THE REGULATION OF THE CARDIOVASCULAR SYSTEM OF RED-EARED SLIDERS *(Trachemys scripta)* ACCLIMATED TO EITHER 5 OR 22°C UNDER NORMOXIC OR ANOXIC CONDITIONS.

by

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ABSTRACT

Vertebrates are aerobic organisms that require oxygen to maintain normal homeostasis. Despite this, various aquatic and semi-aquatic vertebrates such as hagfish, goldfish and turtles withstand extended periods of anoxia. To sustain life during prolonged anoxia, certain physiological and biochemical adaptations are required. This thesis examines the effect of anoxia and temperature on the cardiovascular system of red-eared sliders *(Trachemys scripta).* Although oxygen transport is eliminated in anoxia, study of the cardiovascular system provides a means of accurately quantifying the metabolic depression required for anoxic survival.

Anoxia and low temperature are known to depress heart rate and blood pressure. *In vivo* systemic cardiac power output (PO_{sys}) and cardiac output (Q_{sys}) were examined for the first time in turtles acclimated to either 22 or 5°C under normoxic and anoxic conditions. PO_{sys} was 15-fold lower (from 0.81 to 0.053 mW g^{-1}) with 22- versus 5°C- acclimation. Anoxic exposure for 6 hours at 22°C resulted in a 7.4-fold drop in PO_{sys} from 0.81 to 0.11 mW g^{-1}. A comparison of turtles acclimated to 22°C normoxia with those acclimated to 5°C anoxia for five weeks, showed a 312-fold decrease in PO_{sys} (from 0.81 to 0.0026 mW g^{-1}). Therefore, acclimation to cold anoxia caused a 2.8-fold depression of PO_{sys} beyond that expected from the product of cold acclimation and short-term anoxia alone.

Bradycardia was the primary effector in the decline in cardiac performance as heart rate decreased by 25-fold (from 25 to 1 beats min^{-1}), whereas stroke volume fell by only 5-

fold (from 1.33 to 0.27 ml kg^{-1}). Q_{sys} declined 8-fold from 32 at 22°C under normoxia to 4.1 ml min^{-1} kg^{-1} with 5°C-acclimation, 4-fold to 7.6 ml min^{-1} kg^{-1} with acute anoxic exposure at 22°C, and 119-fold to 0.27 ml min^{-1} kg^{-1} with anoxic acclimation at 5°C. Despite these large changes in Q_{sys}, systemic blood pressure deceased by only 1.6-fold with anoxia at each temperature because systemic resistance increased by 2.6-fold at 22°C, and by 11-fold at 5°C under anoxia. These results suggest that heart rate and vascular tone are the major effectors in establishing cardiovascular status during anoxia.

Our working hypothesis proposed that in cold anoxic turtles (a) an increased cholinergic tone would produce the massive decrease in heart rate and (b) an increased adrenergic vasomotor control would elevate systemic resistance. However, *in vivo* injections with the cholinergic antagonist atropine revealed that cholinergic cardiac control was greatly suppressed in 5°C- compared with 22°C-acclimated turtles. This suggests that intrinsic control of heart rate was probably more important under cold anoxic conditions in effecting bradycardia. Also in contrast to the hypothesis, experiments with the adrenergic agonist adrenaline and antagonist nadolol, revealed that the β-adrenergic cardiac and vasomotor controls were blunted by anoxia independent of temperature. Further study into a-adrenergic control of vasomotor tone in cold anoxia is needed to provide greater insight into cardiovascular control under these conditions.

To further investigate anoxic blunting of adrenergic cardiac control, the density of ß-adrenoreceptors was determined in the ventricles of turtles acclimated to 5 and 22°C under normoxia and anoxia. Anoxic exposure significantly reduced ß-adrenoreceptor density by

40% at 22°C and by 33% at 5°C. A portion of the anoxic loss of cardiac inotropy therefore, can be attributed to the reduction in ß-adrenoreceptor density in the ventricles of these turtles.

In conclusion, this study quantified and qualified the profound cardiovascular depression associated with cold anoxic exposure, emphasizing the importance of bradycardia and vasomotor tone and the depression of the normal control mechanisms (vagal chrontrophic and β-adrenergic tone) found in normoxia.

ACKNOWLEDGMENTS

I would first like to acknowledge the guidance and support of my supervisor, Dr. Tony Farrell, throughout the course of this thesis. I am still, as ever, fascinated by the physiological adaptations that underlie the remarkable tolerance of turtles. This is due, in large part, to the interest Tony brought to this project. For their critical review of this thesis and for helpful suggestions along the way I would like to thank my committee members Dr. Glenn Tibbits and Dr. Bill Milsom. Thank-you to my fellow lab mates Holly Shiels, Kurt Gamperl, Kamini Jain and Bill Bennett for their help, creative solutions and for making the Farrell lab truly feral. I would also like to thank my parents for their interest in how I was dealing with Graduate student life and for always being supportive. Finally I would like to thank my wife, Barbara Campbell, not only for marrying me in my crazed thesis writing state but for all her love and support which has made this thesis possible.

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CHAPTER 1

General Introduction and Literature Review

Responses to Acute and Chronic Environmental Stressors

Over evolutionary time the physiology of an organism is shaped by the environment in which it lives. Drastic changes in the Earth's environment are mirrored in the life which inhabit it. As populations of animals proliferate, plateau and disappear it becomes increasingly clear that the degree to which an animal can adapt will determine how successful it will be. Natural selection drives the development of diverse adaptations to the myriad of environments present on Earth. Useful traits are favored and organisms that possess useful traits are more likely to pass them onto the next generation.

Life has been found at great ocean depths, extreme temperatures, and reduced oxygen levels, yet no one organism inhabits all of these environments. A kangaroo rat, *Dipodomys ingens,* though wonderfully adapted to living in an arid desert, would be unsuccessful in the arctic tundra. The physiology of an organism is adapted to a particular subset of the conditions present on Earth and it is this subset which constrains geographic distribution. Within this subset of conditions an organism's regulatory mechanism can maintain its internal environment, but at environmental extremes this becomes increasingly difficult. This relationship is illustrated in Figure 1.1a where

conditions outside the range of tolerance have direct consequences on the survivorship of the organism.

Animals are exposed to a package of environmental factors. One factor often impacts upon another making survival increasingly difficult. Sweating is an effective means of thermoregulation, but only if you are not experiencing water stress. However, interactions of environmental factors can also be beneficial. Many aquatic organisms have exploited the depression of metabolic rate that occurs at low temperatures to greatly extend their tolerance of anoxia. It is this complex interplay which makes it difficult to extrapolate the responses seen in the laboratory to ones seen in nature.

Response to environmental change occurs in two different forms, behavioral and physiological, which have different time scales. Behavioral responses can be rapidly initiated and easily reversed. An animal which finds itself under stress from its environment can move away to a more suitable one. Migratory birds travel vast distances to avoid winter. Cooperative social behavior is also used by some animals. Honeybees may beat their wings to cool the hive if it becomes too hot. These behavioral responses however, have limited scope and physiological adaptations are needed to tolerate to environments that animals cannot escape or alter.

Physiological responses require time to develop and often require a similar amount of time to be reversed. On warm days the vasodilatation of skin blood vessels to dissipate heat can occur quickly while alteration of regulatory enzyme levels can take as long as a month to occur (Hicks *et al.*, 1996). Acute or rapid environmental change often leaves little time for physiological responses to occur resulting in a poor performance by

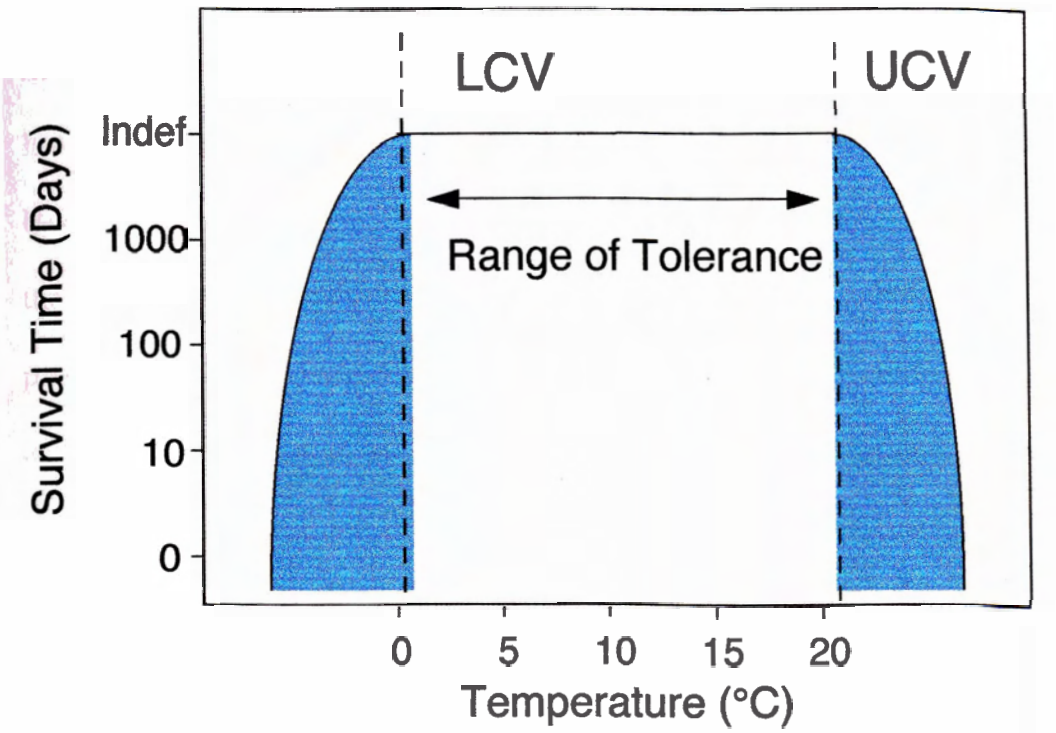
the animal. Circumstances where chronic environmental change is predictable or slow in its onset maximizes beneficial physiological modifications. Figure 1.1b illustrates the effects of chronic and acute environmental change on maximum swimming performance in goldfish.

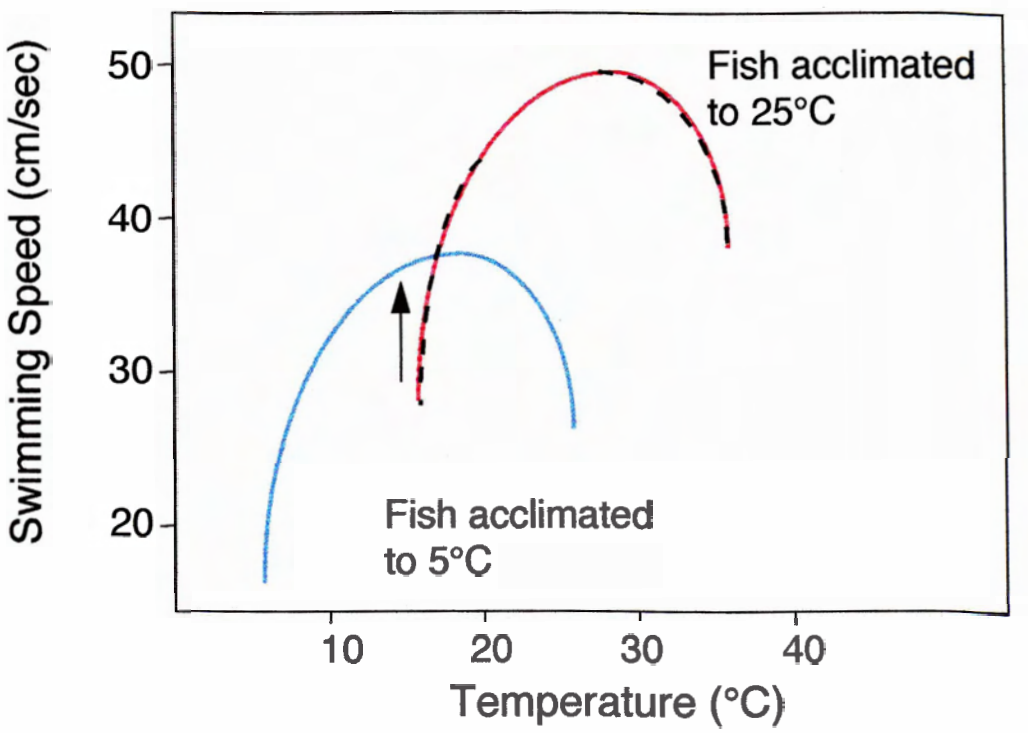
Vertebrates are aerobic organisms that require oxygen to maintain normal homeostasis. Despite this, many are often exposed to wide variations of oxygen availability for extended periods of time through environmental or behavioral changes (Storey, 1996). Life history and degree of exposure are often good indicators of the degree of physiological modification that an organism will go through in response to anoxia. If hypoxic exposure is a daily event, then physiological modifications will be present throughout the life of the organism. With seasonal exposure, environmental cues like photoperiod or temperature will initiate the expression of elaborate physiological changes. For example, turtles over-wintering in the sediments of ponds will experience seasonal anoxia which must be supported by extensive physiological changes. Likewise, Atlantic hagfish *(Myxine glutinosa)* are frequently exposed to hypoxia when they burrow into soft mud or feed inside the body cavity of dead animals.

Temperature is an important modulator of anoxic survival. At 5°C hagfish survive anoxic exposure for periods in excess of 20 hours (Hansen and Sidell, 1983). At elevated temperatures goldfish (*Crassius auratus*) withstand anoxia for a few hours while at 4°C survival is extended to a week (Walker and Johansen, 1977). Freshwater turtles are an extreme example of anoxia tolerance amongst vertebrates tolerating anoxia for periods in excess of four months at 3°C (Ultsch and Jackson, 1982). However at 22°C

Figure 1.1 Physiological responses to environmental variables. (a) The range of any specific variable within which an animal can survive indefinitely (i.e. life span) is the range of tolerance. Beyond the upper (UCV) and lower critical value (LCV) is the range of resistance where the survivorship of the animal is compromised; shown here in blue. Animals have the ability to shift their range of tolerance if stressful conditions persist as shown in figure (b).

(b) Physiological functions have optimal temperatures outside of which performance declines. This graph of swimming speed versus temperature presents data from two different populations of goldfish acclimated to 25 and 5°C. The dotted line indicates acute temperature changes for the fish acclimated to 25°C while the arrow draws your attention to the improvement in performance that is seen at 15°C upon acclimation (chronic effect) to 5°C (modified from Campbell *et al.*, 1993).


LCV
UCV
Indef
1000
100
10
0
Survival Time (Days)
Range of Tolerance
0
5
10
15
20
Temperature (°C)


50
40
30
20
Swimming Speed (cm/sec)
Fish acclimated
to 25°C
Fish acclimated
to 5°C
10
20
30
40
Temperature (°C)

this survival time is limited to a day (Ultsch, 1989).

The closed circulatory system provides effective delivery and removal of oxygen, nutrients and wastes to and from respiring tissues. The circulation of blood requires a heart to generate a pressure gradient that will drive blood through the body. The rate of blood flow, cardiac output, is determined by the rate of contraction and stroke volume of the heart. The work the heart performs is the product of cardiac output and blood pressure as the heart must work harder to eject blood against higher blood pressure. The activity of the circulatory system integrates the function of all organs. The study of the cardiovascular system is very informative since it is a "mirror" of overall physiology. A reduction in the metabolic rate of the liver decreases the amount of perfusion this organ requires so that cardiac output can be lowered. Changes in this system are cues to the action of individual organs, which makes it an excellent model for quantifying down-regulation.

The focus of this thesis is the impact of anoxia and temperature on the cardiovascular system of turtles. In nature, freshwater turtles survive extended periods of anoxia due to a significant metabolic down-regulation. Currently, insight into this metabolic suppression is limited to the scope of biochemical adaptations which curtail energy production and demand. This information, while useful, does not provide a holistic view of the degree of depression of work in cold anoxia. Therefore, the first objective was to quantify the work done by the cardiovascular system of turtles acclimating to these conditions. In addition, little is known about cardiovascular control in turtles acclimated to cold anoxia. This leads to the second objective, to assess the

control of adrenergic and cholinergic regulation of cardiac function in anoxia. In the following section, the central biochemical problem of energy supply and demand in anoxia will be considered with particular emphasis on turtles.

Biochemical Adaptations

Turtles employ fermentative glycolysis, culminating in lactate production, to maintain anaerobiosis. Declining O_2 tension initiates two phases in diving turtles. The first phase, characteristic of most natural dives, is a progressive reliance upon glycolysis to maintain ATP levels as conditions become increasingly hypoxic. The second phase begins when a critical arterial tension of around 2.7 kPa is reached (Lutz *et al.*, 1984). At this point metabolic depression is initiated, conserving metabolic fuel and energy for anoxic exposure that is of indeterminate length (Caligiuri *et al.*, 1981; Storey, 1988b). Strict regulation of glycolysis is required during metabolic depression as energy supply and demand must be lowered in concert.

