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Review

Tribute to P. L. Lutz: cardiac performance and cardiovascular regulation during anoxia/hypoxia in freshwater turtles

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Summary

Freshwater turtles overwintering in ice-covered ponds in North America may be exposed to prolonged anoxia, and survive this hostile environment by metabolic depression. Here, we review their cardiovascular function and regulation, with particular emphasis on the factors limiting cardiac performance. The pronounced anoxia tolerance of the turtle heart is based on the ability to match energy consumption with the low anaerobic ATP production during anoxia. Together with a well-developed temporal and spatial energy buffering by creatine kinase, this allows for cellular energy charge to remain high during anoxia. Furthermore, the turtle heart is well adapted to handle the adverse effects of free phosphate arising when phosphocreatine stores are used. Anoxia causes tenfold reductions in heart rate and blood flows that match the metabolic depression, and blood pressure is largely maintained through increased systemic vascular resistance. Depression of the heart rate is not driven by the autonomic nervous system and seems to arise from direct effects of oxygen lack and the associated hyperkalaemia and acidosis on the cardiac pacemaker. These intra- and extracellular changes also affect cardiac contractility, and both acidosis and hyperkalaemia severely depress cardiac contractility. However, increased levels of adrenaline and calcium may, at least partially, salvage cardiac function under prolonged periods of anoxia.

Key words: reptile, overwintering, hibernation, heart rate.

The anoxia tolerant turtle

The distribution of several species of freshwater turtles extends into temperate climates of North America, where they may overwinter submerged within the mud of ice-covered ponds and lakes. This strategy is employed by a number of freshwater turtle species including the snapping turtle (*Chelydra serpentine*), the painted turtle (*Chrysemys picta*), the spotted turtle (*Clemmys guttata*), the bog turtle (*Clemmys muhlenbergii*) and, to some extent, the red-eared slider (*Trachemys scripta*). The ecology of these freshwater turtles was recently reviewed (Ultsch, 2006). Because of the very low metabolic rate, induced by low temperature and entrance to a hypometabolic state, cutaneous gas exchange can partially or fully suffice to maintain aerobic respiration when the water is normoxic (Belkin, 1968a; Ultsch, 1985; Herbert and Jackson, 1985a; Jackson et al., 2001; Jackson et al., 2004; Ultsch, 2006). However, when the overwintering turtles encounter low oxygen levels or complete anoxia in the water, anaerobic metabolism is pivotal for maintaining physiological functions. Not surprisingly, these species of freshwater turtles are endowed with extraordinary capacities for surviving the lack of oxygen. Indeed, several species of freshwater turtles can survive for months in cold anoxic water (e.g. Ultsch and Jackson, 1982; Ultsch, 1985; Ultsch et al., 1999; Jackson, 2000; Reese et al., 2002; Ultsch, 2006). Thus, together with the Crucian carp, which survives prolonged anoxia during the winter in Scandinavian lakes, freshwater turtles exhibit the highest known anoxia tolerance among vertebrates (Lutz and Nilsson, 1997).

Due to their exceptional anoxia tolerance, freshwater turtles have frequently been used as ‘August Krogh’ animals to investigate mechanisms that confer anoxia tolerance. Most of these studies have been performed on the painted turtle *C. picta* and the red-eared slider *T. scripta*. In contrast to Crucian carp, which maintains physical activity when exposed to prolonged anoxia, the turtles become lethargic and are in a near-comatose state where energy expenditure on many physiological functions is greatly reduced (Lutz and Nilsson, 1997; Jackson, 2000). Turtles reduce their metabolic rate tenfold during anoxia (Jackson and Schmidt-Nielsen, 1966; Jackson, 1968), and a similar anoxic depression of metabolism

has also been characterised in isolated hepatocytes (Buck and Hochachka, 1993). Apart from the direct savings from avoiding physical activity, anoxia is also associated with marked reductions in activities of ion-motive ATPases in the membrane and in protein synthesis (Buck and Hochachka, 1993; Hochachka et al., 1996; Jackson, 2000; Fraser et al., 2001). The heart, nevertheless, continues to pump and transport nutrients, hormones and waste products between the various organs of the body, although heart rate and cardiac work are severely reduced (Herbert and Jackson, 1985a; Hicks and Wang, 1998; Hicks and Farrell, 2000a; Stecyk et al., 2004).

For obvious reasons the nervous and cardiovascular systems have received most interest in the hypoxic turtle. Peter Lutz's research primarily contributed to our understanding of the sustained nervous function in the absence of oxygen. However, the tight correlation to continued cardiovascular function is nicely illustrated by the finding that pulmonary ventilation, reflecting continued function of the central nervous system, continues 14 times longer in anoxic turtles with an intact cardiovascular system compared with turtles where blood flow was artificially stopped (Belkin, 1968b). Here we review the mechanism that allows the turtle heart to continue to function under anoxic conditions.

Comparative hypoxia tolerance of cardiac function

The strategies to postpone or avoid the immediate threats to cellular functions inflicted by anoxia are ultimately based on the ability to balance ATP consumption against ATP production rate without losing cellular viability and function (Hochachka, 1986; Wasser, 1996; Boutilier and St-Pierre, 2000; Boutilier, 2001). While these mechanisms appear qualitatively similar among vertebrates, they differ quantitatively and temporarily, and there are large variations in the extent to which vertebrates can maintain physiological functions during and following severe oxygen lack.

