Fig. 4.8). The lack of casual relationship between myocardial bioenergetic components and cardiac function at 21°C is in agreement with previous findings for warm-acclimated turtles (Wasser et al., 1990b).

Even though our regression analyses do not infer causation, integration of present and previous findings raises plausible hypotheses that could explain the temperature-dependency of the correlations and predict the bioenergetic factors that indeed contribute to a reduction in $f_H$ at 5°C. Firstly, the disparate findings between 21°C- and 5°C-acclimated turtles (Fig. 4.8 and 4.9) could reflect the presence at 21°C and blunting at 5°C of autonomic cardiac control (Hicks and Wang, 1998; Hicks and Farrell, 2000b). Specifically, for 21°C-acclimated turtles, cholinergic inhibition could decrease $f_H$ more quickly than the potential negative effects of increased P$_i$, reduced pH, and $dG/d\xi$. Conversely, $\beta$-adrenergic stimulation may serve to maintain $f_H$ in face of negative effects of increased P$_i$, reduced pH, and $dG/d\xi$. Indeed, negative chronotropic effects of extracellular acidosis on cardiac tissue of warm-acclimated turtles (Yee and Jackson, 1984), which decreases cardiac myocyte intracellular pH (Wasser et al., 1990ab), can be offset with augmented adrenergic stimulation (Stecyk and Farrell, 2006). However, despite the correlation
between $f_H$ and pH, (Fig. 4.9D) and lack of potent in vivo $\beta$-adrenergic stimulation (Hicks and Farrell, 2000b) for 5°C-acclimated turtles, previous findings suggest that intracellular acidosis may not be important in contributing to the reduced $f_H$ at 5°C. This is because cold-acclimation appears to have some form of pre-conditioning effect for the negative chronotropic effect of acidosis exposure (Stecyk and Farrell, 2006). Nevertheless, changes in pH during prolonged anoxia at 5°C may have an important intracellular signaling function (e.g., Ruppersberg, 2000).

Unlike acidosis, it appears highly likely that the elevation of $P_i$ with prolonged anoxia at 5°C does contribute to decreased cardiac activity. In cardiac tissue of mammalian species, elevated $P_i$ diminishes the fraction of activated actin-myosin cross-bridges and reduces the calcium sensitivity of troponin C (Godt and Nosek, 1989; Palmer and Kentish, 1994). Likewise, contraction force and calcium sensitivity of warm-acclimated turtle atrial tissue decreases with increased $P_i$ (Jensen and Gesser, 1999). However, increased ADP concentration to approximately 100 $\mu$M counteracts the negative effects of $P_i$ on turtle cardiac tissue contractility (Jensen and Gesser, 1999). In the present study, calculations of myocardial ADP$_f$ from the creatine kinase equilibrium reaction reveal that with 2.85 h of anoxia at 21°C, ADP$_f$ increases by 3.9-fold from a normoxic concentration of 38 ± 13 $\mu$M to a level similar to that shown to counteract the negative effects of $P_i$ on turtle myocardium (149 ± 95 $\mu$M). By comparison, after 11 d of anoxia exposure at 5°C, the increase in free myocardial ADP is not as substantial. ADP$_f$ only increases 1.5-fold to 56 ± 27 $\mu$M by the 11$^{th}$ day of anoxia. Consequently, if sensitivity of the turtle heart to $P_i$ and potency of ADP to offset the negative effects of $P_i$ are temperature-independent, the low ADP$_f$ concentration with cold anoxia may not be sufficient to offset the negative effects of elevated $P_i$, thus explaining the negative correlation of $P_i$ and $f_H$ at 5°C, but not 21°C.

The stronger correlation between $dG/d\xi$ and $f_H$ than ATP and $f_H$ with prolonged anoxia exposure at 5°C suggests that a change in $dG/d\xi$ may be more important than a change in ATP in
determining anoxic $f_H$. This is intuitive since $dG/d\xi$ need not be correlated with tissue ATP content (Kammermeier, 1987). For anoxia-intolerant mammalian species, a decreased $dG/d\xi$ is reasoned to lead to decreased cardiac activity due to the requirement of cardiac myocytes for a high phosphorylation potential (i.e., ratio of $[ATP]/[ADP][P_i]$) and hence a favourable $dG/d\xi$ to drive ATPase-dependent reactions (Ingwall and Weiss, 2004). Therefore, a decrease of $dG/d\xi$ to below the energy level required for a particular process involved with cardiac performance could lead to a diminished level of function for the process and a decreased cardiac performance (Kammermeier, 1987). Nonetheless, experimental support for this theory is contradictory in mammalian models (Kammermeier et al., 1982; Kentish and Allen, 1986). The close matching of $f_H$ and $dG/d\xi$ for 5°C turtles after 1.17 h of anoxia exposure (Fig. 4.9E) suggests that depression in $dG/d\xi$ contributes to decreased cardiac activity at this acclimation temperature. However, the specific cellular processes affected remain to be investigated. In the anoxia-tolerant mammalian species, it has been proposed that a reduced $dG/d\xi$ decreases cardiac performance by diminishing Ca\(^{2+}\) pumping capacity of the sarcoplasmic reticulum, which ultimately leads to less Ca\(^{2+}\) that can be released from the SR to activate the contractile system upon excitation (Griese et al., 1988). A similar mechanism seems unlikely for the turtle since the SR plays a small role in Ca\(^{2+}\) cycling on a beat-to-beat basis in this species (Galli et al., 2006ab).

Finally, it should be noted that no correlations existed between $f_H$ and key cardiac bioenergetic components during the first 1.17 h of anoxia exposure at 5°C (Fig. 4.9). This finding suggests that a threshold level of $P_i$, pH, and/or $dG/d\xi$ must be reached to initiate bradycardia. Alternatively, the lack of correlation between $f_H$ and myocardial high-energy phosphate metabolism, pH, and $dG/d\xi$ could be the result of a transient stress response similar to that displayed by some ectothermic vertebrates at the onset of hypoxia exposure (Beamish, 1964; Pörtner et al., 1991). An intriguing possibility deserving of future attention is the possibility that
lactate production during this time period (Arthur et al., 1997) functions as an alarm signal for the stress response (Pörtner et al., 1994).

Concluding remarks

In summary, we have provided the first *in vivo* measurements of turtle myocardial high-energy phosphorous metabolites and pH, during prolonged exposure anoxia. Further, we have demonstrated that MRI techniques can be used to obtain reliable measurements of turtle cardiac activity during prolonged anoxia exposure for both warm- and cold-acclimated animals. Our results indicate that at 21°C, cardiac energetic status is continuously decreased with anoxia exposure, despite the maintenance of myocardial ATP at the normoxic control level, and that the anoxic bradycardia is not correlated to changes in myocardial high-energy phosphate metabolism, pH, or dG/dξ. In contrast, results from 5°C-acclimated turtles, indicate that alterations of high-energy phosphate metabolism, pH, and dG/dξ may be important dictators of the depression of cardiac activity with anoxia exposure at this temperature. Further our results show that at 5°C, turtle cardiac energetic state is maintained at a new, reduced steady state with prolonged anoxia.

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 Company of Biologists Travel Fund and Journal of Experimental Biology Travelling Fellowship to J. A. W. S., a Response of Higher Life to Change grant (within MARCOPOLI) to H.-O. P., and Danish Research Council funding to T. W. Special thanks to Rolf Wittig for his post-processing of 31P-NMR spectra and flow-weighted images.
References


Figure Legend

Figure 4.1 Representative (A) coronal, (B) sagittal and (C) transverse scout images of a turtle within the MR magnet. The cross-hairs (darkened lines) indicate the center of the magnet, within which lies the turtle heart. Morphological features: abv, abdominal vein; bd, bladder; cbv, central blood vessels; cp, carapace; gb, gall blader; gt, gut; lg, lung; pl, plastron; v, ventricle.

Figure 4.2 Representative in vivo $^{31}$P-NMR spectra of (A) a 21°C-acclimated normoxic turtle, (B) a 21°C-acclimated turtle at 2.85 h of anoxia exposure, (C) a 5°C-acclimated normoxic turtle and (D) a 5°C-acclimated turtle on day 11 of anoxia exposure. Peaks designated in panel A: PME, phosphomonoester; P, inorganic phosphate; PDE, phosphodiester; PCr, phosphocreatine; α-, β- and γ-ATP correspond to the three phosphates of ATP; α- and β-ADP correspond to the two phosphates of ADP.

Figure 4.3 Total $^{31}$P-NMR signal of (A) 21°C-acclimated and (B) 5°C-acclimated turtle hearts during prolonged anoxia exposure. Note the different time-scale between temperature acclimation groups. Values are means ± S.E.M.; N= 5-7.

Figure 4.4 Typical flow-weighted MR images of the central blood vessels of a 5°C-acclimated turtle during (A) normoxia and (B) following ~12 h of anoxia exposure. The magnitude of blood flow through each vessel is proportional to mean signal intensity. Note the absence of flow in the right and left pulmonary arteries and reduced blood flow in the right and left aortic arches with anoxia exposure. V, ventricle; R, right atria; L; left
atria, RPA, right pulmonary artery; LPA, left pulmonary artery; RAo, right aortic arch; LAo, left aortic arch.

Figure 4.5 Chronological changes of aortic and pulmonary blood flow of (A) 21°C- and (B) 5°C-acclimated turtles during prolonged anoxia exposure. Note the different time-scale between temperature acclimation groups. Values are means ± S.E.M.; N = 5-7.

Figure 4.6 Chronological changes of (A) fH, myocardial phosphorous metabolites [(B) PCr; (C) P; and (D) ATP], (E) cardiac pH; and (F) cardiac dG/dξ of 21°C-acclimated turtles during 2.85 h of anoxia exposure. Statistically significant changes (P<0.05) from normoxic control (t = 0) for each variable are indicated by a solid line above the trace. Statistically significant differences (P<0.05) from final recording time are indicated by a dotted line above the trace. Values are means ± S.E.M.; N = 5-7.

Figure 4.7 Chronological changes of (A) fH, myocardial phosphorous metabolites [(B) PCr; (C) P; and (D) ATP], (E) cardiac pH; and (F) cardiac dG/dξ of 5°C-acclimated turtles during 11 d of anoxia exposure. Statistically significant changes (P<0.05) from normoxic control (t = 0) for each variable are indicated by a solid line above the trace. Statistically significant differences (P<0.05) from final recording time are indicated by a dotted line above the trace. Values are means ± S.E.M.; N = 5-7.
Figure 4.8  Relationships between myocardial (A) PCr, (B) P_i, (C) ATP, (D) pH, and (E) dG/dξ and f_H for 21°C-acclimated turtles exposed to 2.85 h of anoxia. Values are means ± S.E.M.; N= 5-7.

Figure 4.9  Relationships between myocardial (A) PCr, (B) P_i, (C) ATP, (D) pH, and (E) dG/dξ and f_H for 5°C-acclimated turtles exposed to 11 d of anoxia. Values are means ± S.E.M.; N= 5-7.
Figure 4.1
Figure 4.2

**A** Normoxia

- PCR
- Pi
- PDE
- PME
- γ-ATP
- β-ADP
- α-ATP
- β-ATP

**B** Anoxia

**C** 21°C-acclimated

**D** 5°C-acclimated
Figure 4.3

A

Total $^{31}$P-NMR signal (arbitrary units)

Time (h)

B

Total $^{31}$P-NMR signal (arbitrary units)

Time (d)
Figure 4.4
Figure 4.5
Figure 4.8
Figure 4.9
CHAPTER V:
EFFECTS OF EXTRACELLULAR CHANGES ON SPONTANEOUS HEART RATE OF NORMOXIA- AND ANOXIA-ACCLIMATED TURTLES (TRACHEMYS SCRIPTA)

Introduction

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

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

Although the bradycardia and associated depression of cardiac activity exhibited by anoxic turtles is well documented and quantified in vivo, its determinants are not fully elucidated. In warm-acclimated turtles, a simultaneous cholinergic, vagal cardiac inhibition and $\beta$-adrenergic cardiac stimulation contribute to the setting of anoxic $f_H$ (Hicks and Wang, 1998; Hicks and Farrell, 2000b), but $\alpha$-adrenergic (Stecyk et al., 2004) and adenosinergic cardiac inhibition do not (Stecyk et al., 2006). However, cholinergic cardiac inhibition only accounts for $\sim$30% of the reduction in $f_H$ that occurs during warm anoxia (Hicks and Wang, 1998; Hicks and Farrell, 2000b). In cold-acclimated, anoxic turtles, autonomic cardiovascular control is blunted and does not account for the large bradycardia (Hicks and Farrell, 2000b; Stecyk et al., 2004). Similarly, there is no adenosinergic cardiac inhibition during prolonged, cold anoxia (Stecyk et al., 2006). Thus, other determinants must contribute to the depression of $f_H$ in both warm- and cold-acclimated turtles during anoxia, i.e., in addition to autonomic cardiovascular control in warm-acclimated turtles and instead of the autonomic control that is turned off in cold-acclimated turtles. The purpose of the present study was to examine the contribution to this anoxic bradycardia made by the significant changes in the extracellular milieu that accompanies prolonged anoxia.

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

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

**Materials and methods**

*Experimental animals*

Sixty-four red-eared sliders (*Trachemys scripta*, Gray) with body masses ranging between 83 and 506 g (228 ± 88 g, mean ± S.D) were used in this study. Turtles were obtained from Lemberger Inc. (Oshkosh, WI, USA) and airfreighted to Simon Fraser University. We used a 2 x 2 exposure design (normoxia and anoxia exposure x 21°C- and 5°C-acclimation). Turtles studied at 21°C were held indoors in glass aquaria under a 12 h:12 h L:D photoperiod, had free access to basking platforms and diving water and were fed several times a week with a mixture of commercial trout feed pellets, cat food, and fresh vegetables. The turtles studied at 5°C were kept in glass aquaria with shallow water (3-4 cm) under a 12 h:12 h L:D photoperiod in a temperature-controlled room set to 5°C for 5 weeks prior to experimentation to allow adequate time for cold-acclimation (Hicks and Farrell, 2000a). The 5°C turtles were fasted during this period. Normoxic turtles were sampled from these conditions. For prolonged anoxia, turtles were exposed to anoxia for 6 h at 21°C or 14 days at 5°C. The required anoxic conditions were achieved by first individually placing turtles into an enclosed, water-containing plastic chamber that still allowed access to air for 24 hours. Then, the plastic chamber was completely filled with water, continuously bubbled with N_2 (water $P_O_2 < 0.3$ kPa) and the turtle denied air access by means of a metal mesh that was suspended below the surface of the water. All procedures were in accordance with Simon Fraser University Animal Care Guidelines.
Tissue preparation

A spontaneously contracting right-atrial preparation was used to investigate the extracellular effects of anoxia, acidosis, hyperkalemia, hypercalcemia and adrenaline on the spontaneous $f_H$ of normoxic- and anoxic-acclimated turtles at 21°C and 5°C. The heart was accessed through removal of a 3 cm x 3 cm piece of the plastron using a bone saw following euthanasia by decapitation, which for anoxic-acclimated turtles occurred underwater in the plastic containers. The vena cava was ligated with 3-0 braided surgical silk, and the entire right atrium separated from left atrium, and from the ventricle at the atrial-ventricular junction, taking care to preserve the pacemaker region of the sinus venosus. The entire procedure lasted approximately 3 to 5 min, after which the surgical silk was immediately fastened to a force-displacement transducer (Grass, FT 10, Quincy, MASS, USA) and the apex of the atrium was hooked to a fixed arm such that both sides of the atrial wall would be in direct contact with saline solutions. The preparation was then suspended in a water-jacketed organ bath containing the starting saline solution that approximated the in vivo extracellular conditions (i.e. atria from normoxic turtles were placed in simulated normoxic saline while atria from anoxia-acclimated turtles were placed in simulated anoxic saline; Table 5.1). The length of the mounted atrial preparation was adjusted with a micrometer screw to produce ~90% of maximal contraction force to limit inter-preparation variation due to the chronotropic effects of cardiac stretch (Cooper and Kohl, 2005) and the preparation allowed 20 - 25 min to stabilize to allow for washout of any inherent adrenergic agents. No further adjustments were made to the length of the atrial preparation during the experiment.
Experimental protocol

Normoxic preparations

Following the stabilization period, atrial preparations from normoxic turtles were subjected to either a control or treatment protocol. The control preparations remained in the simulated normoxic saline (Control Normoxic protocol), although the saline solution was refreshed at the same time interval as saline changes in the treatment protocol. The treatment protocol (Normoxic Treatment protocol) involved a series of saline solutions that progressively simulated \textit{in vivo} anoxic extracellular conditions (i.e., atria were sequentially and additively exposed to anoxia, acidosis, hyperkalemia, hypercalcemia and increased adrenaline concentration; see Table 5.1 for details).