Of central importance to anoxia tolerance is the ability to supply energy demand from glycolysis, the sole energy production pathway in anoxia. Glycolysis yields only 2 moles of ATP for each mole of glucose, far below the 36 moles of oxidative metabolism. This represents an 18-fold reduction in the energy yielded from one mole of glucose. Animals that cannot endure long periods of anoxia attempt to match normal ATP demands with high rates of glycolysis, a compensatory mechanism known as the Pasteur effect. The low energy yield of glycolysis is counteracted by increasing glucose influx by

at least 18-times to maintain the same aerobic ATP level. Obviously the Pasteur effect would not be an effective option for animals exposed to prolonged periods of anoxia as they would exhaust their energy stores rapidly and metabolic acidosis would quickly ensue. Instead, these animals employ a "reverse Pasteur effect" where glycolytic rate is reduced and ATP demand is suppressed in anoxia (Storey, 1991). Glycolytic rate is only depressed beyond the aerobic rate when ATP production has dropped by 18-fold. Beyond the energy shortfall, the reliance on glycolysis for anaerobiosis requires the availability of sufficient fuel, the depression of metabolic rate (Storey, 1988b), and a buffering capacity capable of neutralizing the resultant acidosis (Hochackha *et al.*, 1993).

Fuel for Anaerobiosis

Consistent with the importance of glycolysis, a striking difference is seen between the size of glycogen stores between anoxia-tolerant and anoxia-sensitive vertebrates. Fresh water turtles and fish of the genus *Carassius* possess the largest glycogen stores of any vertebrate, comprising 15% and 30% of the liver, respectively (Hochachka and Somero, 1984). Their cardiac glycogen reserves are from 4-7% of the wet weight. Similarly the brain of turtle, carp and goldfish has glycogen concentrations (12.8-19.5 μM/ g) 6-times larger than anoxia-sensitive species (2.2-3.7 μM/g; Lutz and Nilsson, 1993). The large glycogen reserves of the brain and heart are, however, quickly depleted in anoxia and it is the large liver stores that sustain long-term survival.

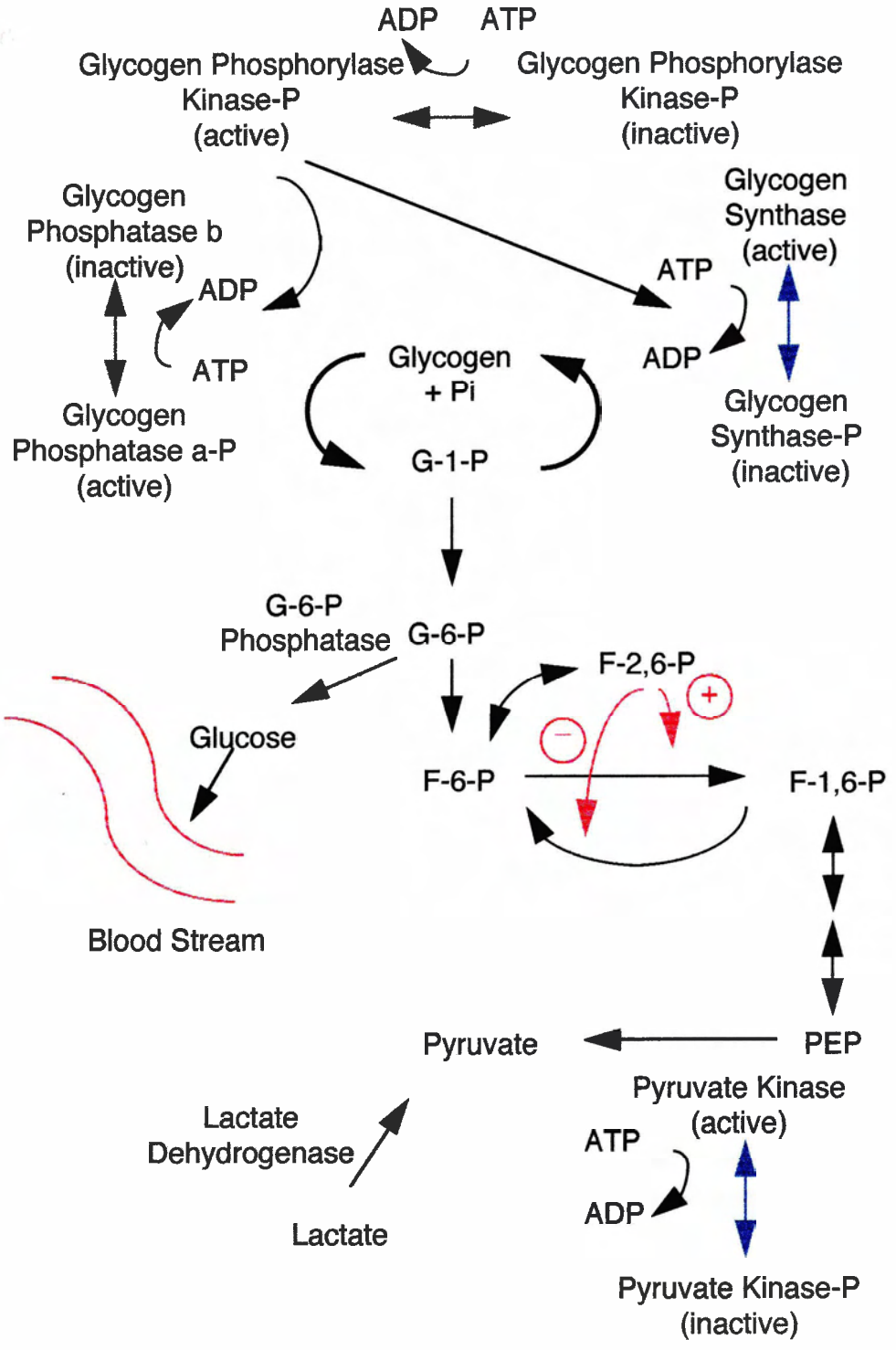
Reversible phosphorylation controls the mobilization of glucose (Figure 1.2). In turtles exposed to anoxia for one hour at 7°C, the active catalytic subunit of liver cAMP-

dependent phosphorylase kinase (PKA) increased 2.3-fold and cAMP levels increased by 60% (Mehrani and Storey, 1995b). After 5 hours, glycogen phosphorylase *a* (GP) levels increased by 12% (Mehrani and Storey, 1995a). GP is required to convert glycogen to glucose 1-phosphate. After five hours, however, activity levels were no longer elevated and PKA activity had returned to control levels (Mehrani and Storey, 1995b). This observation is consistent with the depression of metabolic rate that is seen during prolonged anoxia. Blood glucose levels rise from 5 to 45 mM after 24 hours of anoxia in the turtle (Daw *et al.*, 1976). GP action is opposed by glycogen synthase (GS). PKA acts to inhibit GS activity while protein phosphosphatase type 1 (PP-1) converts GS to its active form. In turtle liver, PP-1 activity fell by 40% within 1 hour and remained suppressed for an additional 20 hours of anoxia. Likewise in red skeletal muscle and brain tissue, PP-1 activity was suppressed over the same time period (Mehrani and Storey, 1995c). Therefore, changes in enzyme activities favor glycogen breakdown to glucose early in anoxic exposure.

Metabolic Depression

The transition from normoxia to anoxia is followed by a suppression of metabolic rate which is essential for the conservation of glycogen and to extend anoxic survival. The initial response of a diving turtle is to maintain ATP levels by increasing glycolysis so a mechanism is required to reduce glycolytic rate and energy demand during prolonged anoxic exposure. The degree of metabolic depression can be significant, to 10-20% of the resting aerobic rate (Herbert and Jackson, 1985b; Jackson, 1968). The control of metabolic depression utilizes reversible protein phosphorylation, and substrate level

Figure 1.2 The complex pathway of glucose mobilization into the blood stream in the liver. Areas of allosteric modulation are indicated in red while covalent modification is shown in blue. Abbreviations as follows: G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-2,6-P, fructose-2,6-phosphate, PEP, phosphoenolpurvate; Pi, inorganic phosphate. General description given in text.


ADP
ATP
Glycogen Phosphorylase Kinase-P (active)
Glycogen Phosphorylase Kinase-P (inactive)
Glycogen Phosphatase b (inactive)
ADP
ATP
Glycogen Phosphatase a-P (active)
Glycogen + Pi
G-1-P
Glycogen Synthase (active)
ATP
ADP
Glycogen Synthase-P (inactive)
G-6-P Phosphatase
G-6-P
F-2,6-P
+
−
Glucose
F-6-P
F-1,6-P
Blood Stream
Pyruvate
PEP
Pyruvate Kinase (active)
Lactate Dehydrogenase
ATP
ADP
Lactate
Pyruvate Kinase-P (inactive)

inhibition, both of which have been shown to control glycolysis in turtles (Brooks and Storey, 1989).

Covalent Modification of Glycolytic Enzymes

Studies focusing on the enzymatic responses of turtles have found covalent modification is important for regulation of carbohydrate metabolism. The kinetic properties of glycogen phosphorlase, PFK-1 and PK were found to be altered in five organs of the red-eared slider *Trachemys scripta* after anoxia exposure (Brooks and Storey, 1988, 1989). The time course of metabolic depression in the heart, brain, liver, and red and white muscle of *T. scripta* submerged in anoxic water was monitored in a study performed by Kelly and Storey (1988). At one hour, glycolysis had been activated in all tissues except the heart. However by five hours, glycolysis and lactate production were reduced indicating that metabolic depression had taken effect. An important factor is that, throughout this time, a constant energy charge was maintained so this transition from normoxia to anoxia was not associated with energy stress.

Covalent modification of key enzymes controlling glycolysis in the organs of anoxia-tolerant species coordinates the overall reduction of carbohydrate flow through glycolysis. Metabolic depression is essential for sustaining long-term anoxia tolerance since it reduces ATP demand and curtails the production of harmful by-products. Covalent modification is not the sole means of attaining metabolic depression as substrate level inhibition also plays a key role, particularly by decreasing the number ATP requiring processes.

Substrate Level Inhibition

Substrate level inhibition (allosteric modulation) represents a regulatory control mechanism that is always in effect in maintaining normal homeostasis. Glycolysis is continuously regulated so that the production of pyruvate and ATP is adjusted to meet the needs of the cell. The principle rate-limiting step of the pathway is the third reaction the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate catalyzed by phosphofructokinase (PFK-1). Under normoxic conditions, inhibition of PFK-1 is initiated by elevated levels of NADH, citrate and ATP so that glycolysis is coordinated with the citric acid cycle. PFK-1 activity is enhanced by high ADP levels. Hexokinase (HK) and pyruvate kinases (PK) are also regulated by their reaction products. HK is inhibited by elevated glucose 6-phosphate levels and PK activity is reduced by high ATP levels. These three glycolytic enzymes are located at key steps in glycolysis which have the most free energy released. As such they are very difficult to reverse and represent a good site for modulation (Darnell *et al.*, 1990).

Early in anoxic exposure glycolytic rate is increased and PFK-1, HK and PK all operate at close to maximal velocity during this period of time. Allosteric modulation is important as PFK-1 and HK are stimulated by a decrease in creatine phosphate levels while PK is possibly activated by a feed-forward mechanism via increases in fructose 1,6-bisphosphate (Driedzic and Gesser, 1994).

The most potent activator of PFK-1 is fructose 2,6-bisphosphate (F 2,6-P_2). F 2,6-P_2 levels are controlled by the enzyme 6-phosphofructo-2-kinase (PFK-2) which is a

phosphoprotein regulated by cAMP and Ca^{2+}-calmodulin dependent covalent modification. F 2,6-P_2 acts as an indicator of the abundance of glucose and in high concentrations will favor the movement of carbon towards biosynthetic pathways. Reduced levels of F 2,6-P_2 would be an appropriate mechanism for metabolic depression as it restricts carbohydrate use to glycolysis (Storey, 1988a).

Covalent Modification beyond Glycolysis

Protein phosphorylation is not limited to glycolytic enzymes but may play a role in the re-establishment of ion homeostasis during anoxic exposure. Covalent incorporation of ^{32}P into proteins from aerobic versus anaerobic turtle tissue was investigated in juvenile *Trachemys scripta elegans* by Brooks and Storey (1993). Anoxic exposure was found to increase the total ^{32}P activity associated with precipitable proteins from brain, heart and liver tissue by 60, 140 and 30%, respectively. Further examination of the subcellular fractionation of liver and brain extracts revealed differences in the location of phosphorylation. In the brain, the increase in phosphorylation occurred in both the plasma membrane and cytosolic proteins indicating that covalent modification is not only important for the regulation of glycolysis in this tissue. In the liver, cytosolic proteins were the primary source of labeled proteins. This difference in the pattern of covalent modification may reflect the importance of ion channel arrest in metabolic rate depression during anoxia.

Studies with the brain, where the maintenance of membrane potential difference and neuronal excitability is the single major energetic cost, provide the best evidence for

ion channel arrest. Ultimately all ATP-dependent ion pump activity has to be supported by glycolysis. Mammals are unsuccessful at effectively suppressing these processes during anoxia, leading to a rapid depolarization of the brain that is fatal (Storey, 1996). Turtle brain tissue has been shown to effectively maintain ion gradients despite reductions in oxygen tension. Rates of K^+ leakage were significantly lower in anoxic, versus normoxic turtle brains (Chih *et al.*, 1989) and Edwards *et al.* (1989) determined that the major factor in reducing anoxic energy consumption was the modulation of ion channel conductance. In other systems, channel phosphorylation has been shown to be a principal mechanism of channel regulation (Catterall, 1984; Reuter, 1987). Whether this mechanism is present in the cardiac tissue of turtles is likely but remains an area for future research.

Protein metabolism is a major energy cost for the cells and as such would be a predictable target for anoxia-induced suppression. Land *et al.* (1993) calculated the suppression of ATP-dependent proteolysis in turtle hepatocytes to be 93% under anoxia. To depress proteolysis one or more cellular proteases could be modulated. Willmore and Storey (1995) found a 68% increase in liver multicatalytic proteinase complex (MPC) during recovery from anoxia. MPC is known to degrade a number of natural peptides *in vivo* and plays a key role in protein turnover. This increased activity has been linked to the degradation of stress-related proteins and proteins damaged by oxygen free radicals generated during reperfusion (Storey, 1996).

Dealing with Acidosis

The genus *Carassius* has developed a novel means of dealing with the acidic by-products of glycolysis. Goldfish survive weeks of anoxia while carp survive months at 4°C (van den Thillart and van Waarde, 1985). The lactate produced by glycolysis is further broken down into ethanol and CO_2 by the red muscle of these fish (Shoubridge and Hochachka, 1980). This is likely a reflection of the ability of these fish to exchange CO_2, ethanol and ammonia with their environment (Hochachka, 1980) and the lack of a source of strong cations (Cameron, 1985). This is really a last resort mechanism as the loss of ethanol to the environment is a loss of chemical potential energy (Hochachka, 1980).

The end product of glycolysis is lactate. Lactate cannot be metabolized in the absence of oxygen so its concentrations rise as a function of the glycolytic rate decreasing the pH of bodily tissues and fluids. Metabolic depression reduces the rate of lactate accumulation. However after months of anoxia, even with metabolic depression, turtles must tolerate the extremely high levels of lactate in their blood (~ 200 mM) compared to normoxia (< 1.0 mM). Lactate is still a useful fuel source after the anoxic bout, but the resultant acidosis is the critical factor that limits anoxic survival (Jackson, 1987).

Turtles tolerate acidosis by using a high buffering capacity and compensatory ion changes. Bicarbonate is a major plasma buffer, of which turtles have very high levels amongst vertebrates, reaching 40-45 mM (Jackson, 1993). However, plasma lactate during extended anoxia exceeds the decrease in bicarbonate concentration. Robin *et al.*

(1981) attributed this "cation gap" as being due to the titration of anionic sites on plasma proteins. Subsequent studies have shown that the lactate load that results in anoxia is counter-acted by large quantities of Ca^{2+} and Mg^{2+} that are mobilized from the shell of the turtle in association with carbonate. The carbonate buffers the acidosis and the high lactate concentrations complex with calcium to form calcium lactate. This enhances the export of lactate from the cell resulting in lactate levels being 40% lower in the cell than in the plasma (Jackson and Heisler, 1983). The rise in calcium is also helpful in counteracting the negative inotropic effects of acidosis on cardiac function (Lagerstrand and Poupa, 1980).

Conclusion

Anoxic exposure places profound energy constraints upon an organism since aerobic respiration must be abandoned in favor of glycolysis. Tolerance of anoxia is then determined by how long the organism can match energy demand and glycolytic potential. For prolonged anoxia exposure sufficient amounts of glucose must be mobilized and compensatory mechanisms must neutralize the resultant acidosis. Numerous adaptations by turtles involve the circulatory system to underlie their extreme anoxia tolerance through the mobilization of glucose reserves to the compensatory ion changes which reduce acidosis. The following section will focus on the cardiovascular system of turtles with particular emphasis on anoxia-induced changes.

Impact of Temperature and Oxygen Availability on Cardiovascular Function in Fishes and Turtles

Thermal Effects on the Cardiovascular System

Ectothermic animals are in thermal equilibrium with their environment. Variations in environmental temperature presents a major challenge for physical activity and survival. Decreases in temperature reduces the kinetic energy of molecules which slows reaction rates and hinders the activity of biological systems. The effect of temperature on a physiological or biochemical process is characterized by a Q_{10} value which describes the effect a 10°C change would have on the rate of that process. Most physiological or biochemical process have a Q_{10} value in the range of 2-3; values greater than these implicate down-regulation while Q_{10} values less than 2 indicate compensation.