The variability in the effects of anoxia on cardiac contraction

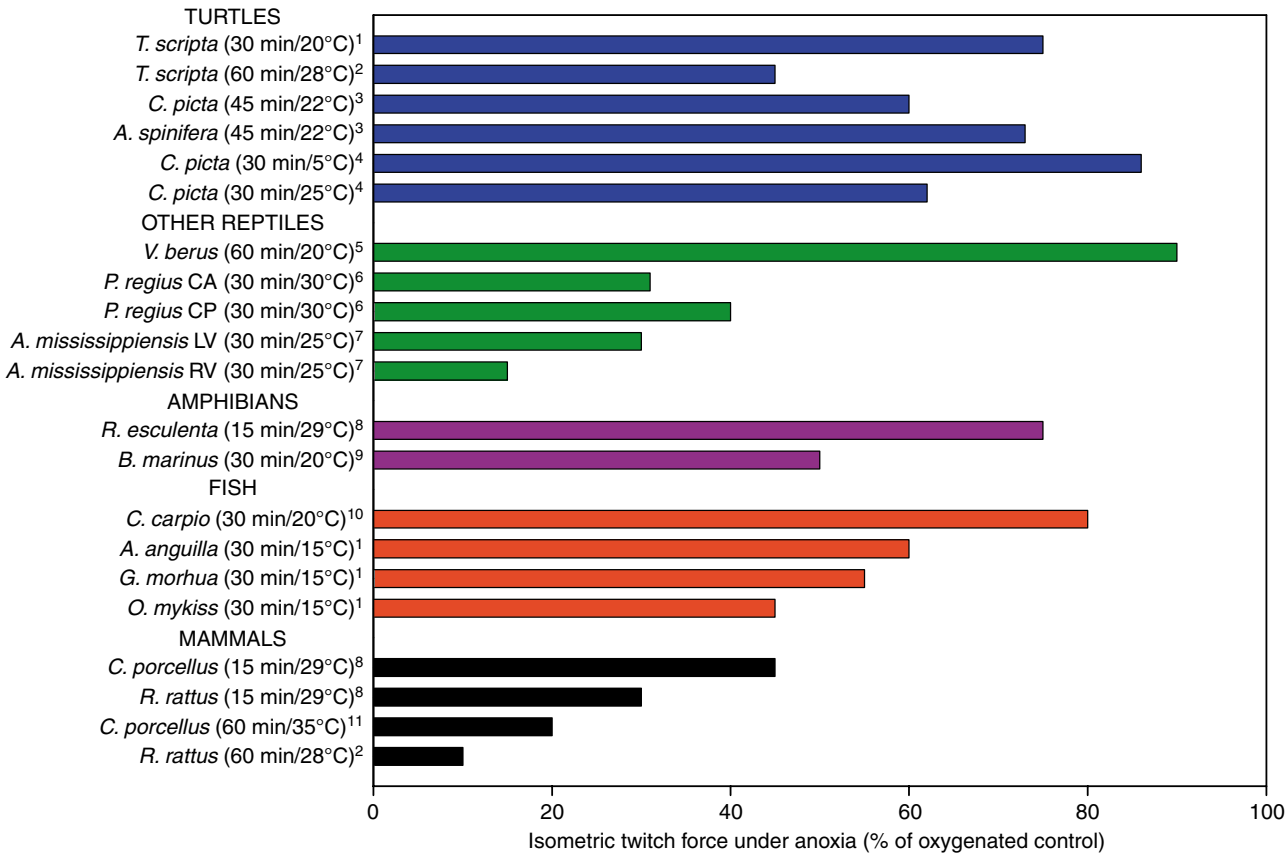

Fig. 1. Anoxic twitch force relative to twitch force under oxygenation (%) in isolated and electrically paced cardiac strips from: red-eared slider (*Trachemys scripta*); painted turtle (*Chrysemys picta*); softshelled turtle (*Apalone Spinifera*); European viper (*Vipera berus*); ball python (*Python regius*); American alligator (*Alligator mississippiensis*); edible frog (*Rana esculenta*); marine toad (*Bufo marinus*); common Carp (*Cyprinus carpio*); European eel (*Anguilla Anguilla*); Atlantic cod (*Gadus morhua*); rainbow trout (*Oncorhynchus mykiss*); guinea pig (*Cavia porcellus*); rat (*Rattus rattus*). Data are obtained from: [1](Hartmund and Gesser, 1996), [2](Bing et al., 1972), [3](Bobb and Jackson, 2005), [4](Overgaard et al., 2005), [5](Gesser and Poupa, 1978), [6](Zaar et al., 2007), [7](W.G. and T.W., unpublished), [8](Joseph et al., 2000), [9](Andersen et al., 2004), [10](Gesser, 1977), [11](McDonald and Macleod, 1971). For *Python regius*, CA is a preparation from Cavum arteriosum, and CP is a preparation from Cavum pulmonale. For *Alligator mississippiensis*, LV is left ventricle and RV is right ventricle.

is illustrated in Fig. 1, where the relative reduction in twitch force developed by isolated and electrically paced cardiac strips during anoxia is shown for various vertebrates. The turtle is among the best at preserving contractility during anoxia. However, the European viper, which hibernates at low temperature, also possesses a heart muscle with a pronounced anoxia tolerance, although it is unlikely that it experiences hypoxia during hibernation (Gesser and Poupa, 1978). In contrast, the heart muscle from tropical species, pythons and alligators, is much more sensitive (Zaar et al., 2007) (H.G. and T.W., unpublished results). It might therefore be speculated that the tolerance to oxygen lack in turtle and viper hearts represents an adaptation to hibernation at low temperature, as it does not seem to be a general feature of the reptilian heart. Similarly, the anoxia tolerance of the edible frog was higher than that of the tropical *Bufo marinus*, and within fish, the best-preserved performance was found in species known to have a high hypoxia tolerance. A comparison within reptiles also indicates that diving reptiles, where hypoxia may develop during prolonged breath-hold diving, are not necessarily endowed with a high cardiac anoxia tolerance, since the ventricle of the American alligator exhibits a low tolerance to oxygen lack (H.G. and T.W., unpublished results). Clearly, the anoxia tolerance of hearts from more reptiles with different natural histories needs to be studied and analysed within an appropriate phylogenetic context. Interestingly, in a comparison of the anoxia tolerance of more than 60 species of reptiles, assessed as the time in which ventilation persisted in an oxygen-free atmosphere, Belkin (Belkin, 1963) found that turtles were much more tolerant than other reptiles. However, because anoxia tolerance was common to both terrestrial and aquatic species, he concluded that the exceptional anoxia tolerance of turtles is of taxonomic origin rather than being based on natural history, and he also suggested that the high plasma buffer values of turtles could be of importance.

Within a single species, differences in anoxia tolerance may also exist between the two sides of the ventricle because oxygen-poor blood from the systemic veins largely enters the cavum pulmonale, while oxygenated blood returning from the lungs predominately enters the cavum arteriosum. The differences in *in vivo* oxygenation of the two parts of the ventricle should be particularly pronounced in animals with anatomical or functional separation of the ventricles, but in neither pythons nor alligators was it possible to discern differences in the hypoxia tolerance in isolated ventricular strips from the two sides of the heart (Fig. 1) (Zaar et al., 2007) (H.G. and T.W., unpublished).

Energetic and cardiac requirement during anoxia

Resting non-contracting and working preparations of turtle myocardium both depress energy turnover during hypoxia (Overgaard and Gesser, 2004). This may be due to cessation of protein synthesis during anoxia (Fraser et al., 2001), but other mechanisms may also contribute. The metabolic depression of the cardiac myocytes is accompanied by a comparable reduction of functional demand on the heart, which lowers the amount of energy required for the contractile proteins and ATPases. There is probably a cellular hierarchy so that some energy demanding processes such as protein synthesis might be downregulated to a disproportionately large extent or even put on stand-by during anoxia (Hochachka et al., 1996; Fraser et al., 2001; Boutilier, 2001; Jackson, 2002), whereas processes crucial for cell survival such as the Ca^{2+} homeostasis are excellently defended (Wasser, 1996). Fig. 2 illustrates how energy producing and energy consuming processes are affected by the transition from oxygenated to anoxic conditions. Overall, it seems that energy consumption of the anoxic turtle is depressed sufficiently to compensate for the tenfold reduction in ATP production mol^{-1} glucose that accompanies the transition from aerobic to anaerobic metabolism. Accordingly, it has been suggested that downregulation of energy consumption rather than the capacity for anaerobic metabolism explains the hypoxic tolerance of the turtle heart (e.g. Farrell et al., 1994).

In vivo cardiovascular performance in anoxic turtles

Turtles were already often being used as experimental animals for various cardiovascular experiments in the 19th century. Apart from practical aspects such as availability and ease of handling, the turtle was probably a favoured animal model because the anoxia-tolerant heart enabled prolonged experiments with little or no effort needed for appropriate ventilation of the lungs. Similar experimental approaches would have been impossible in mammals. While the pronounced anoxia tolerance of the turtle heart has long been established, the continued cardiac function during prolonged anoxia was first quantified by Penney (Penney, 1974), who documented a slow onset of bradycardia upon enforced submersion in anoxic water over 24 h at 22°C. The slow decline in heart rate differs from voluntarily diving turtles, where heart rate decreases immediately upon submersion (e.g. White and Ross, 1966; Wang and Hicks, 1996), and can most likely be ascribed to physical activity and stress. Despite the differences between voluntary and enforced diving at the onset of submersion, most studies on the effects on cardiovascular function during anoxia have been performed on forcefully submerged turtles. While the cardiovascular status manifested when the turtles have become inactive or even comatose is unlikely to differ between voluntary and forced submersion, the initial phase is undoubtedly influenced by the manner in which the experiment is performed.