Anoxia-acclimated preparations

Like the normoxic preparations, atria from anoxia-acclimated turtles were subjected to either a control or treatment protocol (Table 5.1). The anoxia-acclimated control preparations remained in simulated anoxic saline (Control Anoxic protocol) with refreshment of the saline occurring at the same time interval as saline changes in the treatment protocol. The purpose of the Anoxia-acclimated Treatment protocol was to return the atria to a simulated \textit{in vivo} normoxic extracellular condition from the simulated \textit{in vivo} anoxic extracellular condition. Therefore, the Anoxia-acclimated Treatment protocol was the exact reverse order of saline solutions as the Normoxic Treatment protocol (see Table 5.1 for details).

Saline compositions were devised to closely mimic the changes in blood plasma that occur \textit{in vivo} with 6 h (21°C) or 14 days (5°C) of anoxia (the anoxia-acclimation times of our turtles) and not the changes that occur with several months of anoxia (see Ultsch and Jackson, 1982; Jackson and Heisler, 1982; Jackson and Ultsch, 1982; Herbert and Jackson, 1985a; Keiver and Hochachka, 1991; Wasser and Jackson, 1991; Keiver et al., 1992; Warren et al., 2006)
Therefore, some of our changes in saline ionic composition differ from those utilized in previous studies that have examined the effects of extracellular factors on turtle cardiac inotropy and chronotropy. Further, it should be noted that we utilized both hypercapnic and lactic acidosis to depress pH. Consequently, the concentration of ionized calcium (Ca\(^{2+}\)) in the saline solutions would be slightly less than indicated in Table 5.1 due to the binding of calcium and lactate to form a calcium-lactate complex (Jackson and Heisler, 1982). Moreover, the anoxic plus acidotic saline solution was simultaneously made hypermagnesemic in order to accurately simulate the changes in blood plasma that accompany anoxia (see Table 5.1), but no attempt was made to distinguish unique effects of hypermagnesemia from those of acidosis.

**21°C-acclimated experiments**

For experiments with warm-acclimated preparations, exposure time to each saline solution in the Normoxic Treatment and Anoxia-acclimated Treatment protocols was 15 min. Likewise, simulated normoxic and simulated anoxic saline solutions were refreshed every 15 min during the 1.5 h Control Normoxic and Control Anoxic protocols, respectively. Additionally, at 21°C, the Control Normoxic, Normoxic Treatment, and Anoxia-acclimated Treatment protocols were conducted with two levels of tonic adrenergic stimulation (1 nM and 10 nM; Table 5.1).

**5°C-acclimated experiments**

For experiments with cold-acclimated preparations, exposure time to each saline solution in the Normoxic Treatment and Anoxia-acclimated Treatment protocols was 20 min. Likewise, simulated normoxic and simulated anoxic saline solutions were refreshed every 20 min during the 2 h Control Normoxic and Control Anoxic protocols, respectively.
The exposure times of 15 min at 21°C and 20 min at 5°C were chosen to obtain an effective balance between reaching new steady state with a saline change and maintaining tissue integrity for the duration of the experiments. These times were based on a wide range of previous studies reporting that, if present, inotropic and chronotropic responses of turtle myocardium to anoxic, acidotic and/or hyperkalemic changes occur within approximately 5 min to 15 min of exposure for warm-acclimated turtle heart (Poupa et al., 1978; Gesser and Poupa, 1978; Gesser and Jørgensen, 1982; Yee and Jackson, 1984; Wasser et al., 1990a; Wasser et al., 1990b; Wasser et al., 1997; Nielsen and Gesser, 2001), and within 20 min of exposure for 5°C acutely exposed turtle heart (Farrell et al., 1994).

Data analysis and statistics

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

\textbf{Results}

\textit{Stability of spontaneously contracting right-atrial preparations}

Control experiments conducted to assess the stability of spontaneously contracting right-atrial preparations over time revealed that our preparations were viable throughout the durations of experimental protocols. At 21°C, Control Normoxic (both 1 nM and 10 nM tonic adrenaline groups) and Control Anoxic preparations maintained a stable $f_H$ throughout the control experiments (Fig. 5.1). Similarly, no statistically significant change in $f_H$ occurred over time in 5°C Control Normoxic preparations. However, spontaneous $f_H$ of 5°C Control Anoxic atrial preparations increased significantly from an initial rate of $2.1 \pm 0.2 \text{ min}^{-1}$ to a stable rate of $\sim 3 \text{ min}^{-1}$ at 40 min (Fig. 5.1).

\textit{Chronotropic effects of extracellular changes on normoxic hearts}

\textit{21°C-acclimated preparations}

Exposure to anoxic saline had no effect on spontaneous $f_H$ of normoxic preparations at 21°C (Fig. 5.2). Combined anoxia and acidosis significantly decreased spontaneous $f_H$ by 22% with 1 nM tonic adrenaline and by 12% with 10 nM tonic adrenaline (Fig. 5.2). Subsequent exposure to combined anoxia, acidosis and hyperkalemia further decreased $f_H$ (to a 33% decrease) with 1 nM tonic adrenaline, but not with 10 nM tonic adrenaline (Fig. 5.2). Hypercalcemia did not reverse these negative chronotropic effects (Fig. 5.2). However,
increasing adrenaline concentration from 1 nM to 60 nM adrenaline completely reversed the negative chronotropic effects and returned $f_H$ to 1) the simulated normoxic saline $f_H$, 2) the comparable Control Normoxic $f_H$, and 3) the $f_H$ of the 10 nM tonic adrenaline group under simulated anoxic conditions (Fig. 5.2). Therefore, increasing adrenaline from 10 nM to 60 nM did not have any effect on the already elevated $f_H$ (Fig. 5.2). Consequently, at least for normoxia-acclimated preparations at 21°C, a tonic adrenaline concentration of 10 nM in concert with hypercalcemia appears strong enough to produce a maximal adrenergic response and compensate for the negative chronotropic effects of anoxia, acidosis and hyperkalemia.

5°C-acclimated preparations

The 5°C-acclimated preparations revealed an important temperature-dependency to extracellular effects on spontaneous $f_H$. In contrast to 21°C, $f_H$ at 5°C was not affected by combined anoxia and acidosis (Fig. 5.3). But, like at 21°C, combined anoxia, acidosis and hyperkalemia with 1 nM tonic adrenaline significantly depressed $f_H$ from the simulated normoxic saline rate (by 23%) as well as from the comparable Control Normoxic rate; Fig. 5.3). Nevertheless and contrary to 21°C, this negative chronotropic effect at 5°C was completely offset by increased extracellular Ca$^{2+}$, whereas subsequent exposure to 25 nM adrenaline had no further effect on spontaneous $f_H$ (Fig. 5.3). Therefore, cold-acclimation has some form of preconditioning effect for anoxic and acidosis exposure.

Effect of prolonged anoxia exposure on spontaneous $f_H$

An important finding of this study was that the initial spontaneous $f_H$ of 5°C turtles exposed to prolonged anoxia and placed in simulated anoxic saline was not the same as the spontaneous $f_H$ of 5°C normoxic preparations exposed to the same saline (Fig. 5.3). Specifically, spontaneous $f_H$ of 5°C anoxia-acclimated preparations was approximately half the rate of
normoxic preparations. Moreover, in simulated normoxic saline, spontaneous $f_H$ of 5°C anoxia-acclimated preparations was 47% lower than the initial $f_H$ of normoxic preparations in the same saline (Fig. 5.3), whereas spontaneous $f_H$ of 5°C Control Anoxic preparations at $t = 2$ h was 32% lower than comparable normoxia-acclimated preparations despite the step-wise increase in $f_H$ that occurred at $t = 40$ min (Fig. 5.1). Thus, anoxia acclimation at 5°C re-sets the spontaneous $f_H$ of turtle hearts to about half of that found in normoxia-acclimated preparations.

Anoxia acclimation at 21°C produced a similar re-setting of $f_H$ to a reduced level. Spontaneous $f_H$ of 21°C anoxia-acclimated preparations in simulated normoxic saline was 25% (with 1 nM tonic adrenaline) and 34% (with 10 nM tonic adrenaline) lower than the initial spontaneous $f_H$ of comparable normoxia-acclimated preparations (Fig. 5.2). However, in contrast to 5°C, no statistically significant differences in $f_H$ existed between normoxic and anoxia-acclimated preparations in simulated anoxic saline at 21°C. This indicates that 60 nM adrenaline compensated for the re-setting of intrinsic $f_H$ that occurs with prolonged anoxia at 21°C.

**Chronotropic effects of extracellular changes on anoxia-acclimated hearts**

21°C-acclimated preparations

The reversed exposure of anoxia-acclimated hearts at 21°C to extracellular changes revealed that some important differences existed between anoxia- and normoxia-acclimated preparations in their chronotropic responses. Removal of hypercalcemia significantly reduced $f_H$ of preparations with 1 nM and 10 nM tonic adrenaline, which contrasted with the lack of a protective effect of hypercalcemia in normoxia-acclimated preparations (Fig. 5.2). Thus, prolonged anoxia exposure at 21°C appears to heighten the protective role of extracellular $Ca^{2+}$ on cardiac chronotropy. Further, 21°C anoxia-acclimated preparations were less susceptible to the negative chronotropic effects of hyperkalemia and acidosis than 21°C normoxia-acclimated
hearts. Neither decreasing the extracellular $K^+$ concentration to normoxic levels nor removing extracellular acidosis altered spontaneous $f_H$ of anoxia-acclimated preparations (Fig. 5.2). Finally, spontaneous $f_H$ did increase significantly with the cessation of extracellular anoxia in 21°C anoxia-acclimated preparations independent of the tonic adrenaline concentration, whereas no decrease in $f_H$ with exposure to extracellular anoxia was observed in normoxia-acclimated preparations (Fig. 5.2).

Even so, some chronotropic responses of anoxia-acclimated preparations were consistent with the normoxia-acclimated preparations at 21°C. For instance, reducing the adrenaline concentration from 60 nM to 1 nM, but not to 10 nM adrenaline significantly reduced spontaneous $f_H$ of anoxia-acclimated preparations (Fig. 5.2).

5°C-acclimated preparations

Again, the reversed exposure of anoxia-acclimated hearts at 5°C to extracellular changes revealed that some important differences existed between anoxia- and normoxia-acclimated preparations. Specifically, in 5°C anoxia-acclimated atrial preparations a negative chronotropic effect of combined anoxia, acidosis and hyperkalemia was not present in contrast to 5°C normoxia-acclimated preparations (Fig. 5.3). In fact, chronotropy of 5°C anoxia-acclimated preparations was not affected by any extracellular change and spontaneous $f_H$ was unchanged throughout the entire protocol.

Discussion

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

**Critique of methods**

To make useful extrapolation to the in vivo situation, the spontaneously contracting right-atrial preparations should contract at rates comparable to in vivo intrinsic $f_H$ and be stable for the duration of the experimental protocol. Further, compositional changes in saline solutions should be physiologically relevant and the exposure time should be sufficient to reach a new steady state for $f_H$. This was the case. Spontaneous $f_H$ of normoxia-acclimated preparations at 21°C and 5°C closely matched previously reported in vivo and in vitro intrinsic rates (Yee and Jackson, 1984; Wasser et al., 1990ab; Farrell et al., 1994; Wasser et al., 1992; Wasser et al., 1997; Hicks and Farrell, 2000b) and were stable throughout control experiments (Fig. 5.1). Similarly, spontaneous $f_H$ of 21°C and 5°C anoxia-acclimated right-atrial preparations, which were recorded with tonic adrenergic stimulation, were similar to in vivo $f_H$ for anoxic turtles following cholinergic blockade (Hicks and Farrell, 2000b). 5°C anoxia-acclimated control preparations did exhibit a step-wise increase in $f_H$ at 40 min (Fig. 5.1). However, given that this increase in $f_H$ was an initial step change and not a continuous change, that the increased $f_H$ remained statistically lower than 5°C Control Normoxic $f_H$, and that $f_H$ at 2 h was not statistically
significantly different from the $f_H$ of 5°C Anoxia-acclimated Treatment preparations in simulated normoxic saline, we are confident of our findings for 5°C anoxia-acclimated turtle hearts. Moreover, as described above, our saline compositions were devised to closely mimic the changes in blood plasma that occur in vivo with 6 h (21°C) or 14 days (5°C) of anoxia (see Ultsch and Jackson, 1982; Jackson and Heisler, 1982; Jackson and Ultsch, 1982; Herbert and Jackson, 1985a; Keiver and Hochachka, 1991; Wasser and Jackson, 1991; Keiver et al., 1992, Warren, 2006). Finally, visual inspection of traces at both acclimation temperatures revealed that most of the change in $f_H$ occurred within the first 5 min of a saline switch.

**Chronotropic effects of extracellular changes**

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

**Normoxia-acclimated hearts**

Our results for 21°C normoxia-acclimated preparations were consistent with our predictions. Combined anoxia with acidosis, as well as combined anoxia, acidosis and hyperkalemia were found to depress spontaneous $f_H$, whereas adrenaline reversed or diminished these negative chronotropic effects (Fig. 5.2). These present findings are akin to previous
studies showing that anoxia and acidosis act synergistically to depress turtle $f_H$ (Wasser et al., 1990ab), whereas individually, anoxia and acidosis may (Reeves, 1963; Yee and Jackson, 1984; Jackson, 1987; Wasser et al., 1990b; Farrell et al., 1994; Wasser et al., 1997) or may not induce bradycardia (Wasser et al., 1990b; Wasser et al., 1992). Further, the decreased spontaneous $f_H$ with hyperkalemia reported here for the turtle is similar to the negative chronotropic effect of increased extracellular K$^+$ concentration on the anoxia-intolerant rainbow trout heart (*Oncorhynchus mykiss*; Hanson et al., 2006).