Chronic (seasonal) changes in ambient temperature are often compensated for by metabolic and physiological modifications that reduce temperature sensitivity. Compensatory changes with acclimation to chronic temperature can take may forms at a number of levels. Rainbow trout maintain cardiovascular performance despite lower heart rates at low temperatures by enlarging ventricular size which increases stroke volume (Graham and Farrell, 1989). The skeletal muscle of striped bass *(Morone saxatilis)* shows an increased abundance of mitochondria at low temperatures possibly to overcome the problems of a reduction in the rate of diffusion (Egginton and Siddell, 1986). As well, modification of the ß-adrenergic system can be an important effect of

temperature. Keen (1992) found that adrenergic sensitivity of the rainbow trout heart was 10-fold greater in trout acclimated to 8 versus 18°C. Further investigation revealed that trout acclimated to 8°C had a greater cell surface adrenoreceptor density. These modifications permit these animals to be more active despite the depressive effects of temperature.

Temperature is an important regulator of metabolic rate and therefore anoxic survival. Temperature reduces work which lowers O_2 requirement, metabolite utilization and waste production. At 3°C, painted turtles (*Chrysemys picta belli*) can withstand anoxia for in excess of 91 days (Ultsch and Jackson, 1982). With increasing temperature this survival time is reduced. At 15°C, turtles begin to die after 3 days while at 20°C survival is limited to 24 hours of exposure to anoxia (Herbert and Jackson, 1985a). A profound drop in metabolic rate from 10 to 3°C by a Q_{10} of 8.5 (Herbert and Jackson, 1985b). This evidence is supportive of the metabolic depression that can occur in animals exposed to low temperature.

The reduction of temperature has a powerfully depressive effect on the cardiovascular system of turtles. Turtles acclimated to 3°C for 3 months under normoxia decreased heart rate by 17-fold from 30 to 1.8 beats min^{-1} and mean arterial pressure by 2.4-fold from 3.35 to 1.42 kPa when compared to 20°C-acclimated turtles (Herbert and Jackson, 1985b). Thus, the primary effector in the reduction of cardiac function with falling temperature is chronotropic. Using an *in situ* heart preparation Farrell *et al.* (1994) found that an acute temperature change from 15 to 5°C reduced heart rate by 2.9-fold, maximum output pressure by 1.6-fold and maximum cardiac power output by 3.8-

fold from 1.50 to 0.39 mW g^{-1}. The absence of neural input in the *in situ* preparation suggests that vagal inhibitory control depresses heart rate at low temperature since *in vivo* heart rates are lower than intrinsic ones.

Cardiovascular Changes during Hypoxia and Anoxia

Mechanisms of Short-term Hypoxia Tolerance

Utilization of existing physiological features are usually sufficient for tolerating short periods of moderate hypoxia. These mechanisms include the following: increased ventilation rate, bradycardia, cardiorespiratory synchrony, redistribution of blood flow, altering hemoglobin affinity for oxygen and catecholamine release (Lutz and Storey, 1995; Wasser and Jackson, 1991). Bluegills when exposed to hypoxia increase their ventilation rate from 84 to 107 times min^{-1} which would aid in oxygen delivery from an environment with reduced content (Marvin and Burton, 1973). Trout decrease their heart rate from 45 to 26 beats min^{-1} in response to hypoxia (0.4 kPa O_2). This bradycardia would increase the time for oxygen diffusion across the gill lamellae (Randall and Shelton, 1963) and to the spongy myocardium (Farrell, 1984). In cardiorespiratory synchrony, heart rate becomes coupled to the ventilation cycle so that the pulsatile flows of blood and water coincide and optimize O_2 exchange in fish (Satchell, 1960). Turtles are able to shunt blood away from the pulmonary circuit during diving decreasing pulmonary blood flow by 20%, conserving cardiac energy (White and Ross, 1966). The decrease in blood pH associated with hypoxia releases oxygen from erythrocytes by decreasing hemoglobin affinity for oxygen. Catecholamine release may assist in

maintaining cardiac performance despite the negative inotropic effects of hypoxia and acidosis (Gesser *et al.*, 1982; Farrell *et al.*, 1986). Collectively, these mechanisms are effective for dealing with short-term hypoxia, but further physiological and biochemical modifications are essential for surviving prolonged oxygen limitation.

In vivo & In Vitro Studies

Most fish hearts succumb to oxygen limitation once a critical pO_2 is reached. Moderate hypoxic exposure initiates bradycardia, but cardiac power output is maintained by increased stroke volume and blood pressure in rainbow trout *(Oncorhynchus mykiss)* (Holeton & Randall, 1967; Wood & Shelton, 1980). Farrell (1982) found that at oxygen tensions down to 6.9 kPa there was no change in heart rate or cardiac output in lingcod *(Ophidon elongatus)* despite ventilation having reached its maximum rate. However, when Po_2 reached 4.4 kPa, stroke volume doubled and cardiac output fell by 31%. These modifications are beneficial for short-term protection but more extensive changes are required for long-term survival. Unlike the salmonids, hagfish have a low routine cardiac power and correspondingly better ability to deal with hypoxia.

During hypoxic exposure (pO_2 ~2kPa) heart rate, ventral aortic pressure and cardiac output in the Atlantic hagfish are maintained at normoxic levels for at least 35 minutes (Axelsson *et al.*, 1990). This is possible because routine cardiac performance of these animals (0.05 mW g^{-1}) is low compared to other fishes (eg. ocean pout 2.0, winter flounder 2.85 mW g^{-1}; Driedzic *et al.*, 1987). This difference arises because of a lower

ventral aortic blood pressure (0.65 kPa; Satchell, 1986). Cardiac output in Atlantic hagfish (8.9 ml min^{-1} kg^{-1}) is not unlike that in the spotted dogfish or eel 9.9 and 10.4 ml min^{-1} kg^{-1}, respectively (Axelsson *et al.*, 1990; Short *et al.*, 1977; Hipkins, 1985). The Pacific hagfish *(Eptatretus cirrhatus),* by comparison, is more active than the Atlantic hagfish and has a higher routine cardiac power output, 0.37 compared with 0.05 mW g^{-1} respectively (Forster, 1989). Consequently it is unable to withstand anoxia as well as the Atlantic hagfish. Thus, cardiac power output and locomotory ability are good predictors of anoxia tolerance amongst fish.

Turtles are an example of extreme anoxic tolerance amongst vertebrates. Turtles over-winter in the sediments of ponds for periods in excess of four months (Ultsch and Jackson, 1982). This anoxia tolerance is, however, temperature dependent as turtles can only withstand anoxia for no longer that a day at 22°C (Ultsch, 1989). The onset of anoxia is accompanied by a profound decrease in cardiovascular performance. At 20°C anoxic exposure led to a decrease in heart rate, which fell from 30 to 9 beats min^{-1}, and mean arterial blood pressure dropped from 3.4 to 2.2 kPa. At 3°C this anoxic cardiac depression is even greater (0.4 beats min^{-1} and 0.65 kPa; Herbert and Jackson, 1985a; b). Reeves (1963 a,b) developed isolated turtle heart preparation which he used to study mechanical and metabolic function in turtles. The results revealed that ATP demand was the same in normoxia and anoxia for the same level of work, whether ATP was produced from aerobic or anaerobic means. Glycolysis can therefore provide sufficient energy to power contraction in anoxia. He also found that higher work rates in anoxia required exogenous glucose but that lower work levels could be supplied by intrinsic stores.

Arthur *et al.* (1997) found that anoxia decreases ATP turnover rate by 2-fold at 15°C and by 4-fold at 5°C demonstrating that turtles are exceptional anaerobic performers due to metabolic depression, not exceptionally high glycolytic rates.

Using an *in situ* heart preparation Farrell *et al.* (1994) recorded a 1.9-fold drop in maximum cardiac power output (from 1.5 to 0.77 mW g^{-1}) after 25 minutes of anoxia exposure at 15°C. At 5°C the reduction was more severe as maximum cardiac power output decreased by 2.3-fold (from 0.39 to 0.17 mW g^{-1}) with anoxic exposure. Heart rate dropped by a consistent 2-3 beats min^{-1} regardless of temperature from 23.4 to 20.0 beats min^{-1} at 15°C and from 8.1 to 6.3 beats min^{-1} at 5°C. Since *in vivo* heart rates fall dramatically upon anoxia exposure these data support the conclusion that anoxic bradycardia is largely controlled by inhibitory cardiac vagal tone at temperatures above 15°C. At 5 and 10°C little difference is seen in heart rate between *in vivo* and *in situ* indicating that inhibitory vagal tone maybe less prominent. However the presence or absence of vagal tone has not been tested *in vivo*.

A range exists among animals exposed to extreme hypoxia in the response of the cardiovascular system. At one extreme is the Atlantic hagfish whose energy demand is similar in aerobic and anaerobic conditions. Thus, cardiac performance is normally very low and can be supported by glycolysis during anoxia (Forster *et al.*, 1991). As a result there is no need for serious metabolic adjustment. For the majority of animals, however, their routine cardiac power output exceeds the glycolytic capacity and metabolic down-regulation is necessary for survival. Fishes, for example, depress cardiac function with progressive hypoxia. Arthur *et al.* (1994) found that the hypoxic *in situ* rainbow trout

heart could maintain the subphysiological work regimes of normoxic hearts hearts, but increasing the power demand to normal *in vivo* levels caused rapid failure. As well, frogs and reptiles employ cardiac depression between breaths during hypoxia. However, reduced oxygen requirements of the heart may avoid anoxic exposure to this organ. Even so, the glycolytic capacity is often insufficient to sustain cardiac performance preventing anoxic survival. At another extreme, turtles have a remarkable ability for severe cardiac depression which allows glycolysis to meet cardiac energy demand (Reeves 1963a; Herbert and Jackson, 1985 a; b).

Cellular Effects of Anoxia

Nuclear magnetic resonance spectroscopy has been used to map the energetics and acid base status of isolated working turtle hearts. Analysis of the spectra show that phosphocreatine levels fall to 50% of normoxic control levels and remain stable there for at least four hours of anoxia (Wasser *et al.*, 1990). Inorganic phosphate levels follow an inverse relationship increasing over the first 30-60 minutes of anoxia while ATP levels remain stable. Intracellular pH falls by 0.2 units over the first 60 minutes after which it is maintained. In comparison, after 60 minutes of anoxia rabbit cardiac tissue that has been cooled to 20°C has near zero phosphocreatine levels while intracellular pH plummets to 6.0. A weakness of these results is that the cardiac power output of the isolated heart used in these experiment was quite low compared to *in vivo* values.

At the cellular level, the consequences of anaerobic metabolism can depress contractility of the heart. In the anoxic turtle heart phosphocreatine levels decrease by

50% in the first hour of exposure to maintain ATP supply. This increases inorganic phosphate (Pi) levels which have been implicated in both reduced Ca^{2+} affinity and maximum force development of the myofibrils (Hibberd *et al.*, 1985). As well, the acidosis created by glycolysis reduced the affinity of troponin C (TnC) for Ca^{2+} (Solaro *et al.*, 1989), possibly by reducing the net negative charge of the myofibril, repelling Ca^{2+} (Godt, 1981). This mechanism appears to involve troponin I (TnI) as the addition of TnI increases pH sensitivity of Ca^{2+} binding to cardiac TnC. The binding of H^+ to the histindine residues of TnI may induce conformational changes in TnC depressing Ca^{2+} affinity (El-Saleh and Solaro, 1988). The lowering of intracellular pH (pHi) creates a shorter action potential reducing the time available for Ca^{2+} influx (Orchard & Kentish, 1990). If these events are not counteracted, contractile force can be significantly limited.

The mobilization of Ca^{2+} from the turtle's shell in anoxia supports contractility by creating a rise in intracellular Ca^{2+} levels. Low pHi stimulates the Na^+-H^+ exchanger producing high intracellular levels of Na^+ which causes an influx of Ca^{2+} through the Na^+- Ca^{2+} exchanger. In addition, higher H^+ concentrations will also displace Ca^{2+} from Ca^{2+} buffering sites increasing levels further (Orchard and Kentish, 1990). Through this complex interplay of factors the rise in Pi and drop in pHi may not produce the expected depression in contractility. Such factors should be considered when dealing with the large species specific responses of contractility to anoxia (Driedzic and Gesser, 1994).

Conclusion

Temperature and anoxia profoundly depress cardiac function in turtles primarily through bradycardia which extends their anoxia tolerance. Anoxia, as well, has a negative inotropic effects which may be mediated through acidosis or inorganic phosphate levels. The importance of cholinergic and adrenergic control in mediating these effects will be focused on in the next section.

Cardiovascular Control

The inotropic strength of the heart is reflected in blood pressure and stroke volume while heart rate is a chronotropic response. The control of these three variables is under neural, humoral and intrinsic control. The autonomic system is composed of the adrenergic and cholinergic systems, both of which are powerful control systems of cardiac function. The role of these systems in the response of the cardiovascular system to anoxia and low temperature in turtles are unknown. The following sections will introduce these systems.

Adrenergic Control

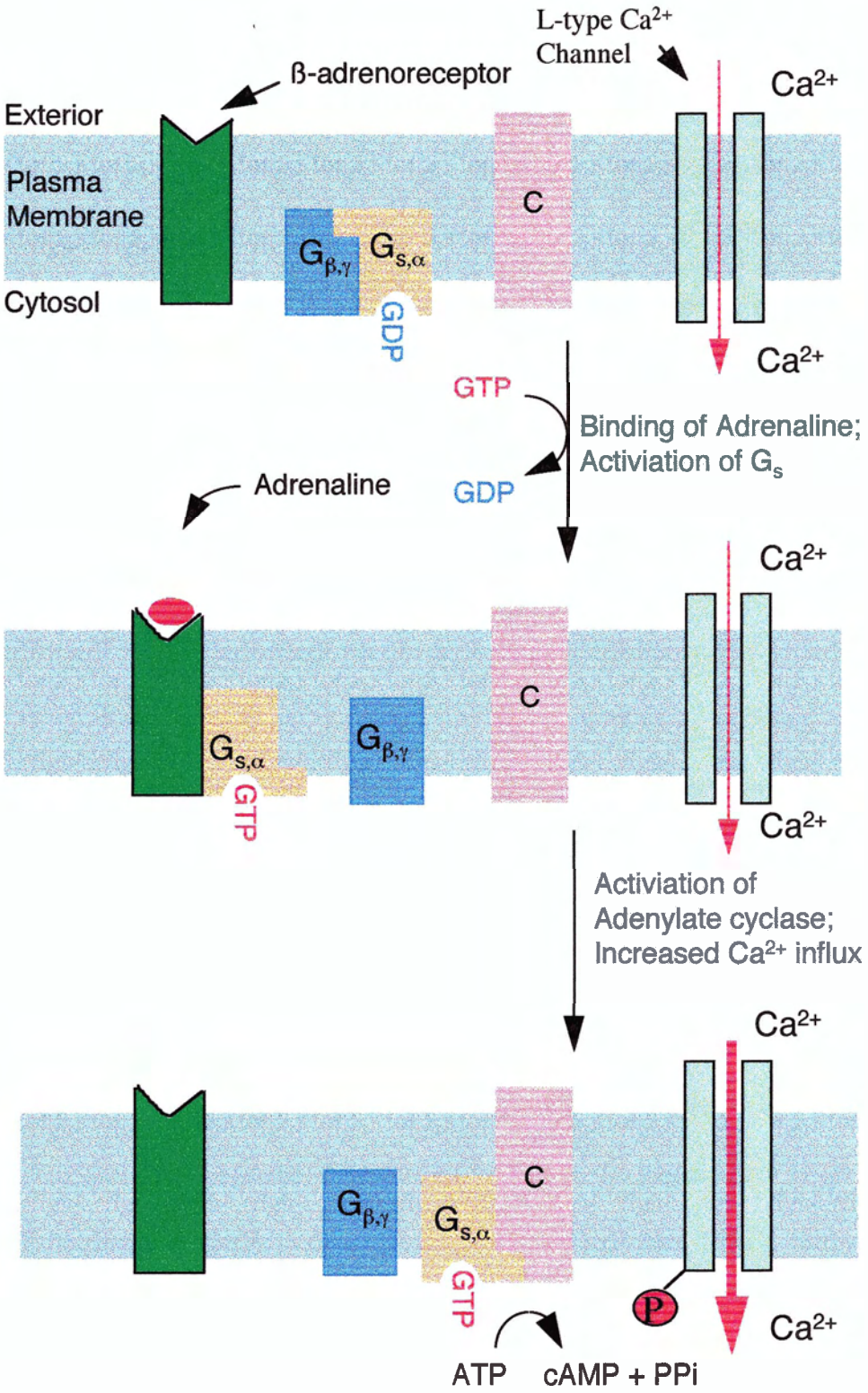
The adrenergic system stimulates the heart through inotropic and chronotropic mechanisms. This can be of significant importance in supporting cardiac function against

the depressive effects of anoxia and low temperature. The 6-15-fold increase in the circulating levels of adrenaline has been implicated in protecting the inotropic capabilities of the heart from exercise-induced acidosis and hypoxia (McDonald and Milligan, 1992). Physiological levels of adrenaline have been shown to restore cardiac performance in trout without altering intracellular pH, indicating that increased Ca^{2+} influx may be involved (Milligan and Farrell, 1986).