There is a progressive decline of the heart rate and arterial pressure in anoxic turtles at temperatures between 3 and 20°C (Herbert and Jackson, 1985a). Heart rate declined at all temperatures, but bradycardia develops slower at low temperatures, which is consistent with the lower metabolism allowing for a longer survival time and a larger anoxia tolerance (Musacchia, 1959; Herbert and Jackson, 1985a; Herbert and Jackson, 1985b). Similar effects of temperature have been reported in subsequent studies on intact turtles

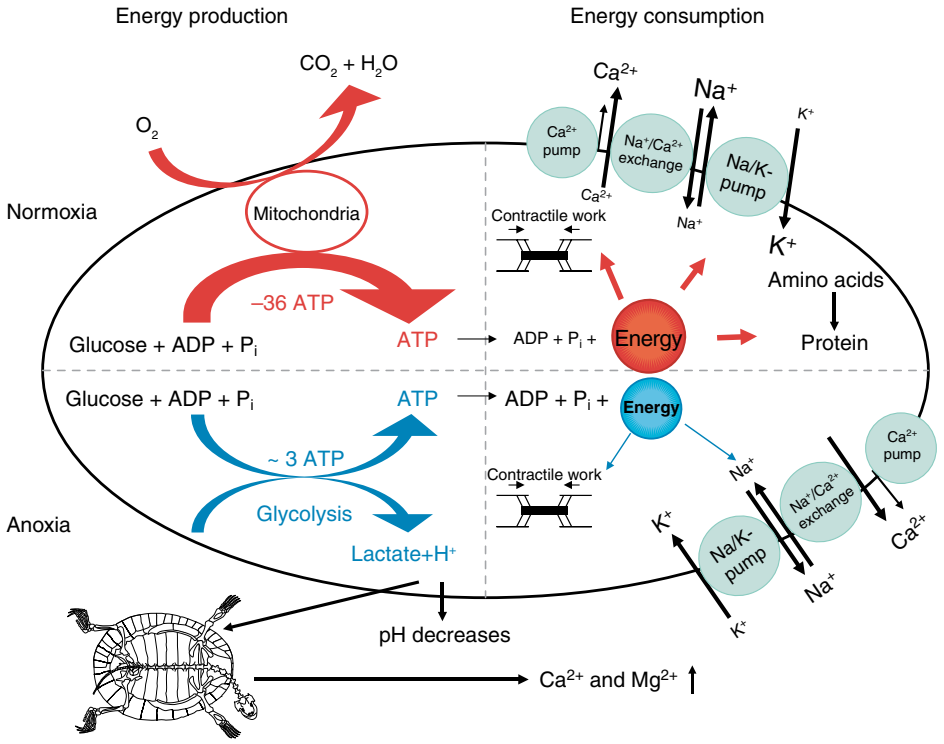

Fig. 2. Major effects of anoxia on cellular energetic turnover and on intra- and extracellular environments of a turtle cardiac cell. Top: normoxic conditions; Bottom: anoxic conditions; Left: ATP production; Right: ATP consumption. Top left: ~36 mol ATP are produced mol^{-1} glucose and waste products in the form of CO_2 and water are lost to the blood; Top right: in the normoxic cell, energy is predominantly used for protein synthesis, contractile work and ion-motive pumps such as the Na^+/K^+ ATPase and the Ca^{2+}ATPase that are both important for contractile performance and Ca^{2+} transport. Thus, the Na^+ gradient established by the Na^+/K^+ ATPase pump is used for the extrusion of Ca^{2+} through the Na^+/Ca^{2+} exchanger during relaxation. The energetic state is high with a high ATP/ADP ratio and low levels of free phosphate. Bottom left: Anaerobic respiration only produces 3 mol ATP mol^{-1} glucose, and the waste product lactic acid accumulates intra- and extracellularly causing intra- and extracellular acidification. The turtle shell buffers a considerable part of the lactic acidosis whereby calcium- and magnesium ions are released. During anoxia, energy state, i.e. the ATP/ADP ratio, decreases and the level of free phosphate increases. Anoxia is associated with a general metabolic depression due to translational arrest, spike and channel arrest. Moreover, mechanical work is reduced. Despite channel and spike arrest, long-term anoxia in turtles is associated with a progressive leak of K^+, which accumulates in the extracellular space and cause a depolarisation.

(Hicks and Farrell, 2000a; Stecyk et al., 2004), and *in situ* perfused hearts of turtles and other ectothermic vertebrates (Farrell et al., 1994; Overgaard et al., 2004). The bradycardia causes reductions in systemic blood flow as stroke volume remains relatively unchanged, and anoxia is associated with a complete cessation of pulmonary blood flow (Millen et al., 1964; Hicks and Wang, 1998; Hicks and Farrell, 2000a; Hicks and Farrell, 2000b; Stecyk et al., 2004). The constriction of the pulmonary vascular bed is not due to increased vagal activity, because atropine is without effect on pulmonary blood flow in the anoxic turtle (Hicks and Wang, 1998). Thus, it is most likely explained by hypoxic pulmonary vasoconstriction within the peripheral circulation of the lungs (Crossley et al., 1998). The benefit of this response may be to save energy otherwise used to perfuse the lungs during the anoxic period where pulmonary oxygen uptake is impossible.

Examples of heart rate as well as flow, pressure and resistance in the systemic circulation of turtles exposed to anoxia at low temperature are shown in Fig. 3. Apart from the hypoxic bradycardia and the decline in systemic blood flow, anoxia only causes a slight hypotension because systemic vascular resistance increases several-fold. This rise in resistance has been shown in several studies and occurs over a broad range of temperatures (Hicks and Farrell, 2000a; Hicks and Farrell, 2000b; Stecyk et al., 2004), and is probably to prevent systemic pressure declining below the critical closing pressure. The mechanisms underlying the systemic vasoconstriction have not been identified and cannot be explained by an increased sympathetic tone on α-adrenergic receptors, although the sympathetic tone may contribute at high temperatures (Stecyk et al., 2004). It is unlikely that the tone of endogenously produced nitric oxide is so high that cessation of NO synthesis during anoxia can explain the marked constriction, and endothelin does not appear to mediate constriction in turtles (Crossley et al., 2000; Skovgaard et al., 2005). Also, while anoxia *per se* clearly leads to constriction of systemic blood vessels from some ectothermic vertebrates (Olson et al., 2001), the systemic vasculature of anaesthetised turtles vasodilates upon short exposure to hypoxia (Crossley et al., 1998). Nevertheless, it would be of considerable interest to study the effects of anoxia on systemic resistance vessels from turtles using a myograph. It is possible that a passive lowering of the diameter of the resistance vessels in response to the lower systemic blood pressure contributes and that the systemic circulation, in that sense, behaves as a Starling resistor. This possibility could be investigated through artificial perfusion of the systemic vasculature.