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

Unlike at 21°C, the 5°C normoxia-acclimated turtle heart was resistant to combined anoxia and acidosis, but when combined with hyperkalemia, $f_H$ decreased by 23%, a negative
effect that was slightly less than at 21°C (Fig. 5.3). Conversely, the negative inotropic effect of hyperkalemia alone is greater in cold-acclimated turtle hearts than in warm-acclimated hearts (Overgaard et al. (2005). Further, unlike at 21°C, hypercalcemia fully reversed the negative chronotropic effect of combined anoxia, acidosis and hyperkalemia in normoxia-acclimated preparations at 5°C, which precluded the positive chronotropic effect of subsequently increasing the adrenaline concentration (to 25 nM) (Fig. 5.3). This heightened importance of extracellular \( \text{Ca}^{2+} \) in protecting chronotropy at 5°C suggests that cold-acclimation modifies cellular calcium cycling in the turtle heart, a preconditioning effect that would make sense giving the normal mobilization of calcium from turtle bone and shell during cold anoxia (Jackson, 2002). The lack of positive chronotropy in response to 25 nM adrenaline is consistent with the attenuation of adrenergic control in cold-acclimated turtle hearts (Hicks and Farrell, 2000b).

Anoxia-acclimated hearts

Our results indicate that intrinsic \( f_h \) is re-set to a level 32% - 53% (at 5°C) and 25% - 34% (at 21°C) lower than with normoxia as a result of prolonged anoxia exposure (Figs. 5.2 and 5.3). In vivo \( f_h \) decreases by 5-fold at 5°C and by 2.5-fold at 21°C with prolonged anoxia exposure (Hicks and Farrell, 2000a, Stecyk et al., 2004). Thus, in cold-acclimated anoxic turtles, when autonomic cardiovascular control is blunted (Hicks and Farrell, 2000b; Stecyk et al., 2004), this re-setting of intrinsic \( f_h \) could contribute to 40% - 66% of the anoxic bradycardia. In warm-acclimated turtles, the re-setting of intrinsic \( f_h \) could contribute to 42% - 57% of the anoxic bradycardia, but the relative contribution of the re-setting of intrinsic \( f_h \) towards the anoxic bradycardia is more difficult to discern. Autonomic cardiovascular control is not blunted at 21°C (Hicks and Farrell, 2000b; Stecyk et al., 2004), and results from this study revealed that with an in vivo level (60 nM) of adrenergic stimulation, spontaneous \( f_h \) of normoxia-acclimated
and anoxia-acclimated preparations were the same (Fig. 5.2). Thus, *in vivo* at 21°C, circulating catecholamines may be able to compensate for the re-setting of intrinsic $f_{IH}$.

Beyond the clear re-setting of intrinsic rate, anoxia-acclimation also resulted in different chronotropic responses to extracellular changes, indicating that the mechanisms underlying the effects of extracellular factors on spontaneous $f_{IH}$ are modified with anoxia. Primarily, extracellular factors do not appear to be important controlling factors of cardiac chronotropy during prolonged, cold anoxia (Fig. 5.3). At 21°C, anoxia-acclimated preparations were less susceptible to the negative chronotropic effects of hyperkalemia and acidosis than 21°C normoxia-acclimated hearts (Fig. 5.2), indicating that extracellular anoxia is a potent trigger for bradycardia in anoxia-acclimated hearts.

*Potential mechanisms underlying the observed chronotropic effects*

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

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

In this regard, the reduced chronotropic sensitivity of 5°C normoxia-acclimated turtle hearts to acidosis and both 21°C and 5°C anoxia-acclimated turtle hearts to extracellular acidosis and hyperkalemia (Figs. 5.2 and 5.3) could be related to respective changes induced by cold acclimation and anoxia in atrial sarcolemmal ion channel densities and kinetics and/or regulation or contractile protein isoforms to offset the slowed functioning of intracellular pH regulation in turtle myocytes function during anoxia compared to normoxia (Shi et al., 1997). However, alteration of pacemaker mechanisms may be more important in facilitating the re-setting of \(f_H\) with cold anoxia than a change in cardiac cycle length since time-to-peak twitch force and time to relaxation does not differ between 5°C normoxia- and anoxia-acclimated turtle ventricular strips (Overgaard et al., 2005). Future studies investigating the effects of cold acclimation and anoxia exposure on turtle cardiac electrophysiology are of course needed to clarify these possibilities and these are underway in our laboratory.

**Adrenaline and chronotropy**

Additionally, this study revealed some important differences in the effect of adrenaline on cardiac chronotropy between warm- and cold-acclimated, as well as normoxia- and anoxia-acclimated turtles. The positive chronotropic effect of adrenaline present at 21°C disappeared
with cold acclimation and at 21°C, anoxia-acclimation modified the interplay between extracellular Ca$^{2+}$ concentration and adrenergic stimulation without affecting the sensitivity of spontaneous $f_H$ to adrenergic stimulation (Figs. 5.2 and 5.3). These findings open the possibility that cold temperature and anoxia-acclimation alter the interplay between adrenaline and excitation-contraction coupling and/or calcium cycling in the turtle heart. Finally, our 21°C experiments with two levels of tonic adrenergic stimulation (1 nM and 10 nM) revealed that adrenergic stimulation protects the turtle heart equally well after as well as concurrent with the anoxic challenge (Fig. 5.2). This finding contrasts recent findings in the anoxia-intolerant rainbow trout heart where concurrent adrenergic stimulation better protected cardiac performance during, rather than following a combined hypoxic, hyperkalemic and acidotic insult (Hanson et al., 2006). Given that ventricular β-adrenoreceptor density decreases with prolonged anoxia in the turtle (Hicks and Farrell, 2000b), but not during hypoxia exposure in the rainbow trout (Gamperl et al. 1998), the possibility exists that changes in turtle cardiac β-adrenoceptor density with prolonged anoxia exposure may be tissue specific.

**Concluding remarks**

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

Acknowledgements

The authors' research was generously supported by NSERC Canada. The authors would like to thank Dr. M. Axelsson and Dr. J. Altimiras for writing the code for the data acquisition and analysis programs and Priscilla Yu for her help in conducting the experiments.
References


Figure Legend

**Figure 5.1** Spontaneous $f_H$ of 5°C and 21°C, normoxic and anoxia-acclimated control preparations during the control experiments. Significant differences ($P<0.05$) in $f_H$ among time points within an experimental group are indicated by dissimilar letters. Values are means ± S.E.M; $N = 6, 6, 6, 5$ and 6 in the 21°C, 10 nM adrenaline (ADR) normoxic, 21°C, 1 nM ADR normoxic, 21°C, 60 nM ADR anoxic, 5°C, normoxic and 5°C anoxic experimental groups, respectively, unless otherwise indicated in parentheses.

**Figure 5.2** Spontaneous $f_H$ of 21°C, normoxic and anoxia-acclimated, 1 nM and 10 nM tonic adrenaline (ADR) right-atrial preparations during exposure to various saline solutions devised to simulate or reverse the changes occurring in turtle blood plasma during 6 h of anoxic submergence. Exposure order for normoxic preparations was from left-to-right, whereas exposure order for anoxia-acclimated preparations was from right-to-left. Significant differences ($P<0.05$) in $f_H$ among saline solutions within an experimental group are indicated by dissimilar letters. Significant differences ($P<0.05$) in $f_H$ from a comparable Control protocol $f_H$ are indicated by the symbol †. Significant differences ($P<0.05$) in $f_H$ between 1 nM and 10 nM tonic adrenaline groups within an acclimation condition (i.e., normoxic or anoxia-acclimated) are indicated by the symbol §. Significant difference ($P<0.05$) in $f_H$ between normoxic and anoxia-acclimated preparations (of the same level of tonic adrenergic stimulation) under simulated *in vivo* normoxic or simulated *in vivo* anoxic conditions is indicated by an asterisk. Values are means ± S.E.M; $N = 6$ in the 1 nM tonic adrenaline normoxic and anoxia-acclimated groups, 5 in the 10 nM tonic adrenaline normoxic group and 6 in the 10 nM tonic adrenaline anoxia-acclimated group.
Figure 5.3  Spontaneous $f_{H}$ of 5°C, normoxic and anoxia-acclimated right-atrial preparations during exposure to various saline solutions devised to simulate or reverse the changes occurring in turtle blood plasma during 14 days of anoxic submergence. Exposure order for normoxic preparations was from left-to-right, whereas exposure order for anoxia-acclimated preparations was from right-to-left. Significant differences ($P<0.05$) in $f_{H}$ among saline solutions within an experimental group are indicated by dissimilar letters. Significant differences ($P<0.05$) in $f_{H}$ from a comparable Control protocol $f_{H}$ are indicated by the symbol ‡. Significant difference ($P<0.05$) in $f_{H}$ between normoxic- and anoxia-acclimated preparations under simulated in vivo normoxic or simulated in vivo anoxic conditions is indicated by an asterisk. Values are means ± S.E.M; $N = 6$ in both experimental groups.
Table 5.1 Composition of saline solutions used for the 5°C and 21°C experiments.

<table>
<thead>
<tr>
<th>Exposure Order</th>
<th>Saline Solution</th>
<th>NaCl (mM)</th>
<th>NaHCO₃ (mM)</th>
<th>NaH₂PO₄ (mM)</th>
<th>MgSO₄ (mM)</th>
<th>KCl (mM)</th>
<th>CaCl₂ (mM)</th>
<th>Lactic Acid (mM)</th>
<th>Glucose (mM)</th>
<th>Adrenaline (nM)</th>
<th>Gas Composition *</th>
<th>pH **</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simulated</td>
<td>normoxic saline</td>
<td>85</td>
<td>40</td>
<td>1</td>
<td>1</td>
<td>2.5</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>1% CO₂/99% O₂</td>
<td>8.00±0.03 (17)</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>(25)</td>
<td>(1)</td>
<td>(1)</td>
<td>(2.5)</td>
<td>(2)</td>
<td>(0)</td>
<td>(5)</td>
<td>(1 or 10)</td>
<td></td>
<td>(2% CO₂/98% O₂)</td>
<td>[7.75±0.02 (36)]</td>
</tr>
<tr>
<td>Anoxia</td>
<td></td>
<td>85</td>
<td>40</td>
<td>1</td>
<td>1</td>
<td>2.5</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>1% CO₂/99% N₂</td>
<td>7.98±0.03 (12)</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>(25)</td>
<td>(1)</td>
<td>(1)</td>
<td>(2.5)</td>
<td>(2)</td>
<td>(0)</td>
<td>(5)</td>
<td>(1 or 10)</td>
<td></td>
<td>(2% CO₂/98% N₂)</td>
<td>[7.74±0.02 (24)]</td>
</tr>
<tr>
<td>Anoxia + Acidosis</td>
<td></td>
<td>85</td>
<td>40</td>
<td>1</td>
<td>3</td>
<td>2.5</td>
<td>2</td>
<td>14</td>
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<td>2% CO₂/98% N₂</td>
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<td>(3% CO₂/97% N₂)</td>
<td>[7.23±0.02 (24)]</td>
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<td>Anoxia + Acidosis + Hyperkalemia</td>
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<td>85</td>
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<td>(3% CO₂/97% N₂)</td>
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<td>(3% CO₂/97% N₂)</td>
<td>[7.22±0.02 (30)]</td>
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Data for the 5°C experiments are presented without parentheses. Data for the 21°C experiments are presented in parentheses. Bold text highlights the saline solution composition difference from the preceding solution for the normoxic-acclimated experimental protocol.

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

** Saline pH was measured (Model 220 pH meter, Corning Science Products, NY, USA) prior to every experiment and values presented are mean ± S.D. (N).
Figure 5.1
Figure 5.2
Figure 5.3
CHAPTER VI:
EFFECT OF TEMPERATURE AND PROLONGED ANOXIA EXPOSURE ON ELECTROPHYSIOLOGICAL PROPERTIES OF THE TURTLE (TRACHEMYS SCRIPTA) HEART

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 (Ultsch, 1985; Warren et al., 2006). 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 massively reduced rate. This reduced cardiac activity during anoxia reflects and matches the reduction of whole-animal metabolic rate and thus whole-body demand for blood flow (Jackson, 1968; Herbert and Jackson, 1985b), and also serves as a strategy to ensure that cardiac ATP demand is well below the cardiac glycolytical capacity to supply ATP (Hicks and Farrell, 2000a; Farrell and Stecyk, 2006). It is a profound anoxic bradycardia that largely reduces systemic cardiac power output and systemic cardiac output by 4.5- to 20-fold following 6 h and 14 – 21 d anoxic exposures in warm- and cold-acclimated turtles, respectively (Herbert and Jackson, 1985b; Hicks and Wang, 1998; Hicks and Farrell, 2000ab, Stecyk et al., 2004a; Stecyk et al., 2006a). Heart rate ($f_H$) decreases from ~25 min$^{-1}$ to ~10 min$^{-1}$ within 1 h during anoxia at 21°C - 25°C and from a normoxic rate of ~5 min$^{-1}$ to less than 1 min$^{-1}$ within 24 h in anoxic turtles at 5°C.

The mechanisms underlying this bradycardia during prolonged anoxia are not fully understood. In warm-acclimated turtles, cholinergic cardiac inhibition contributes to ~30% of the anoxic bradycardia (Hicks and Wang, 1998; Hicks and Farrell, 2000b), but α-adrenergic (Stecyk at al., 2004b) and adenosinergic (Stecyk et al., 2006a) 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 (Hicks and Farrell, 2000b; Stecyk et al., 2004a). Similarly, adenosinergic cardiac inhibition is not involved either (Stecyk et al., 2006a). Instead, the suggestion has been made (Hicks and Farrell, 2000b; Overgaard et al., 2005; Stecyk and Farrell, 2007) that intrinsic electrophysiological changes account for the anoxic bradycardia in cold-acclimated turtles. Certainly, an increased prevalence of atrial-ventricular blocks in isolated turtle hearts during anoxia exposure suggests a reduced ventricular excitability (Jackson, 1987). Intrinsic electrophysiological modifications also are likely involved in the profound slowing of heart rate with cold-acclimation since cholinergic cardiac inhibition is not involved (Hicks and Farrell, 2000b) and rates of contraction and relaxation decrease (Overgaard et al., 2005).

As a first step to understanding these potential electrophysiological modifications with cold acclimation and prolonged anoxia exposure, the present study investigated in the red-eared slider the effects of cold temperature acclimation and prolonged anoxia exposure on: 1) cardiac action potentials (APs); and 2) current densities of four ventricular sarcolemmal ion channels, namely the voltage-gated Na\(^+\)\((I_{Na})\), L-type Ca\(^{2+}\) \((I_{Ca})\), inward rectifier K\(^+\) \((I_{K1})\) and delayed rectifier K\(^+\) \((I_{Kr})\) channels, involved in generating cardiac APs (Roden et al. 2002). A 2 x 2 exposure design was employed to make comparisons among 21°C- and 5°C-acclimated turtles exposed to either normoxia or anoxia. In addition to measuring these electrophysiological characteristics under similar conditions to those of the turtle, recordings were made following an acute temperature change to distinguish the effect of cold-acclimation from passive consequences of low ambient temperature (termed here direct temperature effects) on the rate of these physiological processes. 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., Paajanen and Vornanen, 2004; Shiels et
al., 2006), I reasoned that changes in cardiac APDs would occur with the decreases in $f_{11}$ associated with 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 2 x 2 (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 (Hicks and Farrell, 2000a). The 5°C turtles were fasted during this 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. 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 $N_2$ (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. 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 anoxic-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 P₀₂ 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 and adrenaline degradation. Saline for anoxia-
acclimated hearts was pre-bubbled with N₂.