Since catecholamines cannot penetrate the cell membrane a transduction pathway is responsible for transmembrane signaling (see figure 1.3). ß-adrenergic receptors initiate an enzyme cascade inside the cell once an extracellular ligand is bound. This results in an activation of a GTP-binding protein (Gs protein) which catalyzes adenylate cyclase to convert ATP to cyclic AMP (cAMP), a potent intracellular messenger (Lefkowitz *et al.* 1983). cAMP in turn activates a cAMP-dependent protein kinases in the cytoplasm, which phosphorylate a number of cellular proteins. The result is an increase in the force of contraction as well as an augmentation of the rates of rise and fall of force.

Several key cellular phosphoproteins are involved in the ß-adrenergic response. The L-type Ca^{2+} channel located on the plasma membrane is phosphorylated (Yatani *et al.*, 1987) which results in an increase in the Ca^{2+} current into the heart by increasing the open probability of Ca^{2+} channels during depolarization (Reuter, 1983). The increase in the Ca^{2+} influx creates the positive inotropic response to adrenaline seen in the heart, since more Ca^{2+} is available to the myofilaments (Yue, 1987). As well, the phosphorylation of TnI and TnC increases Ca^{2+} affinity of the myofibril (Kranias &

Figure 1.3 The ß-adrenergic signaling pathway. The binding of adrenaline to the ß-adrenoreceptor stimulates the binding of the transducer protein (Gs,a) to adenylate cyclase (C) which initiates the enzymatic cascade resulting in increased Ca^{2+} influx into the cell. General description given in text (modified from Darnell *et al.*, 1990).


L-type Ca2+ Channel
ß-adrenoreceptor
Ca2+
Exterior
Plasma Membrane
Cytosol
C
Gβ,γ
Gs,α
GDP
Ca2+
GTP
Binding of Adrenaline; Activiation of Gs
Adrenaline
GDP
Ca2+
C
Gs,α
GTP
Gβ,γ
Ca2+
Activiation of Adenylate cyclase; Increased Ca2+ influx
Ca2+
C
Gβ,γ
Gs,α
GTP
P
Ca2+
ATP
cAMP + PPi

Solaro, 1982). The increase in chronotropy is achieved through direct stimulation of the pace-maker cells (Cobb and Santer, 1973). In mammals, adrenergic stimulation also removes the inhibitory influences of phospholamban (PLB) increasing the rate of Ca^{2+} flux in the cytosol by stimulation the SR- Ca^{2+} pump. PLB has been shown to be a factor in the inotropic response to ß-adrenergic stimulation. Luo *et al.* (1994) used isolated hearts from PLB-deficient mice to assess the role of PLB in regulating basal activity and ß-adrenergic stimulation. Time to peak tension and time to half relaxation were significantly shorter in hearts which had PLB deleted. The wild-type heart performed equally well as the PLB-deficient heart when maximally stimulated with the ß-agonist isoproterenol. This indicated that PLB is a critical repressor of basal myocardial contractility and is a key phosphoprotein in mediating the ß-adrenergic response. Wolska *et al.* (1996) found significant increases in twitch amplitude, maximal shortening velocity, and maximum relengthing velocity in myocytes isolated from PLB-deficient mice. This suggested that other phosphoproteins are invovled in regulating the contractility of myocytes responding to ß-adrenergic stimulation.

Cholinergic Control

The stimulatory influences of the adrenergic system is opposed by the inhibitory action of the cholinergic system. The cardiac branch of the vagus nerve is the primary control of the parasympathetic system on cardiac activity (Satchell, 1991). Stimulation of this nerve slows the heart and is implicated in the diving and anoxic response of turtles at warm temperatures (Driedzic and Gesser, 1994; Wang, personal communication). This

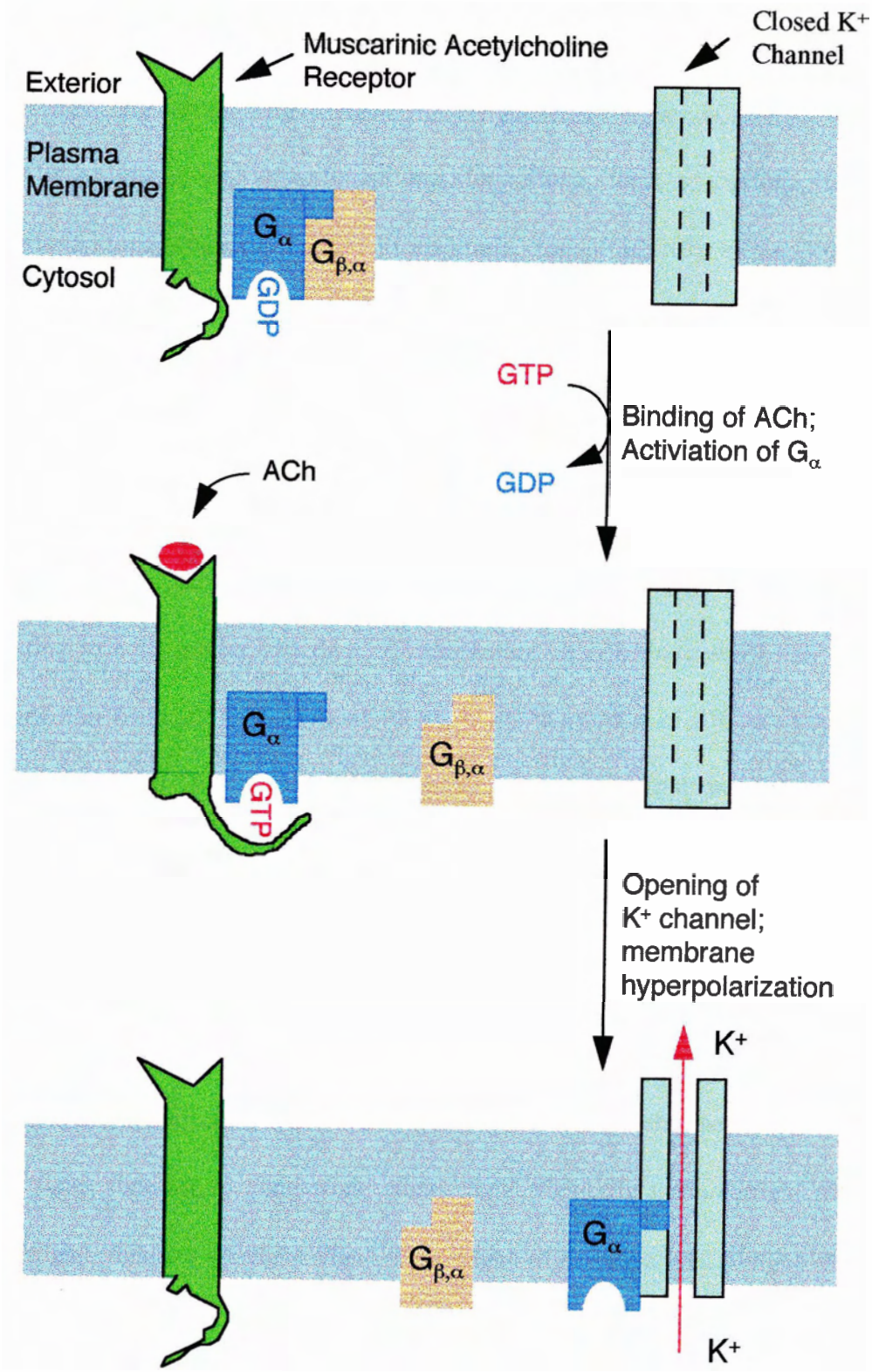
system serves as a potential mechanism for the extreme depression of the cardiovascular system seen in turtles during anoxic exposure at low temperatures.

A proposed mechanism for vagal inhibition of heart rate is shown in Figure 1.4. The binding of acteylcholine to the muscarinic acetylcholine receptor initiates the activiation of the transducing G_α protein by catalyzing the exchange of GTP for GDP on the α subunit. The G_α protein then binds to and opens a K^+ channel which hyperpolarizes the cell reducing heart rate.

Conclusion

It is known that the metabolic rate of turtles decreases sharply in response to anoxia and low temperature, but the effect on systemic cardiac output and the role of control systems in mediating this depression is unknown. This thesis focuses on these areas of turtle cardiac physiology by employing an *in vivo* preparation to measure systemic cardiac output and assess control of adrenergic and cholinergic regulation in turtles acclimated to 22 or 5°C under normoxic or anoxic conditions. In addition, the ß-adrenoreceptor density of acclimated turtle ventricles was examined to determine if anoxia- and temperature-induced changes occur at this level.

Figure 1.4 Proposed mechanism of vagal inhibition of heart rate. The binding of acetylcholine (ACh) to the muscarinic acetylcholine receptor activates the transducing G protein (G_a) which binds to and opens a K^+ channel. The increase in K^+ permeability hyperpolarizes the membrane, which reduces heart rate (modified from Darnell *et al.*, 1990).


Exterior
Muscarinic Acetylcholine Receptor
Closed K+ Channel
Plasma Membrane
Cytosol
Gα
Gβ,α
GDP
GTP
Binding of ACh; Activiation of Gα
GDP
ACh
Gα
GTP
Gβ,α
Opening of K+ channel; membrane hyperpolarization
K+
Gβ,α
Gα
K+

CHAPTER 2

Introduction

Freshwater turtles, over-wintering in the sediments of ponds, can withstand four months of anoxia at 3°C (Ultsch and Jackson, 1982). This remarkable tolerance is supported by a significant reduction in metabolic rate to 10-20% of the pre-dive rate (Herbert and Jackson, 1985b; Jackson, 1968). Our knowledge of this depression of energy supply and demand is limited to the scope of biochemical adaptations, which are incapable of providing a comprehensive view. The measurement of work performed by the cardiovascular system would provide this view since it is easy to quantify and express as ATP demand. However, cardiac power output has not been measured in turtles acclimating to cold anoxia, but there is information on heart rate and blood presssure. The transition from 22°C-acclimated turtles under normoxia to 5°C-acclimated under anoxia resulted in a 76-fold decrease in heart rate and a 5.6-fold drop in mean arterial blood pressure in the turtle *Chrysemys picta belli* (Herbert and Jackson, 1985b). This data combined with the *in situ* reduction of stroke volume in anoxia, predicts a substantial depression of cardiac power output of 500-fold in the transition from warm normoxia to cold anoxia (Farrell *et al.* 1994).

Cardiovascular control is under powerful inhibitory cholinergic control and a much weaker stimulatory adrenergic control at 22°C under normoxia (White, 1976). However, little is know about the effect of temperature and anoxia on this control system. Farrell *et al.* (1994) suggested from their findings using an *in situ* heart preparation that since the intrinsic heart rate was greater than the *in vivo* heart under normoxia that vagal

inhibition could be important a low temperatures. Cholinergic control could be responsible for the reduction of cardiac function that is part of the acclimatory process of over-wintering turtles. Apneoic bradycardia is associated with increased vagal inhibitory tone (White, 1976) while the mechanism for anoxic bradycardia is still, however, unclear.

Anoxic submergence results in extreme variation in the cellular enviroment, particularly with respect to pH. The reliance on anaerobic metabolism, with concomitant lactate and CO_2 production, can reduce blood pH to 6.6-6.8 (Robin *et al.*, 1964) and intracellualr pH to 6.7 (Jackson *et al.*, 1991) during prolonged submergence. The acidosis, along with temperature, has been implicated as a potent intrinsic factor for the depression of cardiac function (Shi and Jackson, 1997). In fact, the defense of cardiac glycogen is improved with acidosis during anoxia, indicating that metabolic rate is depressed further with acidosis (Wasser *et al.*, 1991). Intrinsic factors are therefore important in the reduction of cardiac function during anoxic submergence, and as such, may be an mechanism for cardiovascular control.

In this study, systemic cardiac output and power output were measured *in vivo* in turtles acclimated to either 22 or 5°C under normoxia or anoxia. Once cardiovascular variables had stabilized a drug trial was used to assess the control of cholinergic and adrenergic regulation. The focus of this study was to quantify the degree of down-regulation that occurs with acclimation to cold anoxia as well as identify the control system responsible for cardiovascular regulation.

Methods and Materials

Experimental Animals

Red-eared sliders *(Trachemys scripta)* (mean body mass 724 ± 71g) were obtained from a commercial supplier (Carolina Biological Supply Co.) and held indoors in standing water in polypropylene containers. The turtles were fed on commercial pellets (Wardley Co.), small pieces of fish and lettuce at least three times per week. Turtles were given access to basking platforms as well as deep water. Room and basking lights were set for a 12L:12D photoperiod. Turtles were fasted for one week prior to surgery. Experiments with 5°C-acclimated turtles were conducted from September 1995 to May 1996, while experiments with 22°C-acclimated turtles were conducted from June to September 1996. No significant difference was found between the mean body mass of any experimental group.

Surgical Preparation

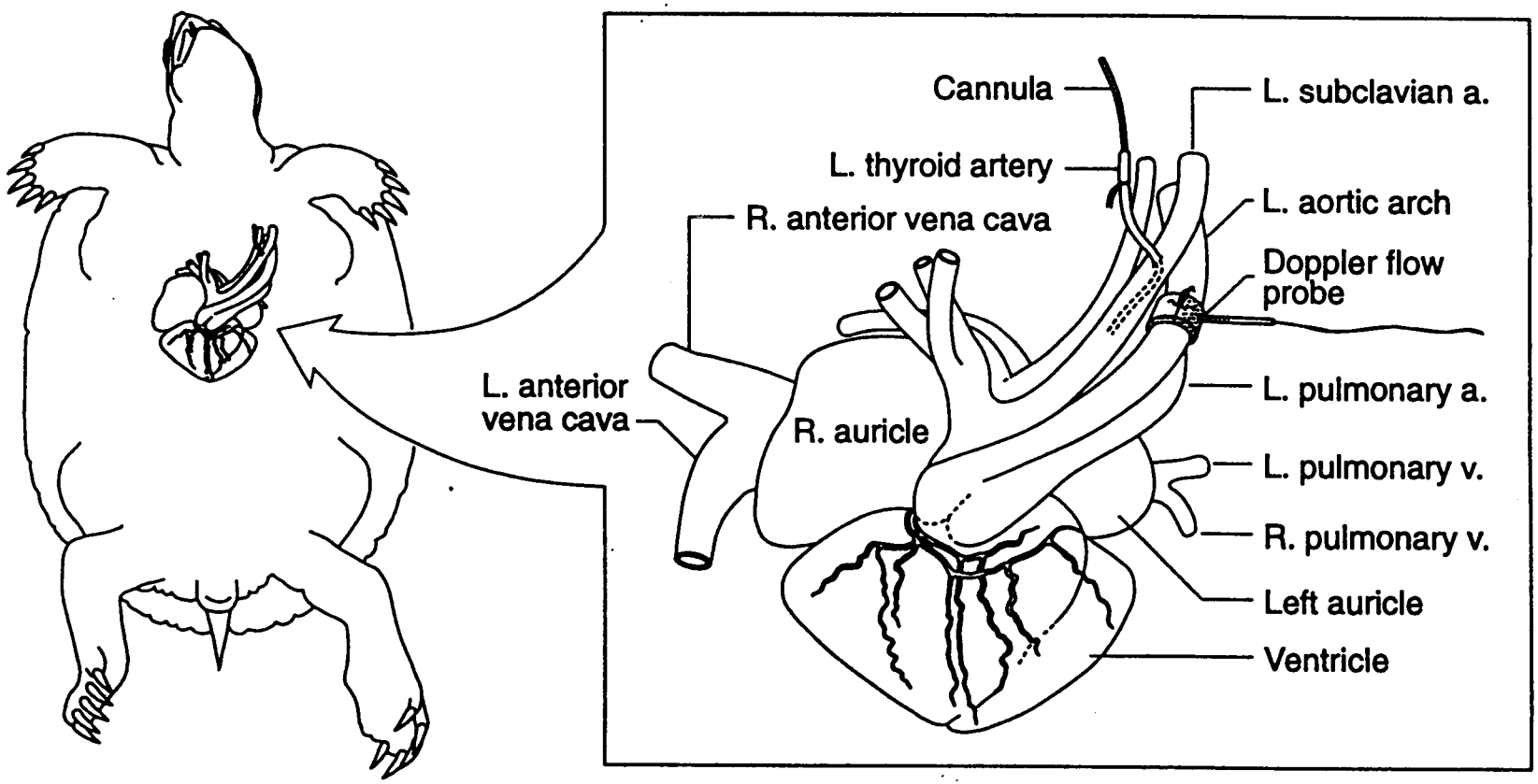
Turtles were anesthetized with 4% Halothane (95% O_2, 5% CO_2), intubated and ventilated with 2% Halothane during surgery (Wang and Hicks, 1996). A Halothane vaporizer (Fluotec Mark 2, Cypran Ltd, UK) was used to control Halothane levels. An electric bone saw (Mopec, Detroit, MI) was used to remove a 4 cm x 5 cm piece of the plastron, thereby exposing the heart and systemic output vessels. A blunt probe was used to gently remove any attached muscle and any bleeding was stopped by cauterization. An occlusive PE 50 polyethylene cannula filled with heparinized saline (50 i.u. mL^{-1}) was inserted into the left subclavian artery via the thyroid artery and advanced towards the heart (~5 cm) before being secured in place with 3-0 gauge silk thread. The cannula was

lead out of the shell through a trocar inserted at the base of the left foreleg, the trocar was then removed and the cannula was secured to the skin with a purse-string suture. A Doppler flowprobe (Iowa Doppler Products, Iowa City, IA) was placed around a portion of the left aorta that had been freed of connective tissue (see figure 2.1). Leads from the probe were carried out through the hole in the plastron and secured along side the cannula onto the carapace with cyanoacrylate and Flexacryl (Lang Dental Mfg Co, Wheeling, IL). Prior to the plastron being closed, a penicillin-streptomycin-neomycin solution (Sigma Chemical Co., St. Louis, MO) was sprayed over the surgical field. The plastron piece was resealed in place with a layer of Gelfoam sponge (Upjohn Co., Kalamazoo, MI) and sealed with bone dust and cyanoacrylate. The entire area was then covered with Flexacryl. Following surgery, turtles were ventilated with air until they could actively withdraw their limbs. The animals were then held individually in 10 gallon glass aquarium for 48 hours prior to any cardiovascular measurements being taken. Hematocrit was monitored for the first week following surgery and ranged from 14% to 26%. Turtles with levels below 13% were euthanised by decapitation.