Apart from the reduction of systemic blood flow, anoxia is associated with a redistribution of blood flow, which was recently described and quantified in anoxic turtles on the basis of infusions of microspheres (Stecyk et al., 2004). Blood flows to digestive and urogenital organs were reduced as previously shown in anaesthetised turtles (Davies, 1989), which is consistent with marked reduction of renal function in anoxia

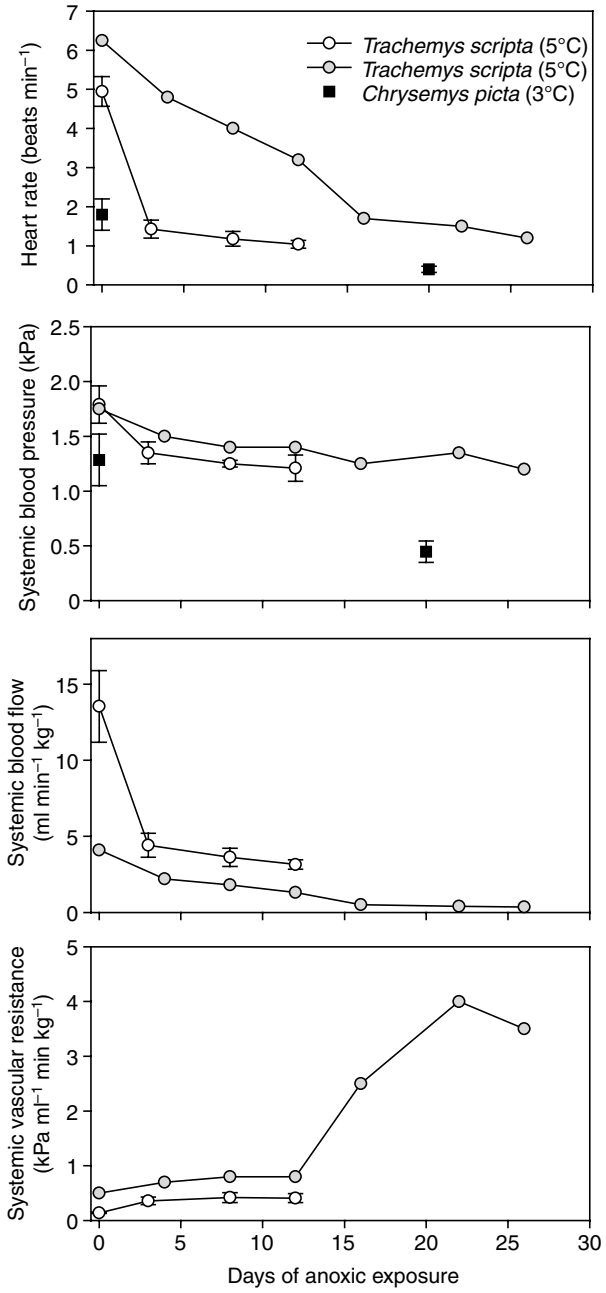

Fig. 3. Haemodynamic changes during submersion in anoxic water at 3 or 5°C in *Trachemys scripta* and *Chrysemys picta*. [Redrawn from Herbert and Jackson (Herbert and Jackson, 1985a); Hicks and Farrell (Hicks and Farrell, 2000a) and Stecyk et al. (Stecyk et al., 2004).]

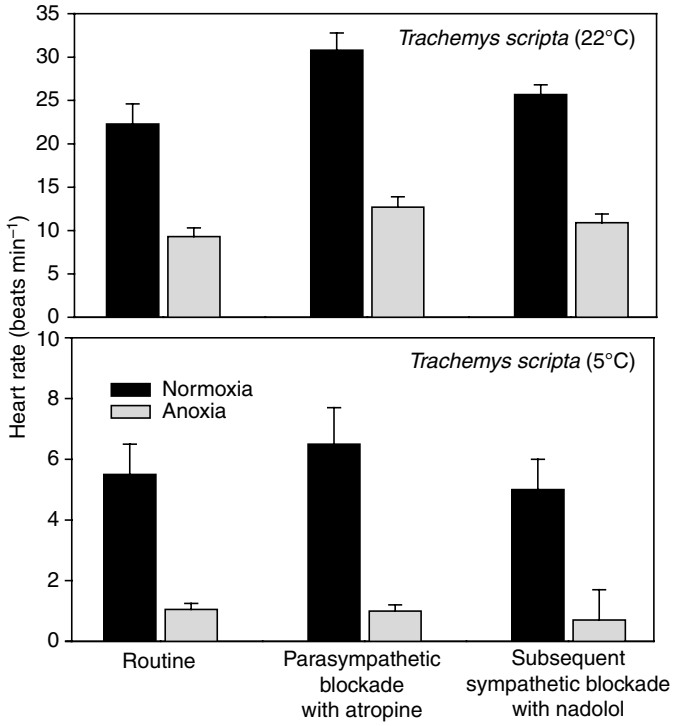

Fig. 4. Heart rates of red-eared slider *Trachemys scripta* in normoxic or anoxic water at 5 and 22°C. The anoxic turtles had been forcefully submerged for 22 days at 5°C and 6 h at 22°C. At this time, the vagal innervation of the heart was blocked by infusion of the cholinergic antagonist atropine, followed by infusion of the β-adrenergic antagonist nadolol, resulting in a complete autonomic blockade of the heart. [Redrawn from Hicks and Farrell (Hicks and Farrell, 2000b).]

(Warburton and Jackson, 1991; Jackson et al., 1996). In cold anoxic turtles the relative proportion of perfusion of the shell increased, and so did the relative perfusion of the liver. These results indicate that perfusion of the liver, which is the major glycogen store, is important for the anaerobic metabolism during anoxia (see Warren et al., 2006). Likewise, the increased perfusion of the shell fits nicely with its role in buffering the lactate acidosis by carbonates and the direct complex binding of lactate within the shell (reviewed by Jackson, 2000; Jackson, 2002) (Fig. 2). The cold anoxic turtles did not increase relative or absolute perfusion of the brain or the myocardium (Stecyk et al., 2004), which differs from previous studies on anaesthetised turtles (Davies, 1989; Davies, 1991; Bickler, 1992; Hylland et al., 1994; Hylland et al., 1996).

Turtles are endowed with both parasympathetic and sympathetic innervation of the heart, and inotropic effects of these innervations was already well established by the end of the 19th century through studies of Gaskell and others. The decline in heart rate during anoxia could, therefore, be due to altered autonomic tones (i.e. lower sympathetic and/or increased parasympathetic tones, respectively) as well as to a direct effect on the pacemaker driving cardiac contraction. The role of the autonomic nervous system has been addressed in a few studies on turtles instrumented with catheters for measurements of heart rate and infusion of appropriate receptor agonists. At high temperatures (20–25°C) the parasympathetic blocker atropine significantly increases heart rate when infused during anoxia, indicating that the increased parasympathetic tone explains part of the anoxic bradycardia (Hicks and Wang, 1998; Hicks and Farrell, 2000b) (Fig. 4). The parasympathetic tones, however, are not larger than in normoxic animals. Similarly, while inhibition of cardiac β-receptors also slows the heart in both normoxic and anoxic turtles, the anoxic bradycardia cannot be

explained by a reduced sympathetic tone. The density of ventricular β-receptors, nevertheless, is reduced during anoxia, but the marked rise in circulating levels of catecholamines may obliterate the effects of this downregulation (Wasser and Jackson, 1991; Keiver et al., 1992; Hicks and Farrell, 2000b). The autonomic modulation of the heart was even less pronounced at low temperature (Hicks and Farrell, 2000b), and the most pronounced contribution to the anoxic bradycardia, therefore, appears to be a reduction in pacemaker activity.