Hearts were allowed to stabilize to the experimental conditions for 30 - 45 min before
recordings were made. For normoxia-acclimated hearts, APs and spontaneous \( f_1 \) were recorded
first at the acclimation temperature of the animal (i.e., 21°C or 5°C) and then the saline was
acutely changed either from 5°C to 21°C, or from 21°C to 5°C and hearts allowed 45 - 60 min to
stabilize before APs and spontaneous $fH$ were re-recorded for that preparation. For anoxia-acclimated hearts, APs and spontaneous $fH$ 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°C and pH 7.55 at 5°C; still continuously bubbled with $\text{N}_2$) that mimicked in vivo blood plasma pH of turtles exposed to anoxia for 6 h (warm-acclimated) or 14 days (cold-acclimated) (Ultsch and Jackson, 1982; Wasser et al., 1991). A 25 - 30 min stabilization period was allowed following the switch to the acidotic saline before APs and spontaneous $fH$ were re-recorded for that preparation.

Cardiac APs were recorded using high-resistance, sharp microelectrodes (6 – 30 MΩ when filled with either 0.3 mol l$^{-1}$ or 3.0 mol l$^{-1}$ 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). 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 $fH$ 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 (Vornanen, 1997; Shiels et al., 2000). The turtle heart was accessed as described above, excised and a cannulated through the left aortic arch into the ventricle. The heart was then perfused retrograde at room temperature (21 ± 1°C) from a height of 50 cm, first with a nominally Ca²⁺-free, low Na⁺ isolation saline solution (containing in mmol l⁻¹: 100 NaCl, 10 KCl, 4 MgSO₄, 1 NaH₂PO₄, 1.2 KH₂PO₄, 50 taurine, 20 glucose and 10 HEPES, with pH adjusted to 6.9 at 21°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 O₂ for normoxia-acclimated hearts or N₂ 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, 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 6°C for up to 12 h.

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 (Vornanen, 1997; Shiels et al., 2000; Vornanen et al., 2002; Paajanen and Vornanen, 2003;
Haverinen and Vornanen, 2004; Haverinen and Vornanen, 2006; Shiels et al., 2006). 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., 21°C or 5°C) and then after an
acute exposure to the common experimental temperature of 11°C (temperature change was
accomplished within 3 – 5 min). This was done to distinguish cold-acclimation effects from
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 N$_2$.

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 3.2 ± 0.7 (N=149; mean ± S. D.) and mean cell capacitance
was 48.6 ± 9 (N=149; mean ± S. D.).
Voltage-gated \( \text{Na}^+ \) current \( (I_{\text{Na}}) \)

\( I_{\text{Na}} \) 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 \( \mu \)l 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 \( \text{K}^+ \)-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; Galli et al., 2006b) 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 (Haverinen and Vornanen, 2004; Haverinen and Vornanen, 2006). \( I_{\text{Na}} \) 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 -120mV 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 \( \mu \)s). 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 \( I_{\text{Na}} \) 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 \(\mu\)l; 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 21°C 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; Hove-Madsen and Tort, 1998), and 10 HEPES; pH was adjusted to 7.4 at 21°C 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 (Galli et al., 2006b). 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 depolarizing 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. 6.1).

**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 5°C) and ~800 ms (at 21°C) 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 (APD₀), 50% (APD₅₀), 90% (APD₉₀) and 100% (APD₁₀₀) 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 Iₙa, Iₖa, Iₖ₁ and Iₖr 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 Iₖ₁ current-voltage plot by dividing the difference in Iₖ₁ 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$10$ 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 normoxic spontaneous $f_h$.

Effect of temperature on cardiac action potentials in normoxia

Like spontaneous $f_h$, the shape and duration of the cardiac AP were extensively 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. 6.2, 6.3AC, 6.4AC, 6.5AC). 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. 6.2, 6.3AC, 6.4AC, 6.5AC; Table 6.1). Further, AP duration was prolonged at least 4-fold in all cardiac chambers compared with 21°C-acclimated hearts (Figs. 6.2, 6.3AC, 6.4AC, 6.5AC). 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. 6.2AC), 4.9-fold in the left atria (590.0 ± 50.5 to 2894.0 ± 198.5 ms; Fig. 6.3AC) and 4.2-fold in the ventricle (850.0 ± 38.5 ms to 3559.0 ± 529.5 ms; Figs. 6.2, 6.5AC). 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 indicate that temperature effects play a predominant role in contributing to the prolongation of AP duration with cold acclimation.

Acute exposure of 21°C-acclimated heart to 5°C, as well as acute exposure of 5°C-acclimated heart to 21°C (Fig. 6.6), could mimic many AP differences observed between the acclimated hearts. For instance, the AP upstroke rate was reduced with an acute 5°C exposure for all cardiac chambers (Fig. 6.6; Table 6.1) such that the AP upstroke rates for the left atria and ventricle were identical to those following acclimation to 5°C. Also, all indices of APD (APD_0, APD_{50}, APD_{90} and APD_{100}) for all cardiac chambers were not significantly different to those of 5°C-acclimated heart (Fig. 6.6ACE). 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. 6.6F). Thus, the changes in AP shape and duration associated with cold-acclimation appear to be predominantly determined by temperature effects rather than acclimation.

Nevertheless, not all changes in AP characteristics with cold acclimation could be attributed solely to a temperature effect, as some changes could not be replicated by acute temperature changes. 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 6.1). But, acute exposure of 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 in all cardiac chambers (Table 6.1). 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. 6.6BD).
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. 6.7). However, the manner through which current density is depressed with cold acclimation differed with each channel type (Figs. 6.8, 6.9, 6.10).

Consistent with the reduced AP upstroke rate at 5°C, in 5°C-acclimated ventricular myocytes, $I_{\text{Na}}$ density was significantly reduced (Figs. 6.7A) and the kinetics of sodium channel activation and inactivation slower (Fig. 6.9) compared to 21°C-acclimated ventricular myocytes. Peak $I_{\text{Na}}$ 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 $Q_{10}$ was 3.4 for the decrease in $I_{\text{Na}}$, 2.5 for sodium channel activation and 1.9 for $I_{\text{Na}}$ inactivation time (the latter were calculated from reciprocal values at -20 mV) (Figs. 6.8A, 6.9). However, when measured at the common temperature of 11°C, peak $I_{\text{Na}}$ density did not differ between 5°C-acclimated (-3.3±0.4 pA pF$^{-1}$) and 21°C-acclimated (-4.1±1.2 pA pF$^{-1}$) ventricular myocytes (Fig. 6.8A). Thus, the density of functional sodium channels on ventricular myocytes is not changed with cold acclimation, but is directly dependent on temperature.

The reduction in $I_{\text{Ca}}$ with acclimation to 5°C (Fig. 6.7B) was more profound than $I_{\text{Na}}$. Peak $I_{\text{Ca}}$ density of 5°C-acclimated myocytes (-0.43 ± 0.03 pA pF$^{-1}$) was approximately 1/13th the peak $I_{\text{Ca}}$ density of 21°C-acclimated myocytes (-5.7 ± 0.5 pA pF$^{-1}$). The $Q_{10}$ for this depression in $I_{\text{Ca}}$ with acclimation to 5°C was 5.0. When measured at the common temperature of 11°C, peak $I_{\text{Ca}}$ density of 5°C-acclimated myocytes (-1.3 ± 0.09 pA pF$^{-1}$) remained significantly lower than 21°C-acclimated myocytes (-2.2 ± 0.3 pA pF$^{-1}$) (Fig. 6.8B). Therefore, in addition to the negative effect of cold temperature and unlike $I_{\text{Na}}$, a component of the decreased $I_{\text{Ca}}$ of 5°C-acclimated myocytes involves the down-regulation of functional L-type Ca$^{2+}$ channels.
The inward $I_{K1}$ density and the inward slope conductance of inward rectifier K$^+$ channels were significantly reduced with acclimation to 5°C (Fig. 6.7C), findings consistent with the depolarized RMP and prolonged APD of 5°C-acclimated ventricular tissue. At -120 mV, inward $I_{K1}$ density of 5°C-acclimated myocytes (-4.6 ± 0.3 pA pF$^{-1}$) was 26% less than the inward $I_{K1}$ 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 the common temperature of 11°C, 5°C-acclimated ventricular myocytes displayed greater inward $I_{K1}$ density at -120 mV (-5.5 ± 0.3 pA pF$^{-1}$ compared to -3.3 ± 0.4 pA pF$^{-1}$; Fig. 6.10), 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. 6.8C) 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 is up-regulated with cold acclimation (Fig. 6.8C). In contrast to inward $I_{K1}$ density, no significant difference in outward $I_{K1}$ density (i.e., at -80 mV and -60 mV) existed between 5°C-acclimated ventricular myocytes and 21°C-acclimated ventricular myocytes (Fig. 6.7C). This finding indicates that temperature does not affect $I_{K1}$ equally at all voltages. However, like inward $I_{K1}$ and inward slope conductance, outward $I_{K1}$ 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. 6.10). This finding further supports the notion that the density of functional inward rectifier K$^+$ channels is up-regulated with acclimation to 5°C to partially compensate for the negative effect of cold temperature on $I_{K1}$.

$I_{Kr}$ was minor and no different for 21°C- and 5°C-acclimated ventricular myocytes (Fig. 6.7D), and this situation was unchanged following acute exposure to 11°C (data not shown).
Effect of prolonged anoxia on spontaneous $f_H$

Spontaneous $f_H$ of 5°C anoxic turtles (3.0 ± 0.7; $N=5$) was significantly 45% lower than the 5°C normoxic spontaneous $f_H$ of 5.5 ± 0.7 min$^{-1}$ ($N=5$). At 21°C, spontaneous $f_H$ of anoxic preparations was 27.4 ± 4.6 min$^{-1}$ ($N=5$), and although 32% lower than the 21°C normoxic spontaneous $f_H$ [35.3 ± 1.3 min$^{-1}$ ($N=5$)], there was no statistically significant difference. These findings are consistent with our other work demonstrating that prolonged anoxia at both 21°C and 5°C re-sets intrinsic $f_H$ to a rate 25% - 53% lower than during normoxia (Stecyk and Farrell, 2007).

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°C, APs for the right and left atria were not significantly modified after 6 h of anoxic exposure (Figs. 6.1A, 6.1B). In contrast, ventricular APD$_{50}$, APD$_{90}$ and APD$_{100}$ were increased significantly by 39%, 49% and to 47%, respectively (Figs. 6.2, 6.5AB). Therefore, the prolongation of the ventricular APD after prolonged anoxia at 21°C is of the same magnitude as the reduction in spontaneous $f_H$.

In contrast, AP shape or duration of all cardiac chambers remained unchanged following 14 d of anoxia at 5°C (Figs. 6.2, 6.3CD, 6.4CD, 6.5CD).

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 ± 0.9 pA pF$^{-1}$ to -16.1±1.7 pA pF$^{-1}$ with 6 h of anoxia at 21°C (Fig. 6.11A), but without affecting activation
and inactivation kinetics of \( I_{\text{Na}} \) (Fig. 6.9). No significant changes in \( I_{\text{Ca}} \), \( I_{K_1} \) or \( I_{K_F} \) density occurred as a result of 6 h of anoxia exposure at 21°C (Fig. 6.11BCD).

Similar to findings at 21°C, peak \( I_{\text{Ca}} \) density as was unaffected by 14 d of anoxia at 5°C (Fig. 6.12B). However, peak \( I_{\text{Na}} \) 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. 6.12A), unlike at 21°C. When recorded with higher (125 mM) extracellular sodium concentration to enhance \( I_{\text{Na}} \) 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 \( I_{\text{Na}} \) under reduced sarcolemmal Na\(^+\) gradient resulted in minimal \( I_{\text{Na}} \) current density at 5°C and thus an inability to detect small changes in \( I_{\text{Na}} \) occurring with prolonged anoxia exposure. At 5°C and similar to at 21°C, prolonged anoxia exposure did not affect activation and inactivation kinetics of the sodium current (Fig. 6.9). Thus, while the effect of prolonged anoxia exposure on \( I_{\text{Na}} \) density is temperature-dependent, the effect of anoxia on sodium channel activation and inactivation kinetics is temperature-independent.

The effects of prolonged anoxia on \( I_{K_1} \) were temperature-dependent. Prolonged anoxia at 5°C significantly reduced inward \( I_{K_1} \) density and inward slope conductance of inward rectifier K\(^+\) channels (Fig. 6.12C), whereas neither effect occurred with anoxia at 21°C (Fig. 6.11C). \( I_{K_1} \) density of 5°C anoxia-acclimated myocytes was significantly 33% and 18% lower than \( I_{K_1} \) 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 \( I_{K_1} \) density occurred with prolonged anoxia exposure at 5°C. \( I_{K_F} \) remained minor after prolonged anoxia at 5°C (Fig. 6.12D).
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 \( f_H \) prior to acidosis was 3.8 ± 0.7 min\(^{-1}\) (\( N = 5 \)) and 4.3 ± 0.3 min\(^{-1}\) (\( N = 5 \)) with acidosis.

In contrast, acute acidosis exposure altered the AP 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. 6.3B), but left atria and ventricular AP shape and duration were unaffected (Figs. 6.4B, 6.5B). At 5°C, APD\(_{50}\) increased by 18% in the left atria with acidosis (Fig. 6.4D), but right atria and ventricular AP shape and duration were unaffected (Figs. 6.3D, 6.5D).

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 ventricular myocyte whole-cell current densities of key sarcolemmal ion channels from turtles acclimated to 21°C and 5°C, and exposed to either normoxia or prolonged anoxia. Our findings revealed that cold temperature effects - both direct and acclimation effects - more so than prolonged anoxia exposure, resulted in substantial modifications of cardiac APs and reduction of ion current densities that contribute significantly to decreased cardiac activity. Thus, the reduction in electrophysiological activity of the turtle
heart associated with cold exposure is presumably critical in preparing cardiac muscle so that
turtles can successfully over-winter under anoxic 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. Nevertheless, we were greatly encouraged by spontaneous $f_{th}$ for
the normoxic 21°C- and 5°C-acclimated heart preparations being close to previously reported *in
vivo* and *in vitro* intrinsic rates (Yee and Jackson, 1984; Farrell et al., 1994; Hicks and Farrell,
2000b). Similarly, spontaneous $f_{th}$ for anoxic 21°C- and 5°C-acclimated heart preparations are
comparable to previous work (Stecyk and Farrell, 2007). Therefore, since APs generally reflect
$f_{th}$, 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 represented an
appropriate $f_{th}$ for the prior exposure history of the animal.

Whole-cell patch-clamp in isolated ventricular myocytes

For turtles, we have provided the first measurements of ventricular sarcolemmal $I_{Na}$, $I_{K1}$,
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*) (Galli et al., 2006b).