Instrumentation and Terminology

Systemic arterial blood pressure (P_{sys}) was recorded through a pressure transducer (Narco LDI-5, Narco, TX) connected to the cannula. The pressure transducer was calibrated against a static water column before each recording and pressures were regularly referenced to the water level of the tank in which the turtle was submerged. The flowprobe was connected to a Doppler flowmeter and the range manipulated for maximum signal strength. This range was recorded and used for all subsequent

Figure 2.1 Ventral view of the surgical area including the sites of cannulation and flow probe placement.


Cannula
L. subclavian a.
L. thyroid artery
L. aortic arch
R. anterior vena cava
Doppler flow probe
L. anterior vena cava
R. auricle
L. pulmonary a.
L. pulmonary v.
R. pulmonary v.
Left auricle
Ventricle

measurements. Each flow probe was individually calibrated *in situ* at the termination of the experiment by delivering known volumes of diluted blood through a cannulated left aorta. The aortic outflow was connected to a pressure head so that calibrations could be made at physiologically representative pressures. Pressure transducer and flowmeter signals were preamplified and displayed on continuously on a Gould chart recorder (model 2400, Cleveland, OH). Heart rate (fH) was measured by counting the number of systolic peaks over a one minute period in the 22°C groups and over a four minute period at 5°C. In the normoxic groups, measurements were taken from post-breath portions of the traces.

Mean systemic arterial pressure (P_{sys}), systemic cardiac output (Q_{sys}), systemic stroke volume (SV_{sys}), systemic power output (Q_{sys}) and systemic resistance (R_{sys}) were calculated as follows:

$$P_{sys} = (\text{systolic pressure} + \text{diastolic pressure})/2$$

$$Q_{sys} = 3.5\ Q_{LAo}$$

$$SV_{sys} = (Q_{sys}/fH)/\ M_b$$

$$MPO_{sys} = (Q_{sys} \times P \times \alpha)/\ M_v$$

$$R_{sys} = P_{sys}/\ Q_{sys}$$

where Q_{LAo} is left aortic blood flow (ml min^{-1} kg^{-1}), fH is heart rate (beats min^{-1}), M_b is body mass (kg), M_v is ventricular mass (g) and α is 0.0167 min/s a conversion to mW. P_{sys} was measured from pressure traces by calculating the average pressure between

systole and diastole. Q_{sys} was estimated from 3.5 Q_{LAo} to approximate the contributions of the right aorta, carotid and subclavian arteries to cardiac output (Wang and Hicks, 1996; Comeau and Hicks, 1994).

Experimental Protocol

During experiments the turtles were allowed to move freely with in an experimental chamber (30 cm x 30 cm x 60 cm) which was covered with black plastic to minimize visual disturbance. Recordings were taken without the experimenter in the room. Turtles acclimated to 22°C were recorded each day for seven days of post-surgery (see Figure 2.2). After this seven-day recovery period, the anoxic group were submerged in water equilibrated with 100% N_2 for 12 hours. A Plexiglas cover was fitted to the top of the tank and plastic mesh grate was attached beneath it which denied the turtles access to the surface. After 6 hours of anoxia, the cardiovascular status was recorded and a drug trial begun. The 22°C normoxic group was treated similarly but the drug trial was begun after the seven-day recovery period. Acclimation to 5°C involved a progressive transition over five days. Turtles were first recorded on day 2 at 22°C then they entered the 5°C exposure regime as follows: day 3, 6 h at 5°C; day 4, 12 h at 5°C; day 5, 18h at 5°C; day 6, no 5°C exposure; day 7, 24 h at 5°C. Cardiovascular variables were recorded at the end of each "re-warming" period prior to the turtles being moved into the 5°C room. The 5°C normoxic group was then acclimated to 5°C for 5 weeks prior to performing a drug trial. The 5°C anoxic group was held at 5°C under normoxic conditions for four days then they were submerged and the water bubbled with 100% N_2 for three weeks prior to a drug trial. Cardiovascular measurements were taken every second day during these

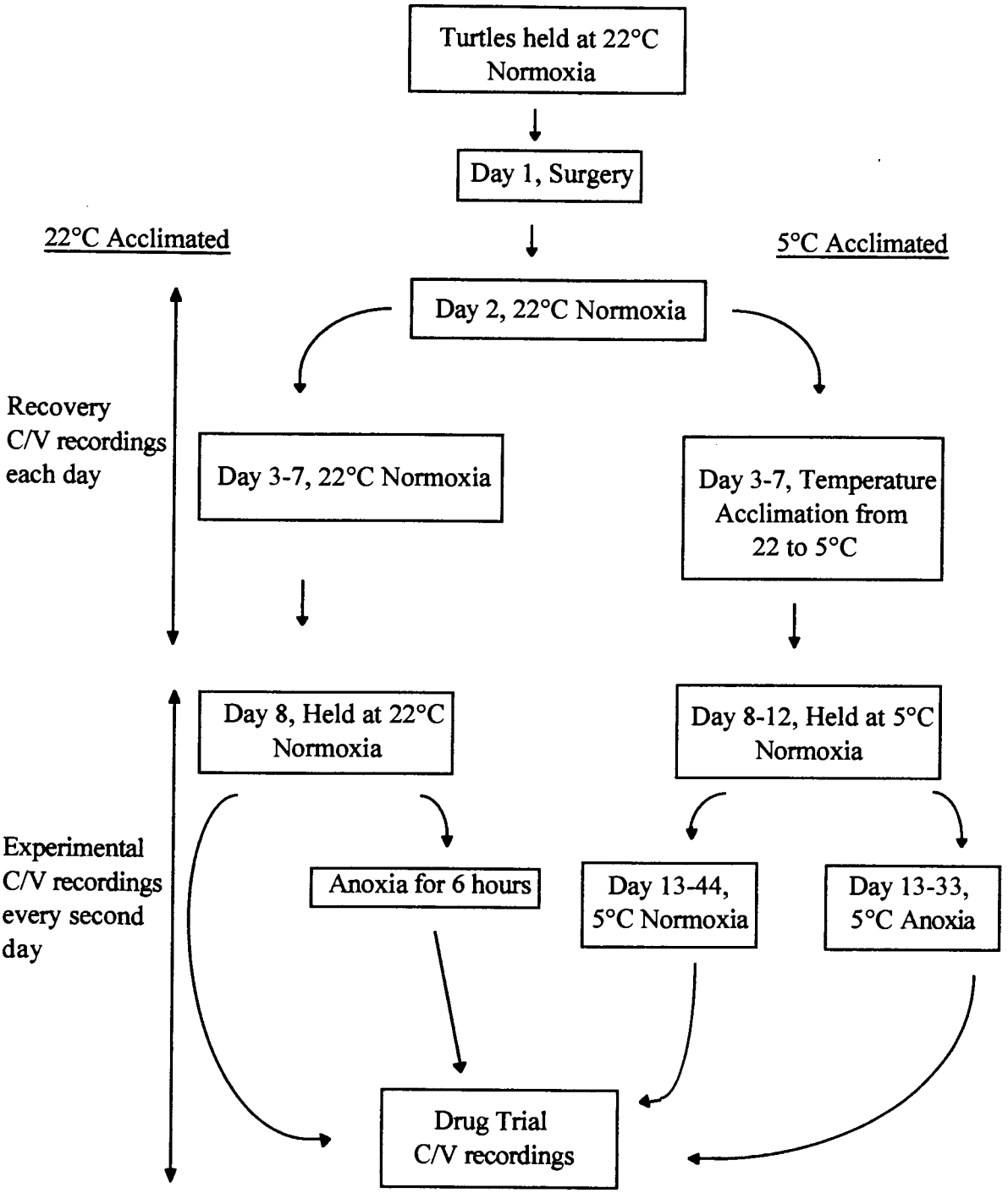
acclimation periods (see figure 2.2). All low temperature experiments took place in a cold room held at 5°C.

A drug trial was used to assess the contribution of cholinergic and adrenergic regulation. It consisted of the following series of injections in 0.5 ml of saline through the aortic cannula: control, 0.5 ml saline injection; 10 μg kg^{-1} adrenaline (α- and β-adrenergic agonist), 1.5 mg kg^{-1} atropine (cholinergic, muscarinic antagonist), 10 μg kg^{-1} adrenaline, 2.0 mg kg^{-1} nadolol (ß-adrenergic antagonist) and 10 μg kg^{-1} adrenaline. Preliminary experiments established the minimum adrenaline dose required to achieve maximum cardiovascular stimulation in turtles held at 22°C normoxia and was found to be representative of the catecholamine levels found in turtles exposed to anoxia (Wasser and Jackson, 1991). Atropine and nadolol doses were based on previous experiments on red eared sliders (Wang, pers. comm.). A suitable recovery period (60 min. for normoxia, 90 min. for anoxia) was allowed between subsequent injections to permit cardiovascular variables to return to resting values. All drug were obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in turtle saline.

Statistical Analysis

In all cases mean values (SEM) for 6 animals are presented (N=5 for 5°C anoxic group). Differences between means of experimental groups were determined using one-way and two-way analyses of variance (ANOVA) for repeated measures, while multiple comparisons were performed using Student-Newman-Keuls tests. Paired t-tests were used to assess the effect of drug infusion. $P < 0.05$ was used as the level of significance.

Figure 2.2 Experimental protocol for the *in vivo* cardiovascular measurement study.



Results

Seven-Day Recovery Period from Surgery

A seven-day recovery from surgery resulted in significant changes in all measured cardiovascular variables, except SV_{sys}, for 22°C normoxia (Figure 2.3). In general, Q_{sys} and *f*H increased while P_{sys} and R_{sys} declined. PO_{sys} changed little. Only *f*H showed a significant change between day 5 and day 7 (Figure 2.3). Changes in cardiovascular variables also occurred with the 22°C anoxia group, but there was less of a progressive pattern as was seen in the 22°C normoxic group (Figure 2.4). There were fewer changes in cardiovascular variables during recovery in the 5°C normoxic and anoxic groups (Figures 2.5, 2.6). This was likely a result of the progressive 5°C exposure they were encountering at this time or may reflect a seasonal difference.

The post-recovery (day 7) cardiovascular values are presented in Table 2.1. The only variable that did not have any significant differences between groups was *f*H. Both normoxic groups had significantly higher Q_{sys} than the anoxic groups, which elevated PO_{sys} and decreased R_{sys}.

Transition to Anoxia at 22°C

All cardiovascular variables showed some significant change during 6 hours of anoxic exposure. Compared with control at time zero, Q_{sys}, P_{sys}, PO_{sys} and *f*H had all significantly declined (Figure 2.7). However, R_{sys} and SV_{sys} returned at the same level. None of the cardiovascular variables were significantly different for the 5^{th} and 6^{th} hour

Figure 2.3 Post-surgery cardiovascular variables during recovery for the 22°C normoxic turtles. Significant differences between columns are indicated by dissimilar symbols.

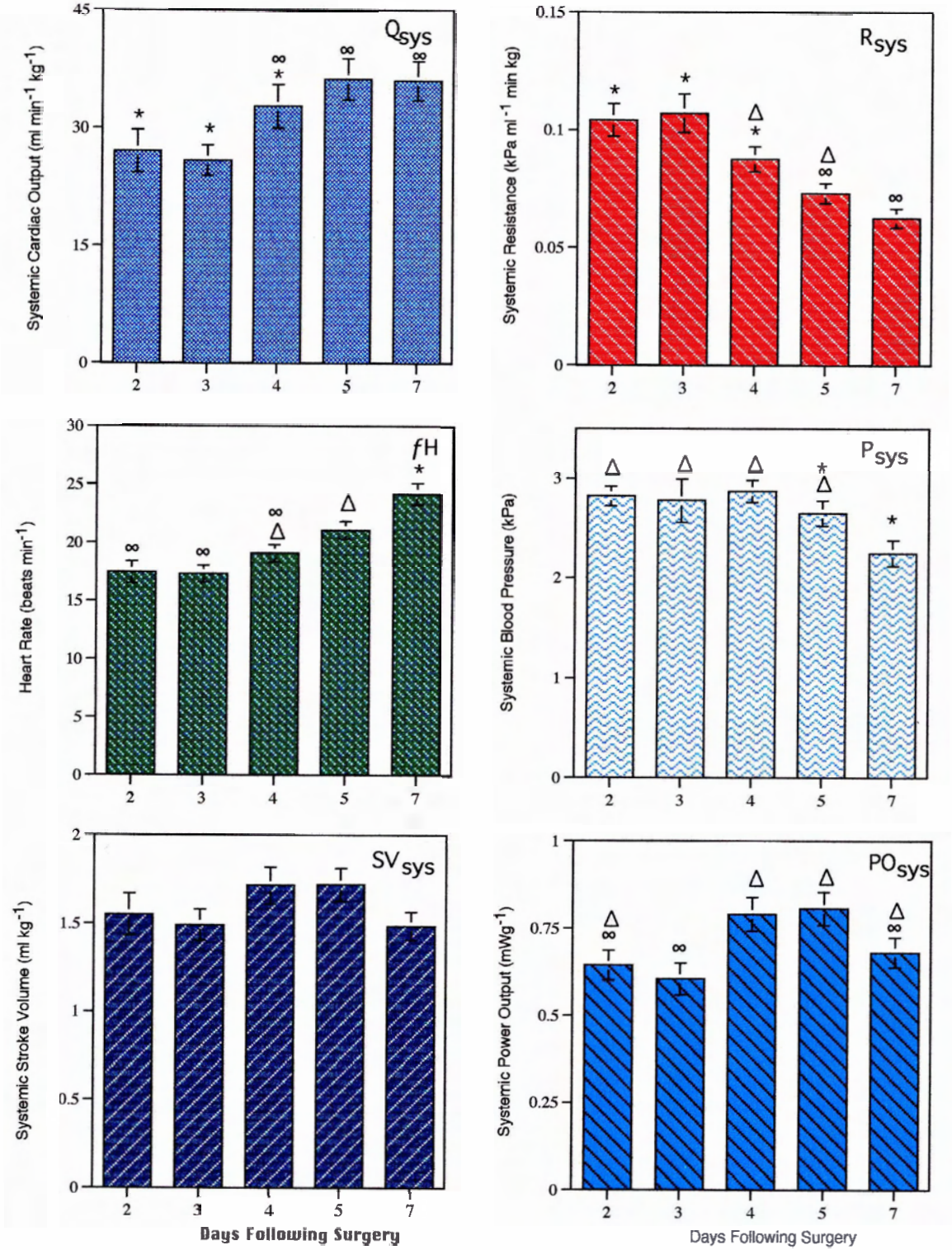

Qsys
Systemic Cardiac Output (ml min-1 kg-1)
Rsys
Systemic Resistance (kPa ml-1 min kg)
fH
Heart Rate (beats min-1)
Psys
Systemic Blood Pressure (kPa)
SVsys
Systemic Stroke Volume (ml kg-1)
POsys
Systemic Power Output (mWg-1)
Days Following Surgery
Days Following Surgery

Figure 2.4 Post-surgery cardiovascular variables during recovery for the 22°C anoxic turtles. Significant differences between columns are indicated by dissimilar symbols.