A direct inhibition of the pacemaker by anoxia is strongly indicated from the marked reduction in the intrinsic heart rate during anoxia (Hicks and Farrell, 2000b). As shown in Fig. 4, heart rate after the combination of parasympathetic and sympathetic blockade is markedly lower in anoxic turtles. This indicates a decline in pacemaker activity. Clearly, the mechanism underlying this reduction in pacemaker activity would be interesting to characterise. The direct inotropic effects of anoxia on the pacemaker have been revealed from recording of heart rates of isolated and *in vitro* or *in situ* perfused hearts where all nerves innervating the heart have been severed. While all such studies concur that anoxia slows heart rate, the variability of the response varies considerably among studies and ranges from having small effects (e.g. Farrell et al., 1994; Bailey and Driedzic, 1995), to reducing heart rate by more than 25% (Reeves, 1963; Wasser et al., 1990b) to reducing heart rate by approximately 75% (Wasser et al., 1997). Some of this variability may be caused by damage to the pacemaker region within the sinus venosus when the hearts are isolated and/or excised for perfusion, but it is also likely that differences in filling pressures and outflow pressures, as well as acid–base status and ionic composition of the perfusate, explain a large part of the variability. Contractions of stimulated isolated ventricular strips, for instance, became unstable and irregular during high extracellular potassium, indicating a loss of excitability (Overgaard et al., 2005). Furthermore, isolated but spontaneously contracting ventricular strips reduce the rate of contraction by approximately 50% when exposed to anoxia, but the slowing of the contractions is greatly alleviated if the Ca^{2+} concentration is increased from 1 to 10 mmol l^{-1} (Wasser et al., 1990b).

The recovery of cardiovascular function after prolonged anoxia has received much less attention. It is evident that blood flows and heart rate return to control levels shortly after the turtles are returned to normoxia, but little is known about the extent to which the heart suffers damage in connection with reperfusion injuries and formation of oxygen radicals (Wasser, 1996; Wasser et al., 1997).

Cardiac performance of the anoxic turtle heart: intra- and extracellular effects

Changes in the extracellular milieu during long term anoxia

In addition to anoxia *per se*, prolonged anoxic submergence is associated with numerous dramatic changes in the extracellular *milieu* that are likely to affect the function of most of the turtle's organs. These changes have been explored and described in great detail by Jackson and co-workers (reviewed in Jackson, 2000; Jackson, 2002). Here the origin for these changes is summarised in Fig. 2 and the progressive development of change *versus* time is shown in Fig. 5. Although metabolic depression is associated with a decreased membrane permeability and lower nervous activity (cf. Hochachka et al., 1996; Jackson, 2002), ion homeostasis is disturbed and leads to a gradual rise in extracellular potassium (Fig. 5), causing a progressive depolarisation (Jackson and Ultsch, 1982; Reese et al., 2002; Nielsen and Gesser, 2001). The continued anaerobic metabolism acidifies the extracellular compartment by more than one pH unit (Fig. 5), and the intracellular pH of most organs, including the heart, also decreases (Ultsch and Jackson, 1982; Herbert and Jackson, 1985a; Herbert and Jackson, 1985b; Wasser et al., 1990a; Ultsch, 2006). Some of the acidosis is buffered by magnesium and calcium carbonate in the shell, which causes a progressive rise in extracellular Ca^{2+} and Mg^{2+} concentrations (Figs 2, 5) (Jackson and Heisler, 1982). All in all, the extracellular disturbance during long-term anoxia includes increased concentrations of H^+, Ca^{2+}, Mg^{2+} and K^+, increased levels of circulating catecholamines, as well as reductions in cellular energy state (Figs 2 and 5).

Several studies have investigated how the many changes in the intra- and extracellular fluids affect cardiac performance during anoxia; some characterised isolated effects, while others investigated simultaneous combinations of the intra- and extracellular perturbations. These studies reveal several clear trends, but it is also obvious that some of the effects vary and

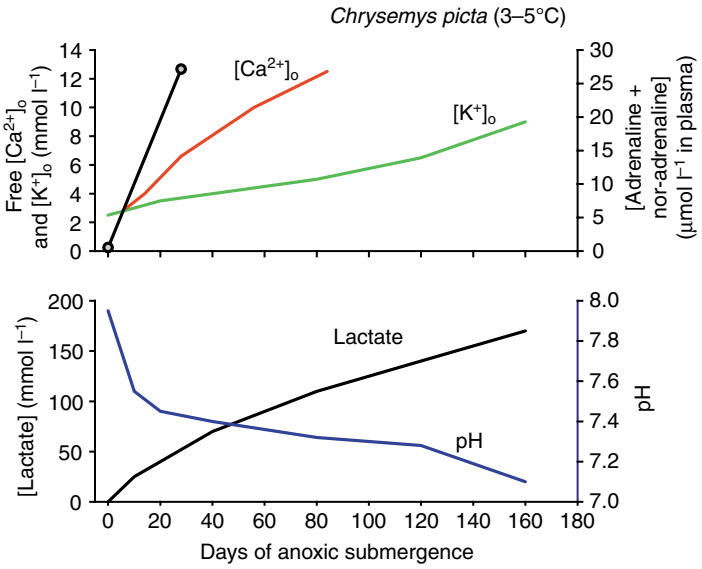

Fig. 5. Progressive changes in the extracellular environment of the cold anoxic turtle (*C. picta*) based on studies using different durations of cold anoxic exposure data. (A) $[K^+]_o$ (Jackson and Ultsch, 1982), free $[Ca^{2+}]_o$ (Jackson and Heisler, 1982) and catecholamine levels (Keiver et al., 1992) and (B) Plasma lactate and pH (Ultsch and Jackson, 1982).

depend on the conditions under which they are examined. Studies on perfused hearts have the clear advantage that the hearts generate real work and therefore mimic the *in vivo* conditions closely. However, if cardiac function is compromised severely, it is more difficult to control the extracellular conditions of the myocardium as reduced cardiac output slows replacement of the buffer to which the heart is exposed. This problem does not exist for ventricular or atrial preparations that are typically bathed in a large volume of homogeneous buffer. In most studies, however, these preparations do not perform external work, and isometric force development must therefore be measured as a proxy for the functional capacity of the heart. Isometrically contracting cardiac preparations do, nevertheless, increase ATP consumption considerably while contracting, and the energetic turnover of cardiac strips and perfused hearts may be quite similar (cf. Farrell et al., 1994; Overgaard and Gesser, 2004).

Anoxia and energy state

Although the lowered ATP production rate during anoxia causes a reduction in energy state (ΔG_{ATP}), ATP concentrations in the turtle heart are relatively well defended and only decreased by 10–20% during short anoxic exposures (Wasser et al., 1990a; Jackson et al., 1995; Hartmund and Gesser, 1996; Overgaard and Gesser, 2004). A decreased energy state is, therefore, more evident from the reduction in the PCr/Cr^2 ratio, which is a more sensitive estimate of the energy state (cf. Meyer, 1988). In comparison with both mammals and other ectothermic vertebrates, the anoxic turtle heart maintains cellular energy state very well (Wasser, 1996; Hartmund and Gesser, 1996). The red-eared slider, for example, was considerably better at maintaining cellular energy state than eel, rainbow trout or cod in electrically paced ventricular strips exposed to either 30 min or 70 min of anoxia (Hartmund and Gesser, 1996; Overgaard and Gesser, 2004). The ability to maintain a high energy state is likely to be important for the preserved anoxic performance of turtle hearts, as the relation of twitch force to cellular energy state seems to be similar between species (Hartmund and Gesser, 1996). Given the ability of the turtle heart to limit the basic cellular energy expenditure during anoxia (i.e. that not relating to contraction), it maintains contractile function at an overall low energetic cost (Overgaard and Gesser, 2004). The apparent reduction in the cost of force production is, however, primarily a consequence of a reduced basal metabolism, as studies have showed that the direct energetic cost of contraction was equal in hypoxia and normoxia (Reeves, 1963; Arthur et al., 1997).