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 (Ruppersberg, 2000), and leads to deterioration (i.e., run-down) of currents over time (Kostyuk, 1984). 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 (Vornanen, 1997; Shiels et al., 2000; Vornanen et al., 2002; Paajanen and Vornanen, 2003; Haverinen and Vornanen, 2004; Haverinen and Vornanen, 2006; Shiels et al., 2006), but were relevant to the freshwater turtle in terms of ionic composition and pH (see Jackson and Heisler, 1982; Jackson and Ultsch, 1982; Herbert and Jackson, 1985a 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 (Wasser et al., 1990; Jackson et al., 1995; Shi et al., 1997, Stecyk et al., 2006b). Further, to minimize exogenous Ca$^{2+}$ buffering of the cytosol (Hove-Madsen and Tort, 1998), either 5 or 10 mM EGTA was included in pipette solutions. In the present study, $I_{Ca}$, a current particularly sensitive to run-down, was -5.7 ± 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. 6.1). The magnitude and degree of repeatability of $I_{Ca}$ is comparable to that recently reported for cardiac myocytes of yellow bellied turtles with the perforated-patch technique (Galli et al., 2006b), 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 Jackson and Heisler, 1982; Jackson and Ultsch, 1982; Herbert and Jackson, 1985a; Wasser et al., 1990; Jackson et al., 1995; Shi et al., 1997, Stecyk et al., 2006b). For instance, adrenergic stimulation of cardiac myocytes likely exists at a tonic level under normoxia and perhaps at an increased level under anoxia because circulating catecholamines are greatly elevated (Keiver and Hochachka, 1991; Wasser and Jackson, 1991; Keiver et al., 1992). Adrenaline increases the open probability of L-type Ca\(^{2+}\) channels (Reuter, 1983), but was not utilized during measurements of \(I_{Ca}\). Since this study was a first step in comparing electrophysiological properties of 21°C- and 5°C-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.

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 intrinsic sarcolemmal cardiac control to 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 (Fozzard and Hanck, 1996), is linked to excitation-contraction coupling of cardiac myocytes via the sarcolemmal Na\(^+\)/Ca\(^{2+}\) exchange (Bers and Despa, 2006), and allows for subsequent activation of other ion channels involved in AP generation (Kleber and Rudy, 2004). \(I_{\text{Ca}}\), the inward Ca\(^+\) current via voltage-gated L-type Ca\(^{2+}\) channels, is responsible for the plateau phase of the AP (Bers, 1991), and is the predominant source of free intracellular Ca\(^{2+}\) needed to bind to the myofilaments and trigger contraction in the turtle heart (Galli et al., 2006a; Galli et al., 2006b). \(I_{\text{K1}}\), 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 (Sanguinetti, 1992; Lopatin and Nicholls, 2001). \(I_{\text{Kr}}\), a repolarizing K\(^+\) current, gradually develops during the plateau phase of the AP, conducts outward current at more positive voltages than the \(I_{\text{K1}}\), and thus is important in balancing \(I_{\text{Ca}}\) and contributing to the plateau of the cardiac AP (Roden, 2002).

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

*In vivo,* cardiac activity of cold-acclimated freshwater turtles is substantially reduced from that of warm-acclimated turtles, with 5- to 15-fold decreases in \(f_H\) following acclimation to 5°C from 21°C-22°C driving similar reductions in \(Q_{\text{sys}}\) and \(P_{\text{O}_{\text{sys}}}\) (Hicks and Farrell, 2000a; Stecyk et al., 2004a, Stecyk et al., 2006a). Similarly, spontaneous \(f_H\) is 6- to 7-times less in *in vitro* heart preparations from 5°C-acclimated turtles compared with 21°C-acclimated turtles (Stecyk and Farrell, 2007). The present findings not only confirm these findings for spontaneous \(f_H\), but also indicate that, at least for ventricular tissue, cold temperature induced both direct and acclimation changes in the density of sarcolemmal ionic currents that serve to alter cardiac AP characteristics and, presumably, contribute to the depression of cardiac activity *in vivo.*
To summarise these differences 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. 6.2, 6.3AB, 6.4AB, 6.5AB). In the ventricle, the increased RMP and APD are consistent with the 50% reduction of $I_{K1}$ conductance (Fig. 6.7C), whereas the decreased AP upstroke rate is consistent with the 7-fold reduction in peak density of $I_{Na}$ (Fig. 6.7A). The 13-fold reduction in ventricular $I_{Ca}$ of 5°C-acclimated turtles (Fig. 6.7B) 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 (Overgaard et al., 2005).

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 dissected from those due to cold temperature acclimation. Specifically, we found that the decreased $I_{Na}$, and thus AP upstroke rate of 5°C-acclimated turtles was due predominantly to a direct temperature effect (Figs. 6.5AB, 6.8A), whereas density of functional L-type Ca$^{2+}$ channels was down-regulated as part of the cold acclimation (Fig. 6.8B). Our findings contrast with anoxia-tolerant crucian carp (Carassius carassius) in that cold acclimation in crucian carp reduced functional sodium channels (Haverinen and Vornanen, 2004), but share the cold acclimation active down-regulation of functional L-type Ca$^{2+}$ channels also shown for crucian carp (Vornanen and Paajanen, 2004). 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. 6.8C). 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 6.1).

Reduced ventricular sarcolemmal ion currents and 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 strategies to cope with the cold. Some ectotherms exhibit 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 (Graham and Farrell, 1989), increased myofibrillar ATPase activity and decreased refractoriness of the heart (Aho and Vornanen, 1999), proliferation of the sarcoplasmic reticulum (Bowler and Tirri, 1990), modulation of Ca$^{2+}$ cycling (Shiels and Farrell, 1997; Shiels et al., 2000; Shiels et al., 2002a; Shiels et al., 2002b), increased $I_{\text{Na}}$ (Haverinen and Vornanen, 2004), and alterations in $K^{+}$ conductances that shorten APD (Vornanen et al., 2002; Paajanen and Vornanen, 2004).

However, for the freshwater turtle that becomes inactive during prolonged periods of anoxia during winter months, physiological processes must be primed to conserve ATP, thus making positive compensatory changes maladaptive (Hochachka, 1986). Accordingly, these organisms reduce activity, metabolic rate and subsequently cardiac activity with cold exposure in anticipation of winter anoxic conditions (Herbert and Jackson, 1985b; Jackson, 2000). Similarly, cold-acclimated crucian carp exhibit reduced $f_R$ (Matikainen and Vornanen, 1992; Vornanen, 1994), reduced rate of cardiac contraction (Tiitu and Vornanen, 2001) a 4.4-fold reduction in maximal conductance of $I_{\text{Na}}$ (Haverinen and Vornanen, 2004) and a 6.1-fold reduction in peak $I_{\text{Ca}}$ density (Vornanen and Paajanen, 2004). Therefore, similar to the findings for crucian carp, the reduced peak density of 5°C-acclimated turtle ventricular $I_{\text{Na}}$ and $I_{\text{Ca}}$ 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 a cell (Hochachka, 1986). In principle, decreased $I_{\text{Na}}$ and $I_{\text{Ca}}$ 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 higher RMP, therefore making myocytes more readily excitability at cold temperature.

Nevertheless, investigation of direct and acclimatory temperature effects on $I_{\text{Na}}$ and $I_{\text{Ca}}$ with pulse protocols representative of physiological APs and in-depth examination of sodium and calcium channel activation/inactivation kinetics are needed to confirm these hypotheses. 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 in evidence for atrial myocytes of rainbow trout (Oncorhynchus mykiss), a cold-active species that exhibits positive thermal compensation, because the charge carried by $I_{\text{Ca}}$ while temperature dependent with square pulses becomes temperature-independent when stimulated with physiologically relevant AP pulse protocols (Shiels et al., 2000). Of course, the reductions in peak $I_{\text{Na}}$ and $I_{\text{Ca}}$ 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 and influences $I_{\text{Ca}}$ (Reuter, 1983,) could play an important role with cold acclimation in turtles the same as it does in cold rainbow trout (Shiels et al., 2003). Future studies investigating the role of adrenaline on $I_{\text{Ca}}$ 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 $I_{Ca}$ and prolonged ventricular APD at 5°C and determine if reversed NCX current maintains the long AP in the cold.

A surprising finding in the present study is the discovery that $I_{Kf}$ is not a predominant current in turtle ventricular myocytes from any experimental group (Figs. 6.7D, 6.11D, 6.12D) because $I_{Kf}$ is the predominant repolarizing current in mammalian myocytes (Roden, 2002). In fact, cold acclimation markedly increases $I_{Kf}$ in rainbow trout (Vornanen et al., 2002), and in the burbot (*Lota lota*), a cold stenothermic fish, $I_{Kf}$ is much larger than $I_{K1}$ (Shiels et al., 2006). This interspecific difference in $I_{Kf}$ does not appear to be related to the different cold survival strategies among these species because $I_{Kf}$ is present in the crucian carp and is up-regulated with cold acclimation (M. Vornanen, personal communication). Whether this phenomenon unique to turtles (or reptiles in general) remains to be determined.

Finally, the longer APD$_{90}$ and APD$_{100}$ of atrial tissue, but not ventricular tissue, of 5°C-acclimated turtles when acutely warmed (Fig. 6.6BD) 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 is reported for rainbow trout where substantial differences in $I_{K1}$ exist between atrial and ventricular myocytes (Vornanen et al., 2002). 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 they might account for the depression of $f_i$ in cold-acclimated turtles. Even the longest tissue APD$_{100}$, i.e., 3559.0 ms measured in the ventricle, would allow a $f_i$ of $\sim17$ min$^{-1}$ if no refractory period existed and clearly does not represent a major restriction on the measured spontaneous $f_i$ of $\sim5$ bpm. Since cholinergic cardiac inhibition is not involved in the depression of cardiac activity with cold acclimation (Hicks and Farrell, 2000b), 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, ventricular refractory period increases by 6-fold with cold acclimation (Tiitu and Vornanen, 2001). If refractory period of ventricular tissue increased in cold-acclimated turtles by a similar amount as the crucian carp, the intrinsic $f_H$ of approximately 4 - 5 min$^{-1}$ at 5°C (Stecyk and Farrell, 2007; present study) could be fully accounted for. Indeed, depolarization of RMP at 5°C (Figs. 6.2, 6.5AC) should theoretically result in slower recovery of voltage-gated Na$^+$ channels from inactivation and increase 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 had few and only small changes in cardiac APs. Nevertheless, the 47% increase in ventricular APD$_{100}$ with prolonged anoxia exposure at 21°C (Fig. 6.5AB) closely matches the reduction of ventricular contraction rate of ~30%. Thus, for warm-acclimated turtles, a prolongation of the cardiac cycle could account for the reduction in spontaneous cardiac contraction frequency with anoxia. At 5°C, 14 d of anoxia exposure did not affect AP characteristics of any cardiac chamber (Figs. 6.2, 6.3CD, 6.4CD, 6.5CD). Therefore, the resetting of intrinsic $f_H$ with prolonged anoxia at cold acclimation temperatures (Stecyk and Farrell, 2007; 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 5°C for normoxia- and anoxia-acclimated turtle ventricular strips (Overgaard et al., 2005). 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 $f_I$ with prolonged, cold anoxia exposure. Present findings that exposure of anoxia-acclimated heart preparations to an extracellular acidosis equivalent to the *in vivo* situation (i.e., pH of 7.55 at 21°C or pH of 7.25 at 5°C) did not result in drastic, chamber-independent alterations of APs (Fig. 6.3BD, 6.4BD, 6.5BD) excludes the prospect of acidosis as one of these possibilities.

Some changes in ventricular sarcolemmal currents were induced by prolonged anoxia exposure without affecting APs. For instance, at 21°C but not at 5°C, peak $I_{Na}$ density doubled with 6 h of anoxia exposure (Fig. 6.11A). 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 effects of increased extracellular K$^+$ concentration (Jackson and Heisler, 1982; Jackson and Ultsch, 1982; Herbert and Jackson, 1985a) on $I_{Na}$ sodium channels. 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. 6.11C, 6.12C). 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 (Golod et al., 1998). Thus, the reduced $I_{K1}$ density and conductance with 14 d of anoxia 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. A low 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. 6.5CD) 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 14 d of anoxia (Fig. 6.12B) 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 (Overgaard et al., 2005).

*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 (Hochachka, 1986). Such a strategy has been demonstrated in freshwater turtle brain and liver, where \( Na^+ \), \( K^+ \) and \( Ca^{2+} \) channel activity are all down-regulated with anoxia (Chih et al., 1989a; Pérez-Pinón et al., 1992; Buck et al., 1993; Bickler and Buck, 1998). 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 tissue for channel arrest, but has not previously been investigated.

For warm-acclimated turtles, present findings indicate that channel arrest during prolonged anoxia exposure 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. 6.11BCD). 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. 6.11A) 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 14 d of anoxia exposure at 5°C. Theoretically, since a proportion of the \( \text{Na}^+ \) 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 \( \text{Na}^+/\text{K}^+ \)-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 \( \text{K}^+ \) channels diminished by almost half with prolonged anoxia at 5°C (Fig. 6.12C). In principle, the down-regulation of \( I_{K1} \) density and conductance with prolonged anoxia could also serve to limit \( \text{K}^+ \) leakage and lower ATP demand. Inward rectifier \( \text{K}^+ \) channels allow for continuous \( \text{K}^+ \) efflux from resting cardiac myocytes and also contribute to phase-3 repolarization of the cardiac AP (Roden et al., 2002). Therefore, during both diastole and contraction, \( I_{K1} \) creates a \( \text{K}^+ \) leakage pathway across the sarcolemma and consequently places demands on the \( \text{Na}^+/\text{K}^+ \)-ATPase. Reducing this \( \text{K}^+ \) 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 \( \text{K}^+ \) channel activity and the number of \( \text{Ca}^{2+} \) channels remains unchanged in the crucian carp heart with prolonged anoxia at 4°C (Paajanen and Vornanen, 2003; Vornanen and Paajanen, 2004). However, crucian carp do not exhibit massive reductions in cardiac activity during prolonged anoxia like 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 (Stecyk et al., 2004b) and the normoxic cardiac ATP demand is likely within the glycolytic capabilities (Farrell and Stecyk, 2006). Further, unlike the turtle, no channel arrest occurs in the brain of crucian carp during anoxia exposure (Johanssson and
Nilsson, 1995; Vornanen and Paajanen, 2006). 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 (Lutz and Nilsson, 1997; Farrell and Stecyk, 2006). Briefly, in contrast to the turtle that enters a comatose-like state during anoxia, crucian carp do not become comatose (Nilsson 2001) and continue to swim, albeit at a reduced level compared to normoxia (Nilsson et al., 1993).

Concluding remarks

In summary, we compared cardiac APs from spontaneously contracting whole-heart preparations as well as $I_{Na}$, $I_{Ca}$, $I_{K1}$ and $I_{K}$, 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-conditioning the heart for winter anoxic 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 channel arrest in turtle cardiac tissue during prolonged anoxia exposure.
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


Figure Legend

**Figure 6.1** 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. 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.

**Figure 6.2** Representative recordings of ventricular action potentials from (A) 21°C normoxia- and anoxia-acclimated turtles and (B) 5°C normoxia- and anoxia-acclimated turtles.

**Figure 6.3** 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.
**Figure 6.4** 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 $\pm$ S.E.M. $N=4 - 5$ turtles.

**Figure 6.5** 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 $\pm$ S.E.M. $N=4 - 5$ turtles.
**Figure 6.6** 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.

**Figure 6.7** 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.

**Figure 6.8** 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 the 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. Values are means ± S.E.M.

**Figure 6.9** (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).

**Figure 6.10** 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).
Figure 6.11  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.