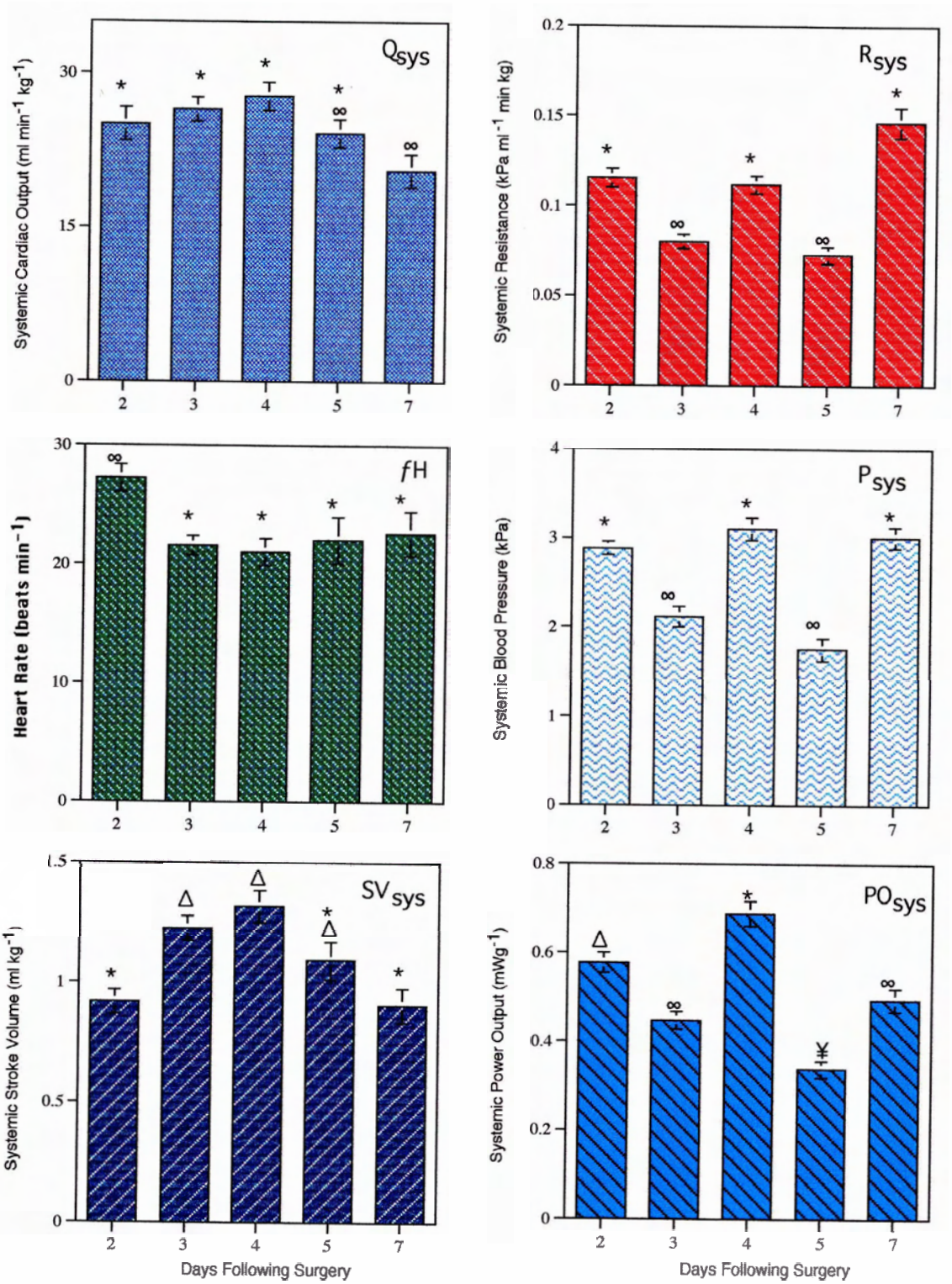

Qsys
Systemic Cardiac Output (ml min-1 kg-1)
Rsys
Systemic Resistance (kPa ml-1 min kg)
fH
Heart Rate (beats min-1)
Psys
Systemic Blood Pressure (kPa)
SVsys
Systemic Stroke Volume (ml kg-1)
POsys
Systemic Power Output (mWg-1)
Days Following Surgery
Days Following Surgery

Figure 2.5 Post-surgery cardiovascular variables during recovery for the 5°C normoxic turtles. Significant differences between columns are indicated by dissimilar symbols.

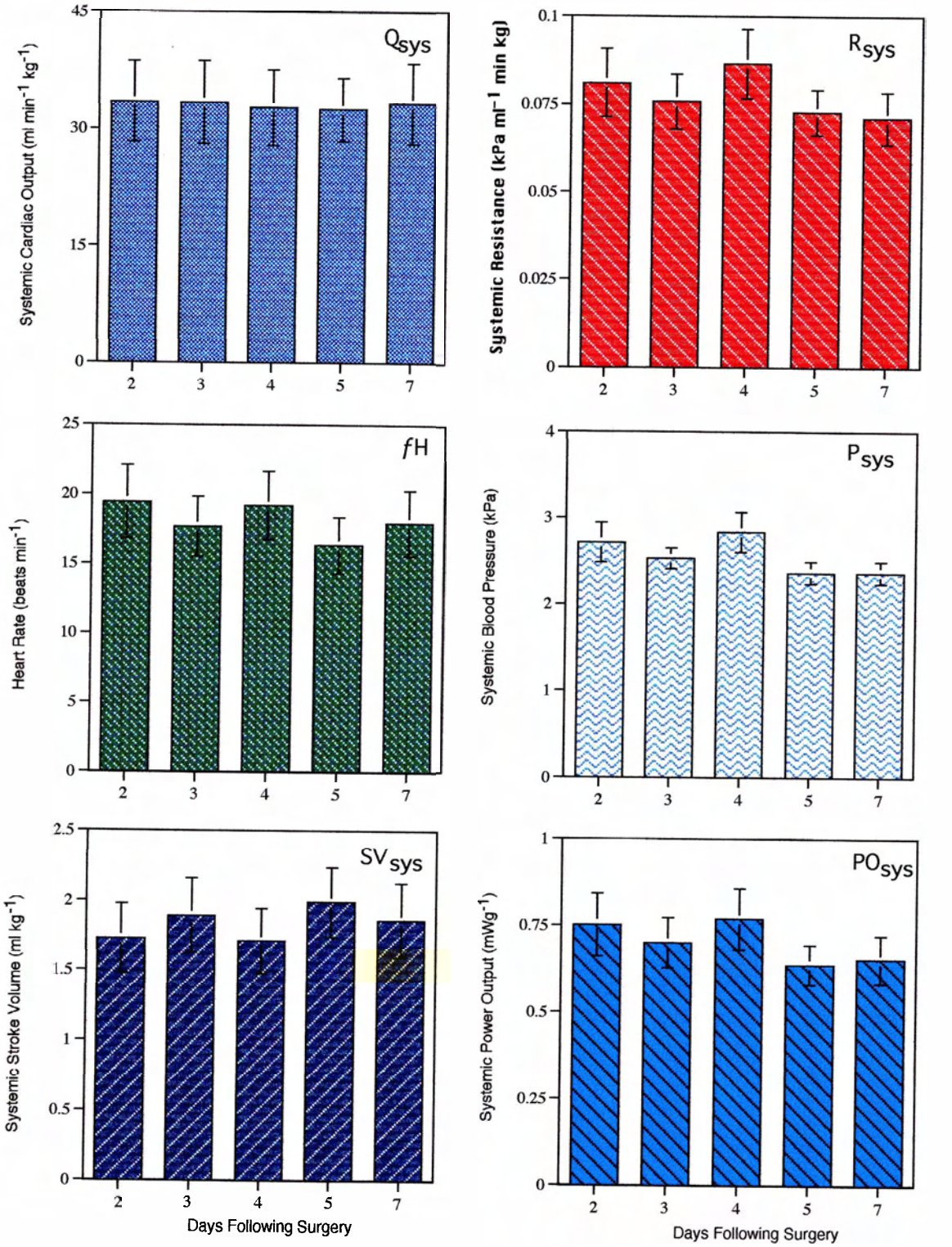

Systemic Cardiac Output (ml min-1 kg-1)
Qsys
45
30
15
0
2
3
4
5
7
Systemic Resistance (kPa ml-1 min kg)
Rsys
0.1
0.075
0.05
0.025
0
Heart Rate (beats min-1)
fH
25
20
15
10
5
0
Systemic Blood Pressure (kPa)
Psys
4
3
2
1
0
Systemic Stroke Volume (ml kg-1)
SVsys
2.5
2
1.5
1
0.5
0
Systemic Power Output (mWg-1)
POsys
1
0.75
0.5
0.25
0
Days Following Surgery
Days Following Surgery

Figure 2.6 Post-surgery cardiovascular variables during recovery for the 5°C anoxic turtles. Significant differences between columns are indicated by dissimilar symbols.

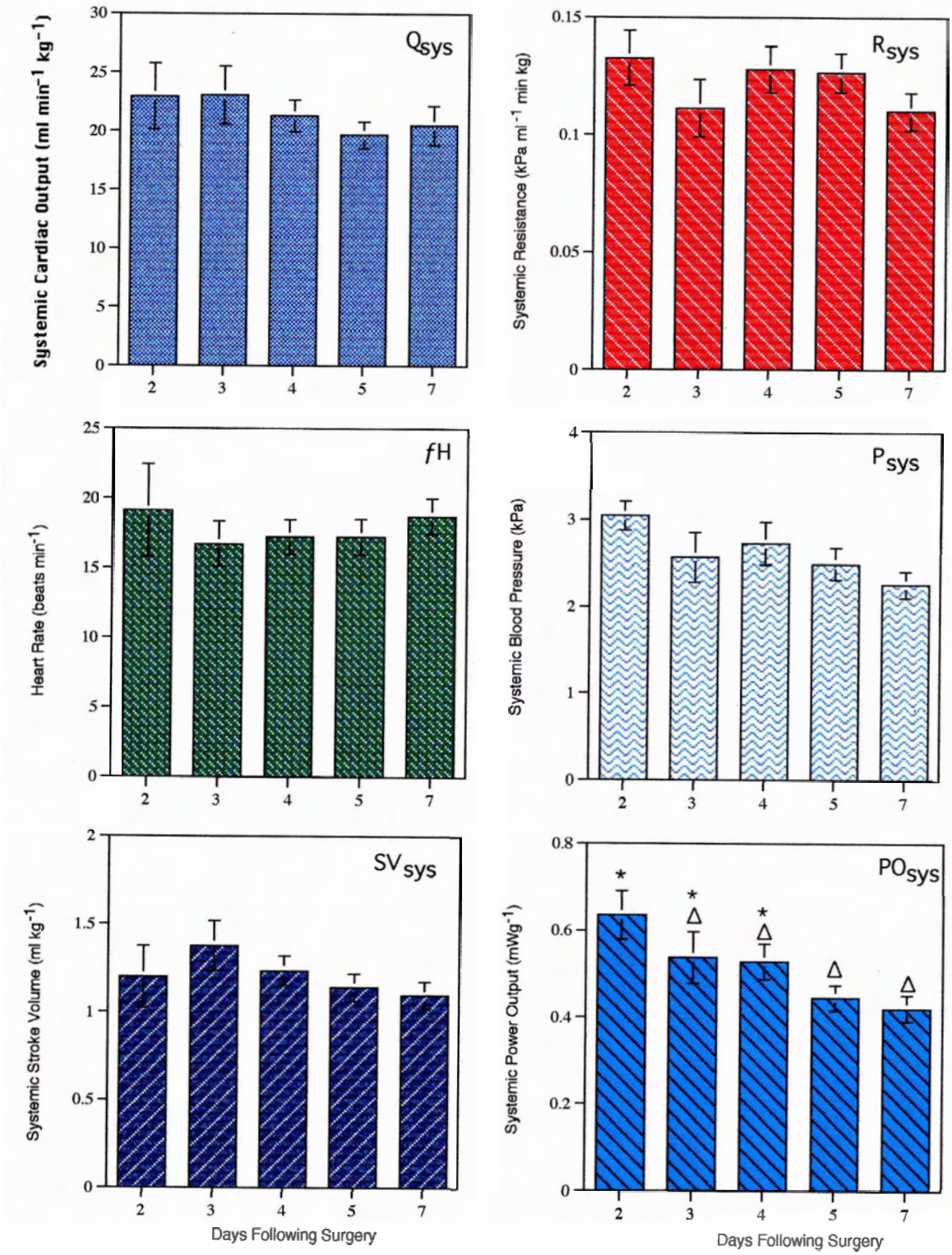

Systemic Cardiac Output (ml min-1 kg-1)
Qsys
Systemic Resistance (kPa ml-1 min kg)
Rsys
Heart Rate (beats min-1)
fH
Systemic Blood Pressure (kPa)
Psys
Systemic Stroke Volume (ml kg-1)
SVsys
Systemic Power Output (mWg-1)
POsys
Days Following Surgery
Days Following Surgery

Table 2.1 Post-recovery (Day 7) *in vivo* cardiovascular variables for turtles acclimated to either 22 or 5°C under normoxic or anoxic conditions. Mean values (SEM) are presented N=6 for all experimental groups, except 5°C anoxic which is 5. Significant differences between groups are indicated by dissimilar letters.

Test Group	Heart Rate (beats min^{-1})	Systemic Stroke Volume (ml kg^{-1})	Systemic Cardiac Output (ml $min^{-1}kg^{-1}$)	Mean Arterial Pressure (kPa)	Systemic Power Output (mW g^{-1} ventricular mass)	Systemic Resistance (kPa ml^{-1} min kg)
22°C Normoxic	24.2 (0.94)	1.49 (0.080) a,d	36.0 (2.49) a	2.25 (0.13) a	0.68 (0.043) a	0.063 (0.004) a
22°C Anoxic	22.7 (1.8)	0.91 (0.071) b	20.5 (1.54) b	3.01 (0.12) b	0.49 (0.025) b	0.15 (0.008) b
5°C Normoxic	17.9 (2.35)	1.86 (0.27) a	33.3 (5.19) a	2.36 (0.13) a	0.65 (0.069) a	0.071 (0.0075) a
5°C Anoxic	18.8 (1.28)	1.09 (0.081) b,d	20.5 (1.63) b	2.26 (0.15) a	0.42 (0.031) b	0.11 (0.0080) c

of anoxia. This evidence indicates that a measure of cardiovascular stability had been reached prior to the drug treatments.

Acclimation to 5°C

The cardiovascular variables recorded during 36 days of acclimation to 5°C are shown in Figure 2.8. Substantial cardiovascular change had already occurred by day 2. By the end of the first 10 days, all significant cardiovascular adjustments were completed. Q_{sys}, SV_{sys} , PO_{sys} and fH all decreased significantly while R_{sys} increased significantly. None of the cardiovascular variables changed significantly during day 20 through day 36.

Acclimation to 5°C Anoxia

The cardiovascular variables recorded during 28 days of 5°C acclimation with 21 days of anoxic acclimation beginning at day 6 are shown in Figure 2.9. Anoxic exposure significantly decreased Q_{sys}, P_{sys}, SV_{sys} , PO_{sys} and fH; while R_{sys} significantly increased. Significant differences during last 10 days of 5°C anoxic acclimation were few and minor.

Routine Cardiovascular Variables

Systemic Cardiac Output

The routine *in vivo* cardiovascular variables are presented in Table 2.2. These were recorded prior to drug infusion on the final day of acclimation for each group. Q_{sys} decreased significantly with cold-temperature acclimation and anoxia exposure. Compared with 22°C normoxic turtles, acclimation to 5°C reduced Q_{sys} by 7.8-fold (from

Figure 2.7 Cardiovascular variables of turtles acclimating to 22°C anoxia for 6 hours. Significant differences between columns are indicated by dissimilar symbols. At time zero turtles are recorded in normoxia while subsequent recording are in anoxia.