Even though turtles perform comparatively well during anoxia, cardiac performance is still reduced by 20–50% of the normoxic level (Fig. 1). This reduction is likely due to the combination of intracellular acidosis, lowered phosphorylation potential and increased levels of free phosphate (P_i) (e.g. Allen et al., 1985; Godt and Nosek, 1989; Hartmund and Gesser, 1996). High levels of inorganic phosphates generally depressed force developed by actin–myosin bridges (Cooke and Pate, 1985) and reduced Ca^{2+} sensitivity of the myofilaments (Bers, 1991; Driedzic and Gesser, 1994; Fukuda et al., 2001), which would be accentuated by the concomitant intracellular acidification. In turtles, however, the effect of free phosphate may be relatively small (Jensen and Gesser, 1999). Furthermore, decreases in energy state in turtles only elicit a minor release of free phosphate as the phosphocreatine stores of turtle hearts are relatively small compared to those of other ectothermic vertebrates (Christensen et al., 1994). Finally, turtles show an excellent defense of the cellular Ca^{2+} homeostasis during anoxia (Wasser, 1996; Wasser and Heisler, 1997) and also have an ability to attenuate formation of rigor complexes, as seen from the very limited increase in resting tension of anoxic ventricular strips from turtle hearts where glycolysis has been blocked (Hartmund and Gesser, 1996).

Metabolic capacities during anoxia and normoxia

The regulation of cellular metabolism in freshwater turtles and other reptiles during normoxia and anoxia has been thoroughly reviewed (Storey, 1996). Myocardial energy liberation is almost exclusively mitochondrial when sufficient oxygen is available, whereas glycolysis becomes dominant during oxygen lack (Reeves, 1963; Arthur et al., 1997; Overgaard and Gesser, 2004) (Fig. 2). In accordance with this, there is a positive correlation between the cardiac anoxia tolerance in different species and their ratio of pyruvate kinase to cytochrome oxidase activities (Gesser and Poupa, 1974). In a separate study, the turtle heart had the highest PK/CO activity ratio among 13 vertebrates studied under identical experimental conditions (Christensen et al., 1994). A high ratio is not a general reptilian trait, and *Python*, for example, has a much lower ratio between pyruvate kinase and cytchrome oxidase activities (Zaar et al., 2007). The hearts of Crucian carp and hagfish, both of which are very hypoxia tolerant, also have high ratios (Christensen et al., 1994). Furthermore, turtle, hagfish and Crucian carp have high cardiac creatine kinase activities relative to that of cytochrome oxidase (Christensen et al., 1994). While the functional importance of this observation remains somewhat elusive, it may relate to the temporal buffering of ATP and the facilitated transport of ATP and ADP within the cell provided by creatine kinase (Meyer et al., 1984; Saks et al., 2001; Birkedal and Gesser, 2003). The role of creatine kinase seems to be of particular importance during long-lasting hypoxia to counteract local reduction in energy state at the ATPase sites as well as formation of rigor complexes (Veksler et al., 1997).

As suggested by studies of the enzyme complement (Christensen et al., 1994), the turtle heart has a glycolytic capacity, which is comparatively high relative to the aerobic capacity. However, the glycolytic capacity in absolute terms is not extraordinarily high and it is, furthermore, questionable if the turtle heart uses this capacity to its full extent during anoxia. As already mentioned, both cardiac work and metabolic rate are reduced by approximately one order of magnitude. As this is similar to the magnitude of the reduction in ATP production

when changing from aerobic to anaerobic metabolism (Fig. 2), it seems that freshwater turtles have no immediate need for an extraordinary glycolytic capacity to support ATP production. Even so, the proportionally high glycolytic capacity of turtles compared to other ectothermic vertebrates may reflect an excess capacity for ATP production during these adverse conditions, and it may also help to maintain a relatively high energetic state locally in the cell.

Acidosis

Acidosis can depress glycolytic activity, impair the excitation–contraction (E–C) coupling and exert negative effects directly at the contractile apparatus (Steenbergen et al., 1977; Orchard and Kentish, 1990; Bers, 1991; Bers, 2002). Thus, acidosis markedly depresses twitch force in most fish species, while the effects on many air-breathing ectotherms are less pronounced and may depend on whether the acidosis is metabolic or respiratory in origin (Driedzic and Gesser, 1994; Gesser and Poupa, 1983). As seen in Table 1 and Fig. 6, the effects of acidosis are generally small in freshwater turtles, ranging from a 35% depression to an unchanged force production in some studies. Overgaard et al. (Overgaard et al., 2005) found no effect of lactic acidosis at 25°C, while a similar reduction in pH significantly reduced twitch force at 5°C. Thus, it is possible that acidosis impairs contractility more during overwintering than at higher temperatures. More importantly, acidosis typically occurs together with anoxia during anoxic submergence. Under such conditions, the extracellular lactic acidosis will exacerbate the intracellular acidosis caused by anaerobic metabolism. Severe acidosis may partially inhibit glycolysis and thereby cause reductions in cellular energy state. Indeed, when acidosis and anoxia occur simultaneously, they depress contractility, intracellular pH and energy state in a synergistic manner so that function is typically depressed to around 25–30% of control values (Table 1) (Shi and Jackson, 1997; Shi et al., 1999; Wasser et al., 1990a; Jackson et al., 1995; Bobb and Jackson, 2005). This force depression can, however, be moderated slightly when the extracellular Ca^{2+} concentration is elevated to mimic the *in vivo* conditions (Yee and Jackson, 1984; Shi et al., 1999; Wasser et al., 1990a).

Hyperkalemia

Several recent studies show that the high extracellular concentrations of potassium occurring *in vivo* after extended periods of anoxic hibernation severely depress twitch force (Nielsen and Gesser, 2001; Kalinin and Gesser, 2002; Overgaard et al., 2005; Gesser, 2006) (Table 1 and Fig. 6). Elevated $[K^+]_o$ depolarises the membrane potential and shortens the ventricular action potential, which reduces Ca^{2+} influx and contractility (Paterson et al., 1993; Nielsen and Gesser, 2001). In turtles an elevation of $[K^+]_o$ from 2.5 to 10 mmol l^{-1} causes a 50–95% reduction of twitch force of ventricular strips (Table 1), and it also results in unstable contractions and a lowering of the maximal frequency at which regular contractions can be produced (Nielsen and Gesser, 2001; Kalinin and Gesser, 2002; Overgaard et al., 2005; Gesser, 2006).

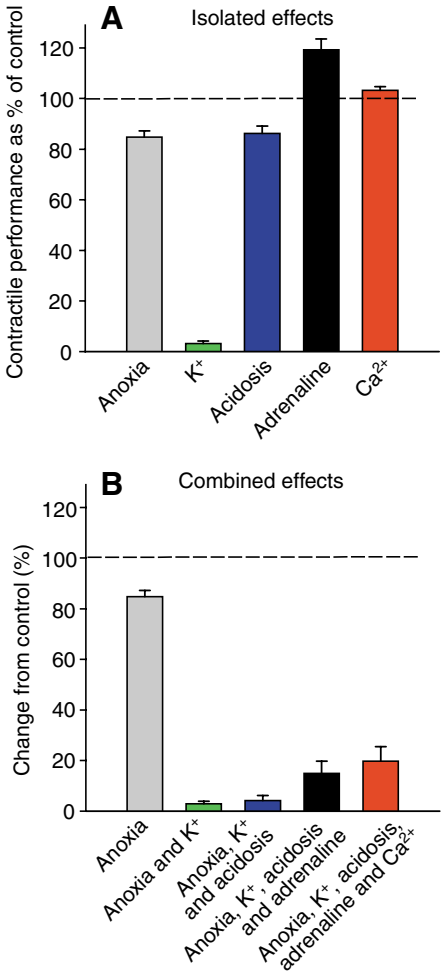

Fig. 6. Effects of anoxia, hyperkalemia, acidosis, adrenaline and hypercalcemia on cardiac performance in cold-acclimated *C. picta*. The experiments were performed at 5°C and show isometric twitch force of ventricular strips as a percentage of untreated control force. The changes in the buffer solution were applied either (A) one by one to show the isolated effects, or (B) in sequence to investigate the combined effects. The experimental changes used were as follows: anoxia; hyperkalemia (K^+ from 2.5 to 10 mmol l^{-1}); acidosis (pH from 7.9 to 6.9); adrenaline (10 μmol l^{-1}) and hypercalcemia (Ca^{2+} from 2 to 6 mmol l^{-1}). Data are from Overgaard et al. (Overgaard et al., 2005).