Figure 6.12  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.
Figure 6.1

A

\[ \frac{I_{Ca}}{\text{% Control}} \]

- \( 21^\circ\text{C} \) Normoxia
- \( 21^\circ\text{C} \) Anoxia-acclimated
- \( 11^\circ\text{C} \) acute exposure (21°C)

B

\[ \frac{I_{Ca}}{\text{% Control}} \]

- \( 5^\circ\text{C} \) Normoxia
- \( 5^\circ\text{C} \) Anoxia-acclimated
- \( 11^\circ\text{C} \) acute exposure (5°C)
Figure 6.2
Figure 6.3
Figure 6.4
Figure 6.5
Figure 6.6
Figure 6.7
Figure 6.8
Figure 6.9
Figure 6.10

- 11°C acute exposure (5°C)
- 11°C acute exposure (21°C)
Figure 6.11
Figure 6.12
CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

Chapter II: α-Adrenergic Cardiovascular Control

In conjunction with the large decrease in metabolic rate that occurs with prolonged anoxia exposure, and thus blood flow demand, $Q_{\text{sys}}$ of the turtle is substantially decreased up to 4.5-fold and 12.5-fold in warm- and cold-acclimated turtles, respectively (Hicks and Farrell, 2000ab). However, $P_{\text{sys}}$ only decreases marginally due to a large (3- to 5-fold) increase in systemic peripheral resistance ($R_{\text{sys}}$; Hicks and Farrell, 2000ab). In most vertebrates, $R_{\text{sys}}$ is predominantly controlled by α-adrenergic innervation of the resistance vessels, and α-adrenergic mediated peripheral vasoconstriction occurs during hypoxia in many species (Lillo, 1979; Fritsche and Nilsson, 1989; Axelsson and Fritsche, 1991; Stecyk and Farrell, 2006). However, α-adrenergic cardiovascular control during anoxia exposure has not been investigated for turtles, and the mechanism of the augmented $R_{\text{sys}}$ remains unidentified.

The increase in $R_{\text{sys}}$ with hypoxia in vertebrate species is usually accompanied by a redistribution of systemic blood flow that reflects differences in metabolic needs among tissues, with critical systems, such as the brain and the heart, receiving a high priority to prevent damage from anoxia (Nilsson et al., 1994; Yoshikawa et al., 1995; Söderström et al., 1999). Indeed, a redistribution of blood flow occurs during short-term anoxia in anaesthetized turtles (Davies, 1989; Davies, 1991; Bickler, 1992; Hylland et al., 1994; Hylland et al., 1996). However, prior to my thesis, it remained uncertain whether these findings could be applied to unanaesthetized turtles and to a prolonged period of anoxia when $Q_{\text{sys}}$ is substantially decreased.

Therefore, the objectives of chapter II were to: 1) examine the α-adrenergic regulation of $Q_{\text{sys}}$, $P_{\text{sys}}$ and $R_{\text{sys}}$; 2) identify whether there are critical tissues that receive a high priority of blood flow during prolonged anoxia when cardiovascular function is depressed; and 3) determine if there is α-adrenergic control of this blood flow redistribution. These objectives were investigated with injections of the α-adrenergic agonist phenylephrine and antagonist
phenolamine in conjunction with the microsphere technique in normoxia- and anoxia-exposed turtles acclimated to 5°C or 21°C. My working hypothesis was that increased $\alpha$-adrenergic tone accounts for the augmented $R_{sys}$ and controls blood flow distribution during prolonged anoxia exposure in the turtle at both 21°C and 5°C. Findings of chapter II are published in The Journal of Experimental Biology (Stecyk et al., 2004a).

**$\alpha$-Adrenergic control**

I found that for normoxic turtles at 21°C, there is no $\alpha$-adrenergic systemic cardiovascular control. $Q_{sys}$, $P_{sys}$ and $R_{sys}$ were unchanged following phentolamine injection. In contrast, for normoxic turtles at 5°C, which exhibited an approximate 2-fold higher $R_{sys}$ than 21°C acclimated normoxic turtles, phentolamine injection significantly reduced $R_{sys}$ to the 21°C normoxic level. This reduction was manifested as a decrease in $P_{sys}$ and augmented $Q_{sys}$ through an increase in systemic stroke volume. Thus, in normoxic turtles, systemic $\alpha$-adrenergic tone is increased with a decrease in acclimation temperature.

For anoxic turtles at 21°C, $R_{sys}$ is increased 2.3-fold from normoxia. $\alpha$-Adrenergic stimulation (i.e., phenylephrine injection) did not have any significant effects on $P_{sys}$, $Q_{sys}$ or $R_{sys}$. However, injection of the $\alpha$-adrenergic antagonist phentolamine elicited a 3-fold decrease in $R_{sys}$ and thus completely abolished the increase in $R_{sys}$ that occurred with anoxia exposure. Therefore, the increased $R_{sys}$ during anoxia at 21°C can largely be ascribed to a maximal $\alpha$-adrenergic systemic vascular tone.

For anoxic turtles at 5°C, $R_{sys}$ is 5-times that during normoxia. Phenylephrine produced a clear dose-dependent increase in $P_{sys}$ and $R_{sys}$, indicating that $\alpha$-adrenergic receptors remained functional. Phentolamine eliminated the effects of phenylephrine, but surprisingly, did not result in a decrease of $R_{sys}$ below the routine anoxia level. Thus, $\alpha$-adrenergic tone does not contribute to the marked increase in $R_{sys}$ accompanying anoxia at 5°C.
The present work can account for the large increase in $R_{sys}$ during anoxia at $21^\circ$C. It is the result of an $\alpha$-adrenergic vasoconstriction. This finding is consistent with the importance of autonomic cardiac control during anoxia at warm temperatures (Hicks and Wang, 1998; Hicks and Farrell, 2000b). The blunting of this response with cold anoxia exposure is consistent with the suppression of autonomic cardiac control during anoxia submergence at $5^\circ$C (Hicks and Farrell, 2000b). The mechanism underlying the increase in $R_{sys}$ with anoxia at $5^\circ$C remains to be determined. Possibilities include a reduction in blood vessel diameters with low blood flows and pressures (Lipowsky et al., 1978), increased blood vessel tension at cold temperature (Friedman et al., 1968; Dinnar, 1981), increased blood viscosity as temperature and flow decrease (Langille and Crisp, 1980) and/or non-adrenergic, non-cholinergic regulation of $R_{sys}$.

**Blood flow distribution**

Results from microsphere injections revealed that prolonged anoxia exposure of both $21^\circ$C- and $5^\circ$C-acclimated turtles results in a redistribution of systemic blood flow away from ancillary vascular beds towards more vital circulations. After 6 h of anoxia exposure at $21^\circ$C when $Q_{sys}$ was reduced by 2.6-fold, relative systemic blood flow distribution (%$Q_{sys}$) decreased by 6.2-fold in the stomach and 3.8-fold in the intestines, but increased significantly in the muscle (1.3-fold) and shell (1.7-fold). Absolute tissue blood flow decreased significantly to the intestines (14.4-fold), stomach (11.8-fold), kidneys (10.7-fold) and muscle (1.9-fold). After 12 d of anoxia exposure at $5^\circ$C when $Q_{sys}$ was reduced by 4.3-fold, %$Q_{sys}$ decreased significantly in the kidneys (2.7-fold) and gonads (2.2-fold) and increased significantly in the liver (1.7-fold) and shell (1.2-fold). Absolute blood flow decreased to all systemic tissues, with the largest decreases occurring in the digestive and urogenital tissues and the smallest decreases occurring in the brain, heart and liver. These findings indicate reduced importance of digestive and urogenital tissues, but increased importance of liver and muscle glycogen stores in fueling anaerobic
metabolism during anoxia. Further, they highlight the crucial role of the turtle shell in bufferering acidosis during anoxic submergence (Jackson, 2000, 2002).

Control of regional blood flow

It is well established that $\alpha$-adrenergic control mediates peripheral vasoconstriction and subsequent redistribution of blood flow among tissues during hypoxia and diving in many groups of vertebrates (Butler and Jones, 1971; Butler, 1982; Lacombe and Jones, 1991; Signore and Jones, 1995). For anoxia exposed turtles, the use of microspheres was unable to resolve many major changes in blood flow distribution between tissues following injection of $\alpha$-adrenergic agonists and antagonists. For anoxic turtles at 21°C, no significant changes occurred in relative or absolute tissue blood flows following injection of phenylephrine or phentolamine. For anoxic turtles at 5°C, phenylephrine injection significantly increased $\%Q_{sys}$ in muscle and liver, while $\%Q_{sys}$ to the shell decreased. Similarly, absolute blood flow to the liver increased following $\alpha$-adrenergic stimulation. However, only the increased liver $\%Q_{sys}$, which occurred with $\alpha$-stimulation, was restored to routine anoxic levels with phentolamine injection.

Nevertheless, major changes in blood flow distribution may not be expected after $\alpha$-adrenergic manipulation. This is because $\alpha$-adrenergic stimulation did not increase $R_{sys}$ during anoxia at 21°C and $R_{sys}$ is not $\alpha$-adrenergically mediated during anoxia at 5°C. Further, the general lack of changes in blood flow distribution may simply reflect a global response of all tissues to $\alpha$-adrenergic manipulation, with the resistances in all tissue beds changing simultaneously such that no overall redistribution occurs.
Chapter III: Adenosinergic Cardiovascular Control

A key to surviving prolonged periods of anoxia is balancing ATP demand to the reduced ATP supply available from anaerobic metabolism. A signaling molecule capable of coordinating this balance is adenosine, a ubiquitous, endogenous nucleoside. Adenosine is primarily formed intracellularly when ATP supply and ATP demand are not matched and is subsequently released into the extracellular space where it elicits, through autocrine and paracrine means, numerous physiological and metabolic effects that promote substrate availability and suppress metabolic demands (Mubagwa and Flameng, 2001; Villarreal et al., 2003). This retaliatory role of adenosine is well documented for cardiac tissues of anoxia-intolerant mammalian species. For instance, adenosine dilates the coronary artery, reduces $f_{H}$, decreases the velocity of impulse conduction in the heart and decreases the force of cardiac contractions (Wyatt et al., 1989; Belardinelli and Shryock, 1992; Mubagwa et al., 1996). In the periphery, adenosine is equally important, regulating vascular tone through relaxations and contractions, depending on vessel type and basal tone (Tabrizchi and Bedi, 2001).

Previous findings on the role of adenosine in mediating cardiovascular function in anoxia-tolerant vertebrates appear to be contradictory. In vitro studies suggest that adenosine can have negative chronotropic and inotropic cardiac effects in the hypoxia-tolerant common carp (Cyprinus carpio; Cohen et al., 1981) and anoxia-tolerant crucian carp (Vornanen and Tuomennoro, 1999) under normoxic conditions. However, results from in vivo studies show that adenosinergic cardiac control during short-term, moderate oxygen deprivation does not exist in the crucian carp (Vornanen and Turmennoro, 1999) or hypoxia-tolerant epaulette shark (Hemiscyllium ocellatum; Stensløkken et al., 2004), but may inhibit cardiac activity during prolonged severe hypoxia in the common carp (Stecyk and Farrell, 2006). Therefore, adenosinergic cardiovascular control is less clear in anoxia-tolerant vertebrates than in anoxia-intolerant mammalian species. In particular, it is unknown what role adenosinergic
cardiovascular control plays in mediating cardiovascular function during prolonged anoxia in an anoxia-tolerant vertebrate such as the freshwater turtle.

The objective of chapter III was to examine adenosinergic regulation of the turtle cardiovascular system. This was achieved through intra-arterial injection of the non-selective adenosine receptor antagonist aminophylline into 5°C- or 21°C-acclimated, normoxia- and anoxia-exposed (6 h at 21°C and 14 days at 5°C) turtles. My working hypothesis was that adenosinergic cardiovascular control inhibits cardiac activity during prolonged anoxia in the turtle. Findings of chapter III have been submitted for publication to Comparative Biochemistry and Physiology Part A, Molecular & Integrative Physiology as part of a comparative study of adenosinergic cardiovascular control in the turtle, crucian carp and common carp.

I found that adenosinergic inhibitory cardiovascular control was present in normoxic turtles at both 5°C and 21°C. Aminophylline injection transiently increased $f_H$ by 25% - 30% within 20 – 30 min of injection, before returning to the pre-aminophylline rate by the conclusion of the 60 min recording period. In contrast to my hypothesis, adenosinergic cardiovascular control was not identified in anoxic turtles. Aminophylline injection did not reverse any of the anoxia-induced changes in cardiovascular status of the turtle, namely decreased $f_H$, $Q_{sys}$ and $P_{sys}$, and increased $R_{sys}$.

Given the acknowledged retaliatory role of adenosine, results for normoxic turtles suggest that breath-hold diving behaviour of turtles may result in temporary hypoxic states and cardiac energy imbalance. The adenosinergic inhibition of $f_H$ during normoxia in both 21°C- and 5°C-acclimated turtles could be associated with such imbalances. Additionally, the tachycardia in normoxic turtles following aminophylline injection could be associated with an increased respiration frequency occurring as a result of adenosinergic blockade. Cardiorespiratory synchrony is present in normoxic turtles (Wang and Hicks, 1996), and adenosine, a neuromodulatory respiratory depressant in mammalian species (Long and Anthonisen, 1994),
and has been shown to also inhibit respiration rate of elasmobranch (Stensløkken et al. 2004) and, potentially, teleost fish (Stecyk and Farrell, 2006).

The absence of cardiovascular responses to aminophylline during prolonged anoxia exposure at 21°C and 5°C suggests that the freshwater turtle can balance cardiac ATP supply and demand during prolonged anoxia, thus precluding the formation of adenosine and an adenosinergic inhibition of cardiac activity and fulfilling one key of surviving prolonged periods of anoxia. Therefore, this finding predicts that turtle cardiac tissue ATP content is maintained at a stable level with prolonged anoxia exposure. However, since adenosinergic inhibition of $f_H$ during normoxia of 21°C- and 5°C-acclimated turtles could reflect energetic imbalances associated with breath-hold behaviour, the lack of cardiovascular responses to aminophylline with prolonged anoxia does not exclude the possibility that transient disruptions in cardiac energetic status at the onset of anoxic exposure, when large and rapid changes in cardiac activity occur (Hicks and Wang, 1998; Hicks and Farrell, 2000a; chapter II), invoke adenosinergic cardiovascular depression. Chapter IV attempted to better comprehend the relationship between cardiac energetic status and cardiac activity in the turtle during prolonged anoxia exposure through simultaneous measurements of in vivo cardiac energetic status and cardiac activity.

**Chapter IV: In vivo Myocardial Energy Status**

Chapter III suggests that the freshwater turtle is able to balance ATP supply and demand during prolonged anoxia, thus fulfilling one key of surviving prolonged periods of anoxia. Indeed, previous in vitro studies on warm-acclimated turtle hearts report that myocardial ATP content can be maintained near control levels (Wasser et al., 1990b; Jackson et al., 1995a; Wasser et al., 1997), and cardiac cellular energy state decreased at a slower rate and maintained at a higher level than for cardiac tissue of anoxia-intolerant vertebrates with severe hypoxia/anoxia exposure (Hartmund and Gesser, 1996; Overgaard and Gesser, 2004).
Nevertheless, *in vivo* extracellular conditions and cardiac performance may differ considerably from *in vitro* experiments. For cold-acclimated turtles, terminal sampling revealed a 35 – 40% decrease for cardiac ATP content after 12 weeks of anoxia (Jackson et al., 1995b). In contrast, the 20-fold reduction of $P_{O_{sys}}$ (i.e., ATP demand) and lack of adenosinergic cardiac inhibition with prolonged anoxia exposure suggest that cardiac ATP supply and demand remain balanced in cold, anoxic turtles (Hicks and Farrell, 2000a; chapter III). No previous study has directly examined *in vivo* cardiac energetic status of the turtle during prolonged anoxia exposure.