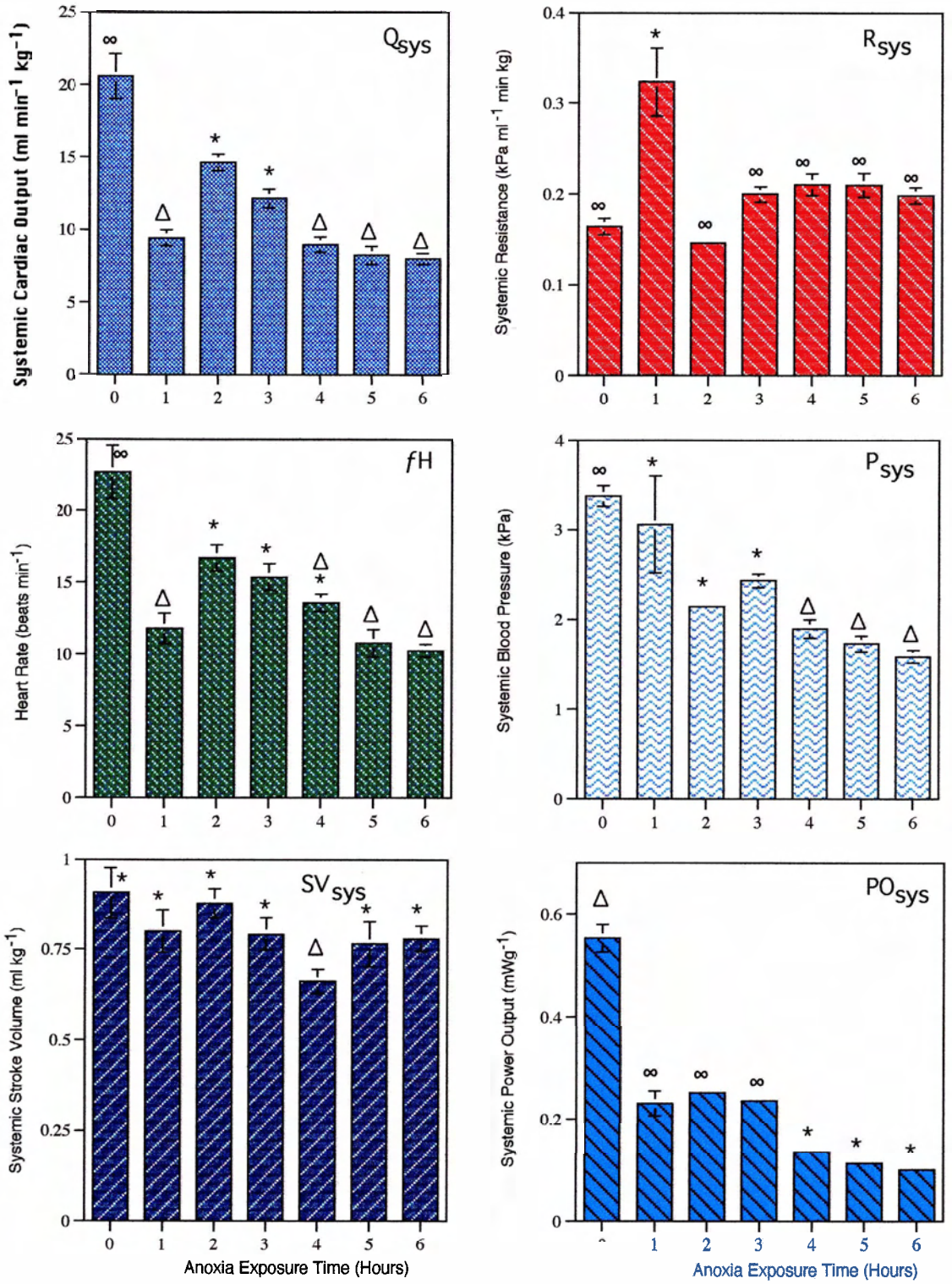

Systemic Cardiac Output (ml min-1 kg-1)
Qsys
Systemic Resistance (kPa ml-1 min kg)
Rsys
Heart Rate (beats min-1)
fH
Systemic Blood Pressure (kPa)
Psys
Systemic Stroke Volume (ml kg-1)
SVsys
Systemic Power Output (mWg-1)
POsys
Anoxia Exposure Time (Hours)
Anoxia Exposure Time (Hours)

Figure 2.8 Cardiovascular variables of turtles acclimating to 5°C normoxia for 5 weeks. Significant differences between columns are indicated by dissimilar symbols.

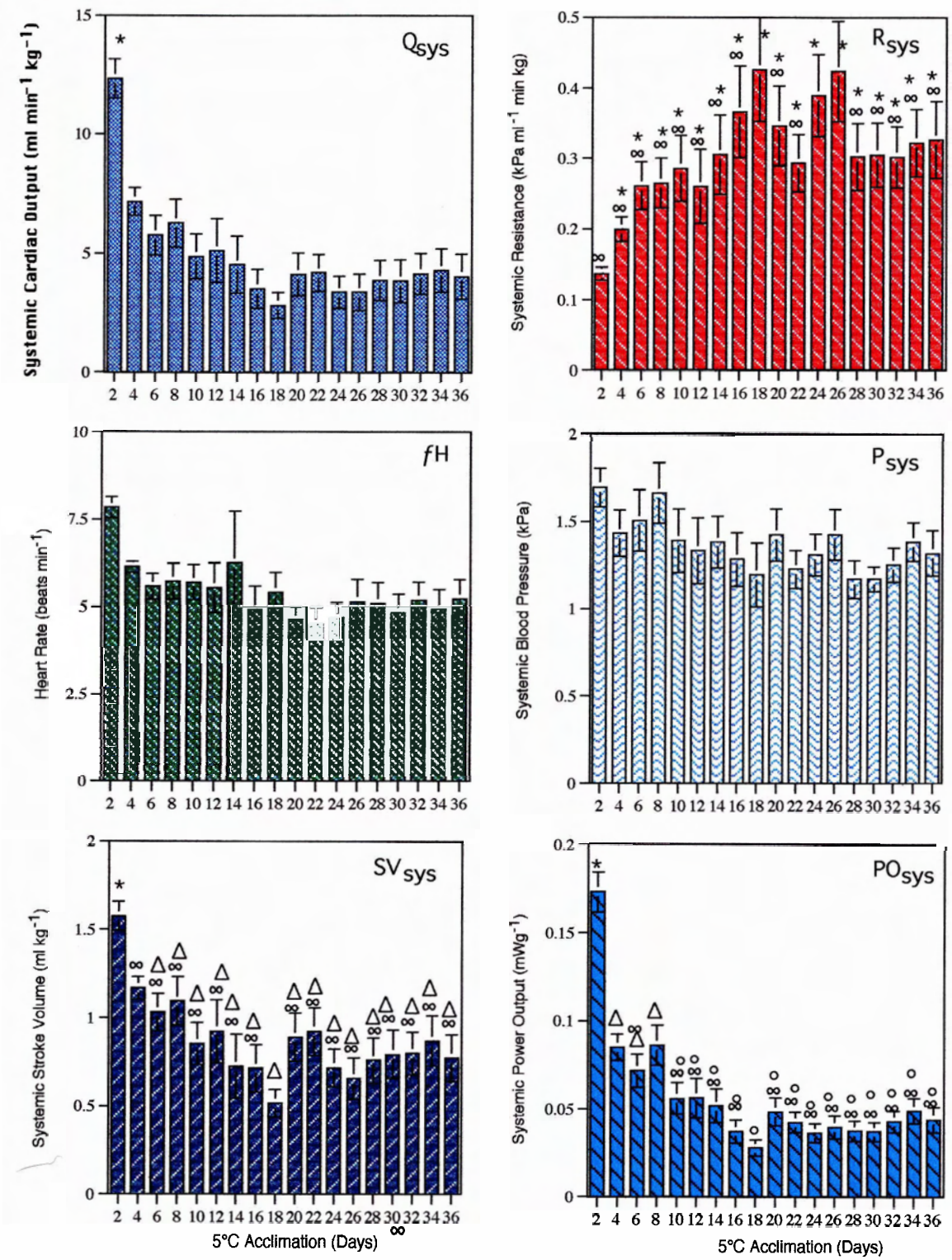
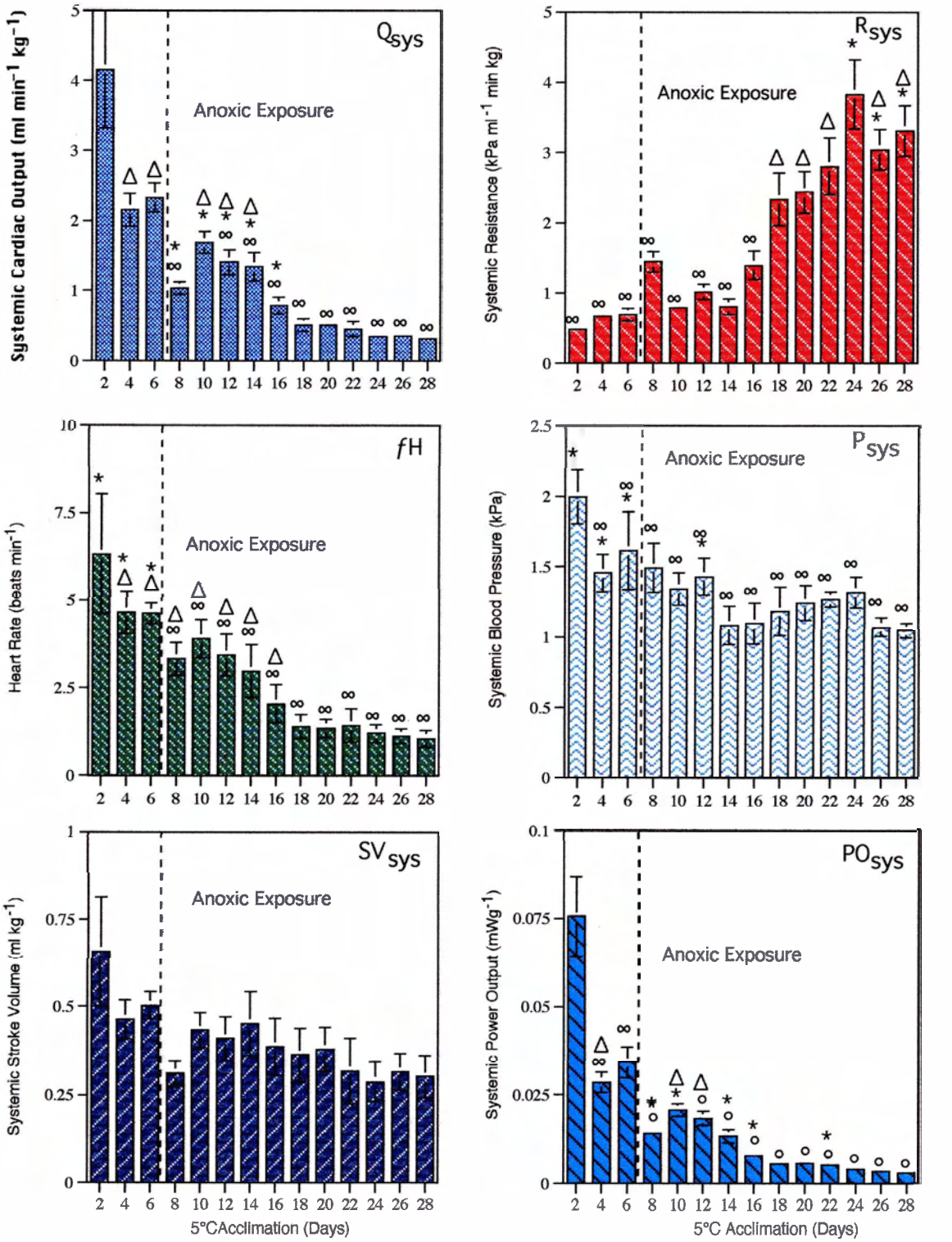

Systemic Cardiac Output (ml min-1 kg-1)
Qsys
Systemic Resistance (kPa ml-1 min kg)
Rsys
Heart Rate (beats min-1)
fH
Systemic Blood Pressure (kPa)
Psys
Systemic Stroke Volume (ml kg-1)
SVsys
Systemic Power Output (mWg-1)
POsys
5°C Acclimation (Days)
5°C Acclimation (Days)

Figure 2.9 Cardiovascular variables of turtles acclimating to 5°C anoxia for 3 weeks. Significant differences between columns are indicated by dissimilar symbols.


Systemic Cardiac Output (ml min-1 kg-1)
Qsys
Anoxic Exposure
Systemic Resistance (kPa ml-1 min kg)
Rsys
Anoxic Exposure
Heart Rate (beats min-1)
fH
Anoxic Exposure
Systemic Blood Pressure (kPa)
Psys
Anoxic Exposure
Systemic Stroke Volume (ml kg-1)
SVsys
Anoxic Exposure
5°CAcclimation (Days)
Systemic Power Output (mWg-1)
POsys
Anoxic Exposure
5°C Acclimation (Days)

32 to 4.1 ml min^{-1} kg^{-1}). Acute anoxic exposure at 22°C depressed Q_{sys} 4.2-fold. Compared with 5°C normoxia, acclimation to anoxia at 5°C decreased Q_{sys} by 15-fold (from 4.1 to 0.27 ml min^{-1} kg^{-1}). Compared with 22°C normoxia, Q_{sys} was 119-fold lower with 5°C anoxic acclimation.

Heart Rate and Systemic Stroke Volume

Bradycardia was the primary effector these profound reductions of Q_{sys}. Compared with 22°C normoxic turtles, acclimation to 5°C reduced fH by 4.5-fold and systemic stroke volume (SV_{sys}) by 1.8-fold. This transition resulted in a Q_{10} of 2.7 for fH. Acute anoxic exposure at 22°C depressed fH 2.5-fold from and SV_{sys} 1.8-fold. Acclimation to anoxia at 5°C decreased fH by 5.5-fold (from 5.5 to 1.0 beats min^{-1}) and SV_{sys} by 2.8-fold (from 0.75 to 0.27 ml kg^{-1}). Compared with 22°C normoxia, fH was 25-fold lower and SV_{sys} 5-fold lower with 5°C anoxic acclimation.

Systemic Blood Pressure

Compared with 22°C normoxic turtles, acclimation to 5°C significantly reduced P_{sys} by 2.0-fold from 2.90 to 1.54 kPa. Acute anoxic exposure at 22°C depressed P_{sys} 1.7-fold from 2.90 to 1.75 kPa. Compared with 5°C normoxia, acclimation to anoxia at 5°C decreased P_{sys} by 1.5-fold from 1.54 to 1.06 kPa. Compared with 22°C normoxia, P_{sys} was 2.7-fold lower with 5°C anoxic acclimation.

Systemic Power Output

Compared with 22°C normoxic turtles, acclimation to 5°C reduced PO_{sys} by 15-fold from 0.81 to 0.053 mW g^{-1}. Acute anoxic exposure at 22°C depressed PO_{sys} 7.4-fold from 0.81 to 0.11 mW g^{-1}. Compared with 5°C normoxia, acclimation to anoxia at 5°C decreased PO_{sys} by 20-fold from 0.053 to 0.0026 mW g^{-1}. Compared with 22°C normoxia, PO_{sys} was 312-fold lower with 5°C anoxic acclimation.

Systemic Vascular Resistance

Compared with 22°C normoxic turtles, acclimation to 5°C increased systemic vascular resistance (R_{sys}) by 4.3-times from 0.087 to 0.37 kPa ml^{-1} min kg. Acute anoxic exposure at 22°C increased R_{sys} by 2.6-fold from 0.087 to 0.23 kPa ml^{-1} min kg. Compared with 5°C normoxia, acclimation to anoxia at 5°C elevated R_{sys} by 11-times from 0.37 to 3.95 kPa ml^{-1} min kg. Compared with 22°C normoxia, R_{sys} was 45-times higher with 5°C anoxic acclimation.

Cardiovascular Control

A series of drug injections was used to assess the contribution of cholinergic and adrenergic regulation once the acclimation period had been completed (Figure 2.2). Saline injection resulted in no significant changes in cardiovascular variables except for a slight decrease in PO_{sys} in the 22°C normoxic group.

Atropine

Atropine infusion had a pronounced effect on cardiovascular variables in turtles acclimated to 22°C under normoxia and anoxia, as presented in Tables 2.3 and 2.4,

Table 2.2 Routine *in vivo* cardiovascular variables for turtles acclimated to either 22 or 5°C under normoxic or anoxic conditions. Mean values (SEM) are presented N=6 for all experimental groups, except 5°C anoxic which is 5. Significant differences between groups are indicated by dissimilar letters.

Test Group	Heart Rate (beats min^{-1})	Systemic Stroke Volume (ml kg^{-1})	Systemic Cardiac Output (ml $min^{-1}kg^{-1}$)	Mean Arterial Pressure (kPa)	Systemic Power Output (mW g^{-1} ventricular mass)	Systemic Resistance (kPa ml^{-1} min kg)
22°C Normoxic	25.0 (1.1) a	1.33 (0.080) a	32 (2.51) a	2.90 (0.076) a	0.81 (0.041) a	0.087 (0.004) a
22°C Anoxic	10.1 (0.45) b	0.76 (0.035) b	7.6 (0.36) b	1.75 (0.066) b	0.11 (0.0038) b	0.23 (0.016) a
5°C Normoxic	5.5 (0.32) c	0.75 (0.095) a,b,d	4.1 (0.80) c	1.54 (0.15) b	0.053 (0.0077) b	0.37 (0.054) a
5°C Anoxic	1.0 (0.05) d	0.27 (0.037) b,d	0.27 (0.028).c	1.06 (0.060) d	0.0026 (0.00021) c	3.95 (0.32) b

respectively. Atropine induced a tachycardia significantly increasing fH by 54% (from 22.6 to 34.7 beats min^{-1}) under normoxia and by 80% (from 9.33 to 16.7 beats min^{-1}) under anoxia. SV_{sys} significantly increased under anoxia by 34% from 0.74 to 0.99. As a result Q_{sys} significantly increased from 6.87 to 16.6 ml min^{-1} kg^{-1}. Under normoxia, Q_{sys} significantly increased from 32.9 to 40.7 ml min^{-1} kg^{-1} despite a drop in SV_{sys} (from 1.46 to 1.17 ml kg^{-1}). P_{sys} significantly increased from 2.35 to 3.82 kPa under normoxia and from 1.39 to 2.00 kPa under anoxia. This significantly increased PO_{sys} under anoxia from 0.076 to 0.265 mW g^{-1} and from 0.65 to 1.31 mW g^{-1} under normoxia. R_{sys} significantly decreased from 0.202 to 0.121 kPa ml^{-1} min kg under anoxia.