Thus, it seems that hyperkalemia also compromises excitability of the cardiac tissue and, as mentioned above, it is possible that this contributes to the lowering of the heart rate during anoxia (Nielsen and Gesser, 2001; Overgaard et al., 2005). The reduced excitability is most likely linked to a depolarisation and inactivation of the Na^+ channels responsible for generating the action potential (Kern et al., 1978; Volkmann, 1983). The negative inotropic effects of hyperkalemia are more pronounced at low temperature and also larger in cold-acclimated turtles than in warm-acclimated turtles, which indicates that the increases in extracellular potassium may severely limit cardiac performance during anoxia in overwintering turtles (Overgaard et al., 2005). In fact, hyperkalemia seems to be far the most potent depressor of contractile force in cold anoxic turtles, and

Table 1. *In vitro performance of cardiac preparations from painted turtle and red-eared slider during extracellular changes simulating those occurring during anoxic hibernation*

Species	Temperature	Treatment	Cardiac performance (% of control)	Cardiac preparation	Reference
Anoxia					
T. scripta	20	Anoxia (30 min)	74	Ventricular strips	1
T. scripta	20	Anoxia (20 min)	61	Ventricular strips	2
T. scripta	42	Anoxia (1 h)	43	Ventricular strips	3
T. scripta	28	Anoxia (1 h)	43	Ventricular strips	3
T. scripta	23-25	Anoxia	100	Perfused hearts	4
T. scripta	15	Anoxia	51	Perfused hearts	5
T. scripta	5	Anoxia	44	Perfused hearts	5
C. picta	25	Anoxia (30 min)	62	Ventricular strips	6*
C. picta	5	Anoxia (30 min)	86	Ventricular strips	6*
C. picta	20	Anoxia	60	Ventricular strips	7
C. picta	20	Anoxia (4 h)	57	Perfused hearts	8
C. picta	20	Anoxia	126	Perfused hearts	9
C. picta	20	Anoxia (1 h)	41	Ventricular strips	10
C. picta	10	Anoxia (1 h)	51	Ventricular strips	10
C. picta	10	Anoxia (30 min)	~50	Ventricular strips	10
Acidosis					
T. scripta	22	pH 7.9→7.0	91	Ventricular strips	11
T. scripta	20	pH 7.7→6.9	73	Ventricular strips	12
C. picta	20	pH 7.8→6.8	64	Atrial strips	13**
C. picta	20	pH 7.8→7.0	104	Perfused hearts	9
C. picta	20	pH 7.8→7.0	97	Perfused hearts	8
C. picta	20	pH 7.8→7.0	41	Ventricular strips	10
C. picta	10	pH 7.95→7.15	51	Ventricular strips	10
C. picta	20	pH 7.8→7.0	65	Ventricular strips	7
C. picta	25	pH 7.8→6.8	99	Ventricular strips	6*
C. picta	5	pH 7.9→6.9	72	Ventricular strips	6*
Hyperkalemia					
T. scripta	20	K^+ 2.5→10 mmol l^{-1}	~50	Ventricular strips	2
T. scripta	20	K^+ 2.5→10 mmol l^{-1}	28	Ventricular strips	12
T. scripta	20	K^+ 2.5→10 mmol l^{-1}	62	Ventricular strips	14
T. scripta	5	K^+ 2.5→10 mmol l^{-1}	50	Ventricular strips	14
C. picta	25	K^+ 2.5→10 mmol l^{-1}	34	Ventricular strips	6*
C. picta	5	K^+ 2.5→10 mmol l^{-1}	18	Ventricular strips	6*
Ca^{2+} and Mg^{2+}					
C. picta	25	Ca^{2+} 2→6 mmol l^{-1}	105	Ventricular strips	6*
C. picta	5	Ca^{2+} 2→6 mmol l^{-1}	101	Ventricular strips	6*
T. scripta	20	Mg^{2+} 1→10 mmol l^{-1}	86	Ventricular strips	14
T. scripta	5	Mg^{2+} 1→10 mmol l^{-1}	96	Ventricular strips	14
Adrenaline					
T. scripta	20	Saturating dose	121	Ventricular strips	2
T. scripta	20	Saturating dose	122	Ventricular strips	12
C. picta	25	Saturating dose	108	Ventricular strips	6*
C. picta	5	Saturating dose	115	Ventricular strips	6*
Combined effects under anoxia					
C. picta	20	Anoxia + pH 7.8→7.0	24	Ventricular strips	10
C. picta	10	Anoxia + pH 7.95→7.15	28	Ventricular strips	10
C. picta	20	Anoxia + pH 7.8→7.0	31	Ventricular strips	7
C. picta	20	Anoxia + pH 7.8→7.0	57	Perfused hearts	9
C. picta	20	Anoxia + pH 7.8→7.0	30	Perfused hearts	8
C. picta	20	Anoxia + pH 7.8→7.0	23	Ventricular strips	15
T. scripta	20	Adrenaline + K^+ 2.5→10 mmol l^{-1}	55	Ventricular strips	12
T. scripta	20	Anoxia + K^+2.5→10 (1 h)	14	Ventricular strips	2
T. scripta	20	Anoxia, adrenaline + K^+ 2.5→10	54	Ventricular strips	2
T. scripta	20	Anoxia + adrenaline (10 μmol l^{-1})	63	Ventricular strips	2
T. scripta	20	Anoxia, adrenaline + K^+ 2.5→10 + Mg^{2+} from 1→10 mmol l^{-1}	3	Ventricular strips	14
T. scripta	5	Anoxia, adrenaline and K^+ from 2.5→10 and Mg^{2+} 1→10 mmol l^{-1}	99	Ventricular strips	14
T. scripta	5	Anoxia, acidosis, adrenaline + K^+ 2.5→10 + Mg^{2+} 1→10 mmol l^{-1}	101	Ventricular strips	14
C. picta	25	Anoxia, K^+ 2.5→10 mmol l^{-1}, pH 7.8→6.8, adrenaline + Ca^{2+} 2→6 mmol l^{-1}	8	Ventricular strips	6*
C. picta	5	Anoxia, K^+ 2.5→10 mmol l^{-1}, pH 7.8→6.8, adrenaline + Ca^{2+} 2→6 mmol l^{-1}	30	Ventricular strips	6*
Combined effects with oxygen					
C. picta	20	pH 7.8→6.8; Ca^{2+} 2→10 mmol l^{-1}	82	Atrial strips	13**
C. picta	25	K^+ 2.5→10 mmol l^{-1}, pH 7.8→6.8, adrenaline + Ca^{2+} 2→6 mmol l^{-1}	98	Ventricular strips	6*
C. picta	5	K^+ 2.5→10 mmol l^{-1}, pH 7.8→6.8, adrenaline + Ca^{2+} 2→6 mmol l^{-1}	37	Ventricular strips	6*

*Average of three acclimation groups; **average of different types of acidosis.