Therefore, one objective of chapter IV was to continuously measure *in vivo* cardiac energetic state during prolonged anoxia in the turtle and establish the time course and stability of the changes in cellular ATP previously reported by other methods. An additional objective of chapter IV was to examine if changes in turtle cardiac high-energy phosphate metabolism, $\text{pH}$, and/or energetic state contribute to the known large depression of cardiac activity with prolonged anoxia. For instance, increased inorganic phosphate ($P_i$) and intracellular acidosis, which have been suggested to be the primary causes of cardiac contractile failure when cellular energy is compromised in mammals (Godt and Nosek, 1989), can have negative inotropic effects on turtle cardiac tissue (Gesser and Jørgensen, 1982; Wasser et al., 1990a; Jensen and Gesser, 1999).

Further, for mammals, it has been suggested that decreased cardiac function with hypoxia is due to a reduction in the amount of free energy released from ATP hydrolysis ($dG/d\xi$), although previous findings are contradictory (Kammermeier et al., 1982; Kentish and Allen, 1986).

Repeated measurements of cardiac high-energy phosphates, intracellular $\text{pH}$ ($\text{pH}_i$), $f_H$ and aortic and pulmonary blood flows of unanaesthetized, warm- and cold-acclimated turtles during prolonged anoxia exposure were obtained by $^{31}$P-nuclear magnetic resonance (NMR) spectroscopy and magnetic resonance imaging (MRI). My working hypothesis was that the depression of turtle cardiac activity with the onset of anoxia exposure correlates with alterations in myocardial high-energy phosphate metabolism, $\text{pH}$, and/or $dG/d\xi$. With prolonged anoxia
exposure, I expected myocardial high-energy phosphate metabolism, pH, and/or dG/dξ to stabilize. Findings of chapter IV have been submitted for publication to The Journal of Physiology.

Cardiac energetic status during anoxia

With 2.85 h of anoxia exposure at 21°C, phosphocreatine (PCr) was reduced by 69%, P\textsubscript{i} increased by 140% and pH\textsubscript{i} reduced by 0.39 units from 7.40 ± 0.10 to 7.01 ± 0.04. However, in contrast to my hypothesis, these changes occurred in a linear fashion and did not stabilize over time. ATP did not change statistically and was maintained above 80% of the control normoxic level, indicating that ATP levels are maintained via the creatine kinase equilibrium.

Nevertheless, dG/dξ continuously decreased in a linear fashion by 5.8 kJ mol\textsuperscript{-1} from -56.5 ± 1.3 kJ mol\textsuperscript{-1} to -50.7 ± 0.9 kJ mol\textsuperscript{-1} with anoxia exposure. This indicates that for the first ~3 h of anoxia exposure, cardiac energetic state of 21°C turtles is not stable.

11 d of anoxia exposure at 5°C resulted in quantitatively similar changes of PCr and P\textsubscript{i}, greater reductions of ATP and pH\textsubscript{i}, and a smaller reduction of dG/dξ than 21°C-acclimated turtles. Specifically, by day 11, PCr was reduced by 63%, P\textsubscript{i} increased by 114%, ATP decreased by 50%, pH\textsubscript{i} reduced by 0.54 units from 7.66 ± 0.06 to 7.12 ± 0.04, and dG/dξ decreased by 4.0 kJ mol\textsuperscript{-1} from -52.3 ± 0.6 kJ mol\textsuperscript{-1} to -48.3 ± 0.9 kJ mol\textsuperscript{-1}. However, in contrast to the continuous, linear changes over time for PCr, P\textsubscript{i} and dG/dξ with prolonged anoxia at 21°C, patterns of change over time for PCr, P\textsubscript{i}, ATP, pH\textsubscript{i} and dG/dξ were all asymptotic with prolonged anoxia at 5°C. This finding suggests that turtle myocardial high-energy phosphate metabolism and energetic state is maintained at a new steady-state with prolonged cold anoxia exposure.
Correlation of \( f_H \) and bioenergetic status

Findings from chapter IV suggest that for 5°C-, but not 21°C-acclimated turtles, alterations in high-energy phosphate metabolism, pH, and/or dG/d\( \xi \) may play an important role in facilitating the depression of cardiac activity with prolonged anoxia. The anoxic bradycardia of 5°C-acclimated turtles closely correlated with changes in PCr, P, pH, and dG/d\( \xi \); whereas for 21°C-acclimated turtles, \( f_H \) was stable despite changing PCr and P, levels, pH, and dG/d\( \xi \). The lack of any correlation between myocardial bioenergetic components and cardiac function at 21°C is in agreement with previous findings for warm-acclimated turtles (Wasser et al., 1990b) and indicates that other mechanisms account for the anoxic bradycardia at this temperature. The significant correlations at 5°C suggest that future study is needed to further examine the inotropic and chronotropic effects of pH, P, and dG/d\( \xi \) on cold-acclimated turtle cardiac tissue.

Chapter V: Extracellular Effectors of Spontaneous Heart Rate

Turtles exhibit a large reduction in \( P_O \) with prolonged anoxia exposure. \( P_O \) is reduced by 6.6-fold and 20-fold with 6 h of anoxia exposure at 21°C and with 3 weeks of anoxia exposure at 5°C, respectively (Hicks and Farrell, 2000b). Bradycardia is the primary determinant of this reduction in \( P_O \), as it contributes to a large decrease in \( Q \). Specifically, \( f_H \) is reduced 2.5-times and 5-times at 21°C and 5°C, respectively. Therefore, control of \( f_H \) is critical to energy management in the heart during anoxia. However, the mechanisms underlying this anoxic bradycardia are not fully understood. At 21°C, cholinergic cardiac inhibition accounts for \(~36-48\%\) of the reduction in \( f_H \), but at 5°C, cholinergic control is blunted and does not contribute to the anoxic bradycardia (Hicks and Wang, 1998; Hicks and Farrell, 2000b). Results from chapters II and III revealed that chronotropic control through \( \alpha \)-adrenergic and adenosinergic mechanisms are not involved during anoxia exposure at either 21°C or 5°C. Results from chapter IV suggest that the anoxic bradycardia is not related to changes in
myocardial high-energy phosphate metabolism, pH, or dG/d\(\xi\) at 21°C. Therefore, for warm-acclimated turtles, other mechanisms must contribute to the depression of \(f_H\) during anoxia. For 5°C-acclimated turtles, alterations in myocardial high-energy phosphate metabolism, pH, or dG/d\(\xi\) may be important dictators of the depression of cardiac activity with anoxia exposure (chapter IV). However, this finding does not exclude the contribution of other mechanisms to the anoxic bradycardia.

Extracellular changes can exert chronotropic effects. In fact, those changes that accompany prolonged anoxia, namely extracellular anoxia, acidosis, hyperkalemia, hypercalcemia and increased adrenaline concentration, are likely candidates, but have not been fully investigated in the turtle. Extracellular anoxia and acidosis alone, as well as anoxia combined with acidosis have negative chronotropic effects on spontaneously contracting cardiac tissue of warm-acclimated, normoxic turtles (Reeves, 1963; Yee and Jackson, 1984; Jackson, 1987; Wasser et al., 1990ab; Wasser et al., 1992; Farrell et al., 1994; Wasser et al., 1997). Likewise, anoxia, acidosis and hyperkalemia exert negative inotropic effects (Yee and Jackson, 1984; Wasser et al., 1990ab; Farrell et al., 1994; Jackson et al., 1995a; Shi and Jackson, 1997; Shi et al., 1999; Kalinin and Gesser, 2002; Overgaard and Gesser, 2004; Overgaard et al., 2005), which can be partially alleviated by increased levels of calcium and/or adrenaline as a result of an enhanced inward Ca\(^{2+}\) gradient and opening of L-type Ca\(^{2+}\) channels (Jackson, 1987; Frace et al., 1993; Nielsen and Gesser, 2001; Overgaard et al., 2005). What remains to be discovered is the role of these extracellular changes on spontaneous \(f_H\) of cold-acclimated turtles and whether these changes can fully account for the prolonged effects of anoxia exposure \textit{in vivo} at any acclimation temperature. The latter requires knowledge and comparison of intrinsic \(f_H\) of anoxia-exposed animals.

Therefore, the objectives of chapter V were to 1) investigate the effects of extracellular anoxia, acidosis, hyperkalemia, hypercalcemia and adrenaline on spontaneous \(f_H\) of normoxia-
and anoxia-exposed turtles and 2) identify temperature acclimation affects on $f_H$ and the chronotropic responsiveness of the myocardium to extracellular changes. A unique experimental protocol exposed atria preparations that were spontaneously contracting at the intrinsic $f_H$ to a series of saline solutions that, in a step-wise manner, either mimicked in normoxia-acclimated preparations or reversed in anoxia-acclimated preparations the expected changes in turtle blood composition during prolonged anoxia at these temperatures. My working hypothesis was that for normoxia-acclimated preparations, extracellular anoxia, acidosis and hyperkalemia would decrease spontaneous $f_H$, and increased concentrations of Ca$^{2+}$ and adrenaline would stimulate $f_H$ to offset these inhibitory effects. Further, I hypothesized that for anoxia-acclimated preparations, the reversed sequence of extracellular changes would restore $f_H$ to that of normoxic preparations. Findings of chapter V have been accepted for publication in the Journal of Experimental Biology (Stecyk and Farrell, 2007).

I found that the spontaneous $f_H$ of 5°C anoxia-acclimated preparations in simulated anoxic saline was approximately half the rate of normoxic preparations in the same saline solution. Similarly, spontaneous $f_H$ of 21°C anoxia-acclimated preparations in simulated normoxic saline was 25% to 34% lower than the initial spontaneous $f_H$ of comparable normoxia-acclimated preparations in the same saline solution. The significance of these findings is that intrinsic $f_H$ is re-set to a reduced level as a result of anoxic exposure at both 21°C and 5°C. This re-setting of intrinsic $f_H$ could account for 42% - 57% (at 21°C) and 40% - 66% (at 5°C) of the anoxic bradycardia displayed in vivo in the freshwater turtle.

Further, I found that the exact nature of the chronotropic effects of extracellular factors was dependent on the acclimation temperature and the prior exposure of the turtle to anoxia. With normoxia-acclimated preparations at 21°C, combined anoxia and acidosis significantly reduced spontaneous $f_H$ by 22% and subsequent exposure to hyperkalemia further decreased $f_H$. With anoxia-acclimated preparations at 21°C, anoxia alone inhibited $f_H$ by ~30%. These
negative chronotropic effects were completely ameliorated in normoxia-acclimated preparations by increasing the adrenaline concentration from the tonic level of 1 nM to 60 nM adrenaline, whereas with anoxia-acclimated preparations, hypercalcemia and adrenaline combined to counteract the negative chronotropic effect of extracellular anoxia. With preparations from normoxia-acclimated turtles at 5°C, only the combination of anoxia, acidosis and hyperkalemia significantly reduced spontaneous $f_H$ (by 23%) and this negative chronotropic effect was fully reversed by hypercalcemia. Thus, cold acclimation has some form of pre-conditioning effect for anoxia and acidosis exposure. Spontaneous $f_H$ of anoxia-acclimated preparations at 5°C was not affected by any of the extracellular changes.

Since intrinsic $f_H$ in turtles is ultimately determined by the spontaneous firing frequency of pacemaker cells located in the sinus-venosus and also by any occurrence or change that affects the length of the cardiac cycle, future electrophysiological studies on turtle pacemaker currents and excitation-contraction coupling are needed to fully comprehend the mechanisms underlying the temperature- and anoxia-dependent differences in chronotropic responsiveness of the turtle heart to extracellular changes. Chapter VI was a first step in comparing electrophysiological properties of 21°C- and 5°C-acclimated turtles exposed to normoxia and anoxia.

**Chapter VI: Turtle Cardiac Electrophysiology**

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 intrinsic
sarclemmal cardiac control to modify cardiac performance in response to a change in whole-body blood flow demand, extracellular conditions and ambient temperature.

Indeed, the suggestion has been made that intrinsic electrophysiological changes account for the bradycardia in turtles during prolonged anoxia exposure (Hicks and Farrell, 2000b; Overgaard et al., 2005; chapter V). Certainly, an increased prevalence of atrial-ventricular blocks in isolated turtle hearts during anoxia exposure suggests a reduced ventricular excitability (Jackson, 1987). Intrinsic electrophysiological modifications also are likely involved in the profound slowing of $f_{hi}$ with cold-acclimation since cholinergic cardiac inhibition is not involved (Hicks and Farrell, 2000b) and rates of contraction and relaxation decrease (Overgaard et al., 2005). However, neither the effect of cold-acclimation or prolonged anoxia exposure on myocardial AP shape or biophysical properties of ion channels responsible for myocardial action potentials have not been directly investigated in the turtle.

As a first step to understanding the potential electrophysiological modifications of the turtle heart with cold acclimation and prolonged anoxia exposure, chapter VI investigated the effects of cold temperature acclimation and prolonged anoxia exposure on: 1) cardiac action potentials (APs); and 2) current densities of four ventricular sarclemmal ion channels, namely the voltage-gated Na+$^+ (I_{Na})$, L-type Ca$^{2+}$ (I$_{Ca}$), inward rectifier K$^+$ ($I_{K1}$) and delayed rectifier K$^+$ ($I_{Kd}$) channels, involved in generating cardiac APs (Roden et al. 2002). In addition to measuring these electrophysiological characteristics under similar conditions to those of the turtle, recordings were made following an acute temperature change to distinguish the effect of cold-acclimation from passive consequences of low ambient temperature (i.e., direct temperature effects) on the rate of these physiological processes. My hypothesis 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., Paajanen and
Vornanen, 2004; Shiels et al., 2006), I reasoned that changes in cardiac APDs would occur with the decreases in \( f_h \) associated with cold-acclimation and with prolonged anoxia exposure. Findings of chapter VI have been submitted for publication to The American Journal of Physiology: Regulatory, Integrative and Comparative Physiology.

### Modification of turtle heart electrophysiology with cold temperature

Findings from chapter VI indicate that, at least for ventricular tissue, cold temperature induced both direct and acclimation changes in the density of sarcolemmal ionic currents that serve to alter cardiac AP characteristics. The reduced ventricular sarcolemmal ion currents and 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 and likely prepare cardiac muscle for winter anoxic conditions.

Specifically, 5°C-acclimated turtle heart exhibited depolarization of resting membrane potential (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, depending on the tissue (i.e., right atria, left atria, ventricle). In the ventricle, the increased RMP and APD are consistent with a 50% reduction of \( I_{K1} \) conductance, whereas the decreased AP upstroke rate is consistent with a 7-fold reduction in peak density of \( I_{Na} \). A 13-fold reduction in ventricular \( I_{Ca} \) of 5°C-acclimated turtles 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 (Overgaard et al., 2005).

Measurements of ventricular sarcolemmal current densities after acutely switching the temperature to a common temperature of 11°C revealed that the decreased \( I_{Na} \), and thus AP upstroke rate of 5°C-acclimated turtles was due predominantly to a direct temperature effect. Density of functional L-type Ca\(^{2+}\) channels was down-regulated as part of the cold acclimation. Further, 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.

**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 had few and only small changes in cardiac APs and ventricular sarcolemmal ion currents. Nevertheless, with prolonged anoxia exposure at 21°C, peak $I_{Na}$ density doubled, and ventricular APD increased by 47%. Although the APD of pacemaker cells is considerably shorter than that of ventricular cells, it is interesting that the 47% increase in ventricular APD is proportional to the ~30% reduction of spontaneous cardiac contraction frequency in warm-acclimated, anoxic turtles.

With 14 d of anoxia exposure at 5°C, AP characteristics were unaffected in any cardiac chamber, and of the ion currents investigated, only the inward conductance via $I_{K1}$ changed significantly (reduced by 46%) with anoxia. This finding indicates that cold temperature effects - both direct and acclimation effects - more so than prolonged anoxia exposure, result in substantial modifications of cardiac APs and reduction of ion current densities that contribute to decreased cardiac activity found when turtles over-winter under anoxic conditions. However, the reduced $I_{K1}$ and unchanged $I_{Na}$ with anoxia exposure at 5°C are consistent with the channel arrest hypothesis (Hochachka, 1986). Briefly, 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 by minimizing the amount of Na$^+$ that enters the myocyte during AP upstroke. The down-regulation of $I_{K1}$ density and conductance with prolonged anoxia could also serve to reduce demands on the Na$^+$/K$^+$-ATPase by reducing the K$^+$ leakage pathway across the sarcolemma that occurs from inward rectifier K$^+$ channels during both systole and diastole (Roden et al., 2002).
To summarize, present findings may account for the reduction of *in vivo* intrinsic $f_h$ with anoxia exposure at 21°C, but the reduction in intrinsic $f_h$ with both cold exposure and cold anoxia still need mechanistic explanations. Since cholinergic cardiac inhibition is not involved in the depression of cardiac activity with either cold acclimation or cold anoxia exposure (Hicks and Farrell, 2000b), future studies are needed to determine if the bradycardia results from changes in cardiac refractoriness, intercellular electrical coupling and/or pacemaker mechanisms.

**Final Perspectives and Future Directions**

The objectives of this thesis were to: 1) investigate what extrinsic, autocrine/paracrine and intrinsic mechanisms contribute to cardiovascular control in 5°C-acclimated, anoxia-exposed freshwater turtles and account for the large depression in cardiovascular status; and 2) examine the effect of temperature acclimation in modulating control of cardiovascular function during anoxia in the freshwater turtle.

Prior to this thesis, *in vivo* studies had revealed that turtles exposed to prolonged anoxia exhibit a large reduction in $Q_{sys}$, which is primarily mediated by bradycardia, and a large increase in $R_{sys}$ (Herbert and Jackson, 1985; Hicks and Wang, 1998; Hicks and Farrell, 2000a). Autonomic cardiovascular control (i.e., cholinergic inhibition and β-adrenergic stimulation) was known to play a role in setting the level of cardiac activity in warm-acclimated, anoxia-exposed turtles. Specifically, at 22°C - 25°C, it was known that ~36-48% of the reduction in $f_h$ occurring with anoxia could be ascribed to cholinergic cardiac inhibition (Hicks and Wang, 1998; Hicks and Farrell, 2000b). However, autonomic cardiovascular control became blunted during prolonged anoxia at 5°C (Hicks and Farrell, 2000b).

Further, previous *in vitro* studies had revealed that extracellular anoxia, acidosis and hyperkalemia, either individually, or combined cause negative inotropic effects in both 21°C and 5°C normoxia-acclimated turtle cardiac tissue (Yee and Jackson, 1984; Wasser et al., 1990ab;
Farrell et al., 1994; Jackson et al., 1995a; Shi and Jackson, 1997; Shi et al., 1999; Kalinin and Gesser, 2002; Overgaard and Gesser, 2004; Overgaard et al., 2005), but that these negative effects could be partially counteracted by increased extracellular concentrations of calcium and/or adrenaline (Jackson, 1987; Nielsen and Gesser, 2001; Overgaard et al., 2005). Similarly, negative chronotropic effects of extracellular anoxia, acidosis or combined anoxia with acidosis on 21°C, normoxia-acclimated turtle hearts (Reeves, 1963; Yee and Jackson, 1984; Jackson, 1987; Wasser et al., 1990ab; Wasser et al., 1992; Farrell et al., 1994; Wasser et al., 1997), that could be (Wasser et al., 1990a) or could not be (Yee and Jackson, 1984) alleviated with increased extracellular Ca²⁺ concentration, had been reported.

Unknown were the mechanisms underlying: 1) the depression in cardiac activity during anoxia at 5°C; 2) the unaccounted portion of cardiac depression during anoxia at 21°C; and 3) the large increase in $R_{\text{sys}}$ during anoxia at both 5°C and 21°C. Further, a large information gap existed for the chronotropic effects of extracellular factors such as anoxia, acidosis, hyperkalemia, hypercalcemia and adrenaline on cold, normoxia-acclimated, as well as both 21°C and 5°C anoxia-acclimated turtle hearts.

When combined with previous findings, results from this thesis reveal that for warm-acclimated turtles, the depression in cardiac activity with prolonged anoxia exposure results from a combination of cholinergic cardiac inhibition (Hicks and Wang, 1998; Hicks and Farrell, 2000b) and a re-setting of intrinsic $f_{\text{H}}$ (chapter V), which account for ~36-48% and up to 57% of the anoxic bradycardia, respectively. Warm-acclimated turtles also increase ventricular APD with anoxia exposure (chapter VI). No evidence of α-adrenergic cardiac inhibition (chapter II) or adenosinergic cardiac inhibition (chapter III) was found in anoxic, warm-acclimated turtles, but tonic adenosinergic inhibition of $f_{\text{H}}$ existed under normoxia (chapter III). Similarly, the anoxic bradycardia did not correlate with alterations in myocardial high-energy phosphate
metabolism, pH, or dG/dξ (chapter IV). Chapter II revealed that α-adrenergic control fully accounts for the increase in $R_{sys}$ with anoxia exposure at 21°C.

Experiments in this thesis also revealed that direct effects of and acclimatory changes to cold temperature provide important pre-conditioning for the turtle heart in preparation for winter anoxic conditions. Chapter VI revealed that a direct temperature affect accounts for the reduction in intrinsic $f_H$ with cold temperature in the absence of a cholinergic cardiac inhibitory mechanism (Hicks and Farrell, 2000b). Further, ventricular $I_{Na}$, $I_{Ca}$ and $I_{K1}$ were found to be reduced in cold-acclimated turtles compared to 21°C-acclimated animals (chapter VI). The depression in $I_{Na}$ occurs as a direct effect of cold temperature, whereas the reduction in $I_{Ca}$ is due to active down-regulation of functional channel density, the mechanism for which remains to be determined (chapter VI). Interestingly, density of functional inward rectifier K⁺ channels was up-regulated with cold acclimation to partially offset the negative direct effects of cold temperature on $I_{K1}$ (chapter VI). The decreased peak densities of $I_{Na}$ and $I_{Ca}$, and the decreased inward slope conductance of $I_{K1}$, could serve to conserve ATP by reducing the cost of ion pumping. Also, cold acclimation was found to have some form of pre-conditioning effect for anoxia and acidosis exposure (chapter V). Chronotropy of 5°C-acclimated hearts was less sensitive to extracellular anoxia and acidosis compared with 21°C-acclimated turtle hearts.

When combined with previous studies, results from this thesis reveal that for 5°C-acclimated turtles, the bradycardia that accompanies prolonged anoxia exposure is not a result of cholinergic (Hicks and Farrell, 2000b), α-adrenergic (chapter II) or adenosinergic (chapter III) cardiac inhibition. Instead, it was discovered that a re-setting of intrinsic $f_H$ with prolonged anoxia exposure can account for up to 66% of the anoxic bradycardia (chapter V). However, unlike at 21°C, anoxia at 5°C did not prolong the cardiac APDs (chapter VI). Additionally, the reduction of in vivo $f_H$ with anoxia at 5°C is significantly correlated with alterations in myocardial high-energy phosphate metabolism, pH, and dG/dξ (chapter IV). Thus, changes in
key cardiac bioenergetic components may dictate the depression in $f_H$ with anoxia at cold acclimation temperatures. Finally, chapter II revealed that autonomic control of peripheral vasomotor tone does not account for the increase in $R_{sys}$ with prolonged anoxia exposure at 5°C. Thus, like the temperature-dependence of autonomic cardiac control during anoxia (Hicks and Farrell, 2000b), autonomic vascular control during anoxia has a temperature-dependency. Therefore, for 5°C-acclimated turtles, other important mechanisms must contribute to the remaining, unaccounted proportion (34%) of the anoxic bradycardia, as well as the increased $R_{sys}$ that occurs with prolonged anoxia exposure.

Logical next steps for this research would be a continuation of the electrophysiological experiments for the turtle heart presented in chapter VI. Specifically, future studies should examine if and how the refractoriness of the turtle heart, NCX current and pacemaker currents are modified with anoxia exposure. Additionally, the experiments of chapter VI should be repeated with physiologically relevant AP pulse-protocols and with extracellular solutions that more precisely mimic the extracellular changes in ionic composition, pH and adrenaline that accompany prolonged anoxia.
References


Maintained Cardiac Pumping in Anoxic Crucian Carp

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Humans, like most vertebrates, die within minutes when deprived of molecular oxygen (anoxia), in part because of cardiac failure. In contrast, freshwater turtles (genera Chrysemys and Trachemys) have the ability to survive anoxia for months at low temperatures, in part because of cardiac fail­ure and autonomic cardiovascular control (1, 2). Although the crucian carp (Carassius carassius) shares this anoxia tolerance, we show here that this fish can maintain normal cardiac performance and autonomic cardiovascular regulation for at least 5 days of anoxia at 8°C. In contrast, its cousin the carp (Cyprinus carpio) survives only 24 hours of severe hypoxia, even though cardiac activity is strongly depressed (3).

Prolonged anoxic survival requires balancing energy supply and demand, as well as coping with the acidosis associated with anaerobic end-product accumulation. Therefore, anoxic freshwater turtles reduce metabolism by 90%, precluding an up-regulation of glycolysis (4), and they buffer lactic acid accumulation with their bone and shell (5). Correspondingly, cardiac output (Q) is reduced by 92% (1), and autonomic cardiovascular control is blunted (2). Conversely, anoxic crucian carp remain active, up-regulate glycolysis to maintain their adenosine triphosphate supply, and avoid acidosis by converting lactate into ethanol (6). Thus, we hypothesized that their cardiovascular status and control might be maintained during anoxia. We examined this hypothesis by measuring Q, heart rate (fH), stroke volume (V̇S), ventral arterial blood pressure (P̅VA), and respiration rate (fR) and by calculating peripheral vascular resistance (R) and cardiac power output (PO) of crucian carp during 5 days of anoxia at 8 ± 1°C (7).

After an initial adjustment period, Q, fH V̇S, PO, and fR all returned to pre-anoxic levels throughout days 2 to 5 of anoxia, whereas P̅VA and R decreased significantly (P < 0.05) by 30% and 40%, respectively, indicating vasodilation (Fig. 1). Furthermore, autonomic controls remained intact during anoxia. Cardiac inhibitory cholinergic and excitatory β-adrenergic tones were revealed by respective increases and decreases in both fH and Q after injections of the pharmacological inhibitors of these control processes (7) (Fig. S1, A and C). Tonic α-adrenergic vasoconstriction was revealed by decreased P̅VA and R after pharmacological α-adrenergic blockade (7) (Fig. S1, B and D).

These responses point to an unusual tolerance of a vertebrate heart and autonomic nervous system to prolonged anoxia. Other anoxic hearts either fail immediately, show strongly suppressed activity (turtle and common carp) (1, 2), or lack autonomic control (hagfish) (8). The crucian carp's maintained Q, reduced R, and conserved autonomic control may permit the rapid distribution of glucose from its unusually large liver glycogen store (6) to metabolically active tissues and the transport of waste lactate to the muscle, the sole site of ethanol production (6). Sustained Q will also allow for ethanol shuttling to the gills for excretion. Using Fick's Principle, we estimate a 23% to 85% loss of ethanol from venous blood with each passage through the gills (7). Because ethanol is freely diffusible across the gill epithelia, ethanol excretion is likely perfusion-limited. Therefore, by maintaining Q during anoxia, the crucian carp may prevent ethanol accumulation and even intoxication in tissues.

References and Notes
7. Materials and methods are available as supporting material on Science Online.
9. Supported by the Natural Sciences and Engineering Research Council of Canada (J.A.W.S. and A.P.F.), the Research Council of Norway (G.E.N.), and a Company of Biologist's Traveling Fellowship (J.A.W.S.).

Supporting Online Material
www.sciencemag.org/cgi/content/full/306/5693/77/DC1
Materials and Methods
Fig. S1
5 May 2004; accepted 5 August 2004

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APPENDIX I

BREVIA
Materials and Methods

Eighteen crucian carp, collected from a pond near Oslo, Norway and with a mass of 0.27 ± 0.06 kg (mean ± S.D.) were used in this study. A Doppler flow probe positioned around the ventral aorta was used to measure cardiac output \( (Q) \), heart rate and stroke volume \( (SV) \). Ventral aortic blood pressure and respiration rate were measured through cannulae (polyethylene 50 tubing) attached to pressure transducers and inserted in the third afferent branchial artery and the buccal cavity, respectively \( (SI) \). Fish were allowed a 48 h post-operative recovery prior to anoxic exposure or pharmacological manipulation. See reference S2 for details on the procedures, which were approved by the Norwegian Animal Research Authority.

Anoxia was induced by continuously bubbling the water in the tank housing the fish with \( \text{N}_2 \). Anoxic conditions \(<0.01 \text{ mg O}_2 \text{l}^{-1}\); regularly monitored with an oxygen electrode) were reached within 3 h. Integrity of autonomic cardiovascular control was assessed through the serial intra-arterial injection \( (1 \text{ mg kg}^{-1}) \) of cholinergic, \( \beta \)-adrenergic and \( \alpha \)-adrenergic antagonists (atropine, propranolol, and phentolamine, respectively) either during normoxia or on the fifth day of anoxia. Cardiovascular status was recorded when it had stabilized 1 h after injections.

Some Doppler flow probes were calibrated at the end of the experiment with crucian carp blood (diluted 10:1) flowing through a polyethylene tubing of similar size as the ventral aorta.
of the fish. These calibrations indicated that normoxic cardiac output was $8.4 \pm 0.8 \text{ ml min}^{-1} \text{ kg}^{-1} (N=5)$.

Using an estimated anoxic $Q$ of $9.2 \text{ ml min}^{-1} \text{ kg}^{-1}$ (110% of $8.4 \text{ ml min}^{-1} \text{ kg}^{-1}$), ethanol excretion rates ($\varphi_{\text{eth}}$) of $10.5 \mu\text{mol kg}^{-1} \text{ min}^{-1}$ (4°C; S3) and $46.7 \mu\text{mol kg}^{-1} \text{ min}^{-1}$ (15°C; S4), and venous blood ethanol concentrations ($C_{\text{veth}}$) of $4.93 \text{ mM}$ (4°C; S3) and $6 \text{ mM}$ at (15°C; S5), arterio-venous ethanol differences of 23% (4°C) and 85% (15°C) were calculated using Fick's Principle, where arterial ethanol concentrations of $3.8 \text{ mM}$ (4°C) and $0.9 \text{ mM}$ (15°C) were calculated as $C_{\text{veth}} - \varphi_{\text{eth}}/Q$. 
Fig. S1  (A) Heart rate, (B) ventral aortic blood pressure, (C) cardiac output and (D) peripheral resistance 1 hour after injection of the indicated antagonists to normoxic (red) and anoxic (blue, five days) carp. Bars marked with the same letter (of the same colour) are not significantly different ($P<0.05$; repeated measures ANOVA, Friedman for percentages; Student-Newman-Keuls post-tests). Values are means ± S.E.M.; $N=10$. 
Supporting References and Notes