References: 1 (Hartmund and Gesser, 1996); 2 (Nielsen and Gesser, 2001); 3 (Bing et al., 1972); 4 (Reeves, 1963); 5 (Farrell et al., 1994); 6 (Overgaard et al., 2005); 7 (Bobb and Jackson, 2005); 8 (Wasser et al., 1990); 9 (Jackson et al., 1995); 10 (Shi and Jackson, 1997); 11 (Poupa et al., 1978); 12 (Kalinin and Gesser, 2002); 13 (Yee and Jackson, 1984); 14 (Gesser, 2006); 15 (Shi et al., 1999).

it is possible that the progressive increase in potassium levels may ultimately compromise sustained cardiac activity in overwintering turtles. It should, however, be noted that *T. scripta* seems to be considerably less responsive to hyperkalemia than *C. picta*. Thus in *T. scripta*, the effect of hyperkalemia is smaller and can be almost completely removed by a high adrenergic tone, while *C. picta* can only recover a small part (Table 1, Fig. 6) (Nielsen and Gesser, 2001; Overgaard et al., 2005; Gesser, 2006).

Adrenaline, Ca^{2+} and Mg^{2+}

The progressive buffering of acidosis by the carapace during long-term anoxia leads to increased levels of free Ca^{2+} and Mg^{2+}. Increased Ca^{2+} levels in the extracellular fluid generally increase cardiac contractility in turtle, in which the sarcoplasmatic reticulum is known to play a minor role for cardiac calcium transport (Galli et al., 2006a,b). In contrast, elevated Mg^{2+} may decrease the inward Ca^{2+} current and thereby impair cardiac performance (Hall and Fry, 1992). Although a considerable amount of the Ca^{2+} released from the shell forms complexes with plasma lactate, the level of free Ca^{2+} also increases quickly in the cold anoxic turtle (Jackson and Heisler, 1982) (Fig. 6). Such an increase may directly improve twitch force at high temperature (20°C), while the effect was absent at low temperature (5°C) (Overgaard et al., 2005) (Table 1). Similarly, the isolated effects of increased Mg^{2+} do not exert strong inotropic effects (Gesser, 2006), and it may be that these changes are more important when they occur together with other changes in the extracellular environment associated with long-term anoxia.

Anoxia is associated with a large increase in circulating catecholamine levels (Wasser and Jackson, 1991; Keiver et al., 1992). Adrenergic stimulation increases Ca^{2+} currents through L-type Ca^{2+}-channels in the sarcolemma and thereby exerts a positive inotropic effect (Bers, 1991; Bers, 2002). Indeed, adrenergic stimulation increases the duration of the action potential in turtles, although this was only significant when the action potential had previously been compromised by hyperkalemia (Nielsen and Gesser, 2001), and adrenaline increases twitch force in ventricular strips from *Trachemys* (Ball and Hicks, 1996; Nielsen and Gesser, 2001; Overgaard et al., 2005; Galli et al., 2006a). These effects are consistent with the rise in stroke volume, which occur upon adrenergic stimulation *in vivo* (Overgaard et al., 2002; Hicks and Wang, 1998). For both adrenergic stimulation and hypercalcaemia, the potential positive effects may be more prominent in alleviating other negative inotropic agents tending to reduce sarcolemmal Ca^{2+} influx. Indeed, hypercalcemia and adrenaline, in particular, have been shown to alleviate negative inotropic effects of hyperkalemia, acidosis and anoxia (Jackson, 1987; Nielsen and Gesser, 2001; Yee and Jackson, 1982; Overgaard et al., 2005; Gesser, 2006).

Contractile performance in the cold anoxic turtle

Most previous studies on cardiac performance in turtles have characterized the isolated effects of temperature, $[K^+]_o$, $[Ca^{2+}]_o$, acidosis, adrenaline and anoxia, or some combination of two or three parameters. The negative effects of acidosis or hyperkalemia are additive to that of anoxia (Wasser et al., 1990a; Wasser et al., 1990b; Jackson, 1995; Shi and Jackson, 1997; Shi et al., 1999; Nielsen and Gesser, 2001; Overgaard et al., 2005; Gesser, 2006), while adrenergic stimulation and hypercalcemia may salvage contractility during hyperkalemia, acidosis or anoxia (Gesser and Jørgensen, 1982; Yee et al., 1984; Shi et al., 1999; Nielsen and Gesser, 2001; Overgaard et al., 2005). In a recent study, we investigated the combined and isolated effects of the major extracellular disturbances encountered by the anoxic turtle heart. The results are summarized in Fig. 6, showing that hyperkalemia, in particular, but also acidosis and anoxia, depress twitch force. When these three treatments are combined, less than 5% of twitch force remains. Addition of adrenaline and high $[Ca^{2+}]_o$ did, however, recover force to approximately 20% of control force. This observation supports the contention that increased Ca^{2+} and catecholamines ensure cardiac performance during long-term anoxic submergence (Jackson, 1987; Shi et al., 1999; Jackson, 2000). While the reductions of contractility during cold anoxia are indeed dramatic, it should be remembered that cardiac power output also decreases dramatically during anoxic submergence, so that 20% of the contractility may suffice to ensure cardiac pumping (Herbert and Jackson, 1985a; Jackson, 1987; Wasser, 1996; Hicks and Farrell, 2000a; Stecyk et al., 2004). Of all the parameters examined, it seems that hyperkalemia is most detrimental in terms of cardiac performance. During long-term anoxic submergence, $[K^+]_o$ actually increases above 10 mmol l^{-1} in painted turtles (Ultsch and Jackson, 1982; Ultsch et al., 1999), and it is possible that the progressive hyperkalemia and the ensuing depolarisation are ultimately important for determining the limit for anoxic survival.

Conclusion

Heart rate may drop below one beat a minute when turtles hibernate in cold anoxic water, but sustained cardiovascular function remains important for the transport of nutrients, hormones and waste products. Most importantly, blood flow to the liver and shell is pivotal for buffering of acidosis and to maintain the supply of glucose to the cells, while the pulmonary circulation is completely shut down. Blood pressure is largely maintained by increased vascular resistance. The bradycardia seems to be caused by direct effects of anoxia and/or hyperkalemia and acidosis on the pacemaker, while the regulation of the systemic vasoconstriction remains unknown. The turtle heart balances the reduced energy turnover during anoxia to maintain energy state. Low cardiac phosphocreatine stores may ensure that the potential increase in free phosphate level is small, and the turtle heart, furthermore, seems to be rather insensitive to increased levels of free phosphate. The turtle heart is endowed with a high glycolytic capacity as well as high levels of creatine kinase that may serve as temporal and spatial buffering of energy state. Despite these adaptations,

cardiac contractility in anoxic turtles is severely challenged by the severe acidification and increases in extracellular potassium and magnesium, which are all known to impair contractile function. It is possible that progressive hyperkalemia that develops over extended anoxic periods may ultimately limit anoxic tolerance in some individuals, as this can lead to cardiac failure. The detrimental changes in anoxia are likely, however, to be partially alleviated by the increased levels of circulating catecholamines and the large rise in free Ca^{2+} that also occurs during anoxia. Future studies should investigate how the passive changes occurring in the extracellular space during anoxia affect *in vivo* performance, and how these affect the excitation–contraction coupling of the heart.

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