The effect of atropine infusion on turtles acclimated to 5°C under normoxia and anoxia is presented in Tables 2.5 and 2.6, respectively. Atropine injection at 5°C had little effect on the 5°C-acclimated groups. Q_{sys} rose significantly from 2.46 to 3.42 ml min^{-1} kg^{-1} while P_{sys} fell significantly from 2.12 to 1.35 kPa under normoxia. Under anoxia, PO_{sys} significantly increased from 0.0029 to 0.0041 mW g^{-1}.

Nadolol

Nadolol infusion had a depressive effect on cardiovascular variables in turtles acclimated to 22°C under normoxia and anoxia, as presented in Tables 2.3 and 2.4, respectively. Under normoxia, fH and SV_{sys} were significantly decreased from 30.8 to 25.7 beats min^{-1} and from 1.48 to 0.96 ml kg^{-1}, respectively. This significantly reduced Q_{sys} from 45.6 to 24.8 ml min^{-1} kg^{-1}. P_{sys} and R_{sys} significantly increased under

normoxia by 61% (from 2.25 to 3.62 kPa) and by 196% (from 0.049 to 0.145 kPa ml^{-1} min kg), respectively. Under anoxia, fH and Q_{sys} declined significantly from 12.7 to 10.9 beats min^{-1} and from 12.0 to 9.08 ml min^{-1} kg^{-1}, respectively. This significantly decreased P_{sys} from 0.157 to 0.118 kPa and significantly increased R_{sys} from 0.137 to 0.179 kPa ml^{-1} min kg.

The effect of nadolol infusion on turtles acclimated to 5°C under normoxia and anoxia is presented in Tables 2.5 and 2.6, respectively. Nadolol injection at 5°C had little effect on the 5°C-acclimated groups. Q_{sys} decreases significantly by 63% (from 3.33 to 1.24 ml min^{-1} kg^{-1}) under normoxia. Under anoxia, no significant changes occurred.

Adrenaline

The effect of adrenaline infusion in 22°C-acclimated turtles under normoxic conditions is shown in Table 2.7. Adrenaline initiated a pressor response, significantly increasing R_{sys} by 135% (from 0.089 to 0.209 kPa ml^{-1} min kg) which significantly increased P_{sys} by 55% (from 2.95 to 4.59 kPa). As a result, SV_{sys} significantly decreased from 1.33 to 0.78 ml kg^{-1}. Q_{sys} was not significantly changed in part due to a tachycardia, which significantly increased fH by 12% from 25.1 to 28.1 beats min^{-1}. Adrenaline injection following atropine infusion induced a pressor response as P_{sys} significantly rose from 2.80 to 4.65 kPa. This significantly increased R_{sys} which significantly reduced SV_{sys} from 1.63 to 0.87 ml kg^{-1} and Q_{sys} from 53.0 to 28.8 ml min^{-1} kg^{-1}. Adrenaline injection following atropine and nadolol infusion induced a pressor response which significantly elevated R_{sys} from 0.077 to 0.141 kPa ml^{-1} min kg and

significantly decreased SV_{sys} from 1.31 to 1.09 ml kg^{-1}. A significant tachycardia significantly increased PO_{sys} from 0.54 to 0.95 mW g^{-1}.

The effect of adrenaline infusion in 22°C-acclimated under anoxic conditions is shown in Table 2.8. The pressor response observed in the normoxic group was blunted in anoxia. R_{sys} significantly increased from 0.19 to 0.25 kPa ml^{-1} min kg which significantly decreased SV_{sys} from 0.90 to 0.69 ml kg^{-1}. Adrenaline injection following atropine infusion did not significantly change any cardiovascular variables. Adrenaline injection following atropine and nadolol infusion significantly increased SV_{sys} (from 0.69 to 0.73 ml kg^{-1}) and PO_{sys} (from 0.10 to 0.13 mW g^{-1}). The effect of adrenaline infusion in 5°C-acclimated under normoxia and anoxic conditions is shown in Tables 2.9 and 2.10. The only significant change was seen in the 5°C normoxic group with the first injection of adrenaline. The injection of adrenaline initiated a pressor response which significantly reduced SV_{sys} and Q_{sys}.

Table 2.3 Summary of cardiovascular results of drug infusion for turtles acclimated to 22°C under normoxic conditions for at least 3 weeks. Mean values (SEM) are presented; N=6 for all variables. Significant differences from control values are indicated by an asterisk.

Drug Injection	Heart Rate (beats min^{-1})	Systemic Stroke Volume (ml kg^{-1})	Systemic Cardiac Output (ml $min^{-1}kg^{-1}$)	Mean Arterial Pressure (kPa)	Systemic Power Output (mW g^{-1} ventricular mass)	Systemic Resistance (kPa ml^{-1} min kg)
Control	25.0 (1.1)	1.33 (0.080)	33.3 (2.5)	2.89 (0.076)	0.81 (0.041)	0.087 (0.0044)
Saline	23.0 (0.82)	1.30 (0.061)	29.9 (1.7)	2.66 (0.12)	0.67 * (0.034)	0.089 (0.0045)
Control	22.6 (0.36)	1.46 (0.056)	33.2 (1.86)	2.35 (0.058)	0.83 (0.037)	0.071 (0.0029)
Atropine after 1 hr.	32.6 * (0.40)	1.63 * (0.027)	53.1 * (1.09)	2.80 * (0.026)	0.85 (0.081)	0.053 (0.00079)
after 1.5 hr.	34.7 * (0.56)	1.17 (0.039)	40.6 * (2.02)	3.82 * (0.12)	0.65 (0.026)	0.093 (0.0038)
Control	30.8 (0.35)	1.48 (0.022)	53.0 (0.86)	2.25 (0.030)	1.25 (0.019)	0.049 (0.00080)
Nadolol & Atropine	25.7 * (0.80)	0.96 * (0.056)	28.8 * (2.08)	3.62 * (0.065)	1.13 (0.035)	0.145 * (0.0074)

Table 2.4 Summary of cardiovascular results of drug infusion for turtles acclimated to 22°C under anoxic conditions for 6 hours. Mean values (SEM) are presented N=6 for all variables. Significant differences from control values are indicated by an asterisk.

Drug Injection	Heart Rate (beats min^{-1})	Systemic Stroke Volume (ml kg^{-1})	Systemic Cardiac Output (ml $min^{-1}kg^{-1}$)	Mean Arterial Pressure (kPa)	Systemic Power Output (mW g^{-1} ventricular mass)	Systemic Resistance (kPa ml^{-1} min kg)
Control	10.1 (0.45)	0.76 (0.035)	7.62 (0.36)	1.75 (0.066)	0.106 (0.0040)	0.229 (0.0098)
Saline	9.95 (0.34)	0.72 (0.033)	7.19 (0.41)	1.78 (0.066)	0.102 (0.0043)	0.247 (0.012)
Control	9.33 (0.36)	0.74 (0.030)	6.87 (0.29)	1.39 (0.053)	0.076 (0.0027)	0.202 (0.0081)
Atropine after 1 hr.	16.7 * (0.87)	0.99 * (0.059)	16.6 * (1.12)	2.00 * (0.11)	0.265 * (0.014)	0.121 * (0.0074)
after 1.5 hr.	13.1 * (0.34)	0.92 (0.023)	12.1 (0.30)	1.64 * (0.031)	0.158 * (0.0031)	0.136 * (0.0030)
Control	12.7 (0.48)	0.94 (0.035)	12.0 (0.43)	1.65 (0.051)	0.157 (0.0047)	0.137 (0.0046)
Nadolol & Atropine	10.9 * (0.36)	0.84 (0.032)	9.08 * (0.40)	1.63 (0.055)	0.118 * (0.0040)	0.179 * (0.0069)

Table 2.5 Summary of cardiovascular results of drug infusion for turtles acclimated to 5°C under normoxic conditions for 5 weeks. Mean values (SEM) are presented N=6 for all variables. Significant differences from control values are indicated by an asterisk.

Drug Injection	Heart Rate (beats min^{-1})	Systemic Stroke Volume (ml kg^{-1})	Systemic Cardiac Output (ml $min^{-1}kg^{-1}$)	Mean Arterial Pressure (kPa)	Systemic Power Output (mW g^{-1} ventricular mass)	Systemic Resistance (kPa ml^{-1} min kg)
Control	5.49 (0.32)	0.75 (0.095)	4.12 (0.80)	1.54 (0.15)	0.053 (0.0077)	0.37 (0.054)
Saline	5.58 (0.39)	0.77 (0.11)	4.29 (0.88)	1.69 (0.20)	0.060 (0.0097)	0.39 (0.064)
Control	5.50 (0.76)	0.45 (0.078)	2.46 (0.52)	2.12 (0.26)	0.043 (0.0072)	0.86 (0.14)
Atropine after 1 hr.	6.63 (0.43)	0.52 (0.070)	3.42 * (0.71)	1.35 * (0.24)	0.038 (0.0074)	0.40 (0.076)
after 1.5 hr.	6.54 (0.43)	0.59 (0.082)	3.85 (0.81)	1.36 * (0.27)	0.043 (0.0088)	0.35 (0.072)
Control	6.50 (0.38)	0.51 (0.067)	3.33 (0.68)	1.59 (0.28)	0.044 (0.0083)	0.48 (0.090)
Nadolol & Atropine	4.96 (0.69)	0.25 (0.062)	1.24 * (0.44)	2.00 (0.40)	0.021 (0.0057)	1.61 (0.45)

Table 2.6 Summary of cardiovascular results of drug infusion for turtles acclimated to 5°C under anoxic conditions for 3 weeks. Mean values (SEM) are presented N=5 for all variables. Significant differences from control values are indicated by an asterisk.

Drug Injection	Heart Rate (beats min^{-1})	Systemic Stroke Volume (ml kg^{-1})	Systemic Cardiac Output (ml $min^{-1}kg^{-1}$)	Mean Arterial Pressure (kPa)	Systemic Power Output (mW g^{-1} ventricular mass)	Systemic Resistance (kPa ml^{-1} min kg)
Control	1.00 (0.18)	0.27 (0.037)	0.27 (0.028)	1.06 (0.063)	0.0026 (0.00021)	3.95 (0.32)
Saline	0.98 (0.17)	0.28 (0.037)	0.27 (0.026)	0.96 (0.097)	0.0024 (0.00023)	3.53 (0.34)
Control	1.05 (0.24)	0.26 (0.050)	0.27 (0.043)	1.18 (0.091)	0.0029 (0.00034)	4.36 (0.51)
Atropine after 1 hr.	1.24 (0.31)	0.31 (0.063)	0.38 (0.061)	1.21 (0.15)	0.0041 * (0.00061)	3.19 (0.46)
after 1.5 hr.	1.04 (0.24)	0.32 (0.060)	0.33 (0.049)	0.99 (0.081)	0.0030 (0.00034)	2.97 (0.34)
Control	1.00 (0.16)	0.32 (0.047)	0.32 (0.042)	0.85 (0.010)	0.0024 (0.00018)	2.69 (0.20)
Nadolol & Atropine	0.67 (0.10)	0.28 (0.040)	0.19 (0.025)	0.94 (0.13)	0.0016 (0.00022)	4.89 (0.67)

Table 2.7 Summary of cardiovascular results of adrenaline infusion (10 μg kg^{-1} body mass) for turtles under cholinergic and adrenergic blockade. Turtles were acclimated to 22°C under normoxic conditions for at least 3 weeks prior to drug infusion. Mean values (SEM) are presented N=6 for all variables. Significant differences from control values are indicated by an asterisk.

Drug Injection	Heart Rate (beats min^{-1})	Systemic Stroke Volume (ml kg^{-1})	Systemic Cardiac Output (ml min^{-1}kg^{-1})	Mean Arterial Pressure (kPa)	Systemic Power Output (mW g^{-1} ventricular mass)	Systemic Resistance (kPa ml^{-1} min kg)
Control	25.1 (0.63)	1.33 (0.060)	33.3 (2.1)	2.95 (0.071)	0.83 (0.032)	0.089 (0.0039)
Adrenaline	28.1 * (0.68)	0.78 * (0.073)	22.0 (3.5)	4.59 * (0.15)	0.85 (0.058)	0.209 * (0.0201)
Control	32.6 (0.56)	1.63 (0.039)	53.0 (2.0)	2.80 (0.12)	1.25 (0.026)	0.053 (0.0029)
Adrenaline after Atropine	35.4 (0.29)	0.87 * (0.020)	28.8 * (1.2)	4.65 * (0.095)	1.12 (0.054)	0.161 * (0.0050)
Control	22.2 (0.67)	1.31 (0.071)	29.0 (2.3)	2.23 (0.030)	0.54 (0.014)	0.077 (0.0042)
Adrenaline after Nadolol & Atropine	25.9 * (0.57)	1.09 * (0.090)	28.4 (4.1)	4.00 * (0.065)	0.95 * (0.039)	0.141 * (0.0146)

Table 2.8 Summary of cardiovascular results of adrenaline infusion (10 μg kg^{-1} body mass) for turtles under cholinergic and adrenergic blockade. Turtles were acclimated to 22°C under anoxic conditions for 12 hours prior to drug infusion. Mean values (SEM) are presented N=6 for all variables. Significant differences from control values are indicated by an asterisk.

Drug Injection	Heart Rate (beats min^{-1})	Systemic Stroke Volume (ml kg^{-1})	Systemic Cardiac Output (ml min^{-1}kg^{-1})	Mean Arterial Pressure (kPa)	Systemic Power Output (mW g^{-1} ventricular mass)	Systemic Resistance (kPa ml^{-1} min kg)
Control	9.89 (0.46)	0.90 (0.043)	8.88 (0.43)	1.71 (0.066)	0.12 (0.0047)	0.19 (0.0084)
Adrenaline	11.2 (0.49)	0.69 * (0.031)	7.75 (0.36)	1.94 (0.10)	0.12 (0.0054)	0.25 * (0.0126)
Control	13.1 (0.34)	0.92 (0.023)	12.1 (0.30)	1.64 (0.031)	0.16 (0.0031)	0.14 (0.0030)
Adrenaline after Atropine	12.7 (0.32)	0.92 (0.026)	11.7 (0.37)	1.67 (0.038)	0.16 (0.0038)	0.14 (0.0039)
Control	10.7 (0.16)	0.69 (0.015)	7.38 (0.20)	1.76 (0.065)	0.10 (0.0030)	0.24 (0.0077)
Adrenaline after Nadolol & Atropine	11.4 (0.18)	0.73 * (0.018)	8.30 (0.28)	1.96 (0.042)	0.13 * (0.0031)	0.24 (0.0065)

Table 2.9 Summary of cardiovascular results of adrenaline infusion (10 μg kg^{-1} body mass) for turtles under cholinergic and adrenergic blockade. Turtles were acclimated to 5°C under normoxic conditions for 5 weeks prior to drug infusion. Mean values (SEM) are presented N=6 for all variables. Significant differences from control values are indicated by an asterisk.

Drug Injection	Heart Rate (beats min^{-1})	Systemic Stroke Volume (ml kg^{-1})	Systemic Cardiac Output (ml min^{-1}kg^{-1})	Mean Arterial Pressure (kPa)	Systemic Power Output (mW g^{-1} ventricular mass)	Systemic Resistance (kPa ml^{-1} min kg)
Control	7.42 (1.6)	0.795 (0.20)	5.89 (1.7)	1.46 (0.12)	0.071 (0.013)	0.25 (0.045)
Adrenaline	6.46 (0.26)	0.372 * (0.035)	2.40 * (0.35)	2.66 * (0.21)	0.052 (0.0060)	1.11 * (0.125)
Control	6.54 (0.43)	0.590 (0.082)	3.86 (0.81)	1.36 (0.27)	0.043 (0.0088)	0.35 (0.072)
Adrenaline after Atropine	6.54 (0.41)	0.523 (0.071)	3.42 (0.71)	1.66 (0.24)	0.047 (0.0082)	0.49 (0.085)
Control	4.87 (0.64)	0.159 (0.047)	0.775 (0.36)	1.85 (0.34)	0.012 (0.0038)	2.38 (0.77)
Adrenaline after Nadolol & Atropine	4.79 (0.61)	0.119 (0.035)	0.568 (0.27)	2.12 (0.41)	0.010 (0.0033)	3.74 * (1.24)

Table 2.10 Summary of cardiovascular results of adrenaline infusion (10 μg kg^{-1} body mass) for turtles under cholinergic and adrenergic blockade. Turtles were acclimated to 5°C under anoxic conditions for 3 weeks prior to drug infusion. Mean values (SEM) are presented N=5 for all variables. Significant differences from control values are indicated by an asterisk.

Drug Injection	Heart Rate (beats min^{-1})	Systemic Stroke Volume (ml kg^{-1})	Systemic Cardiac Output (ml min^{-1}kg^{-1})	Mean Arterial Pressure (kPa)	Systemic Power Output (mW g^{-1} ventricular mass)	Systemic Resistance (kPa ml^{-1} min kg)
Control	1.00 (0.16)	0.278 (0.035)	0.278 (0.026)	0.93 (0.079)	0.0024 (0.00021)	3.37 (0.30)
Adrenaline	1.09 (0.24)	0.275 (0.047)	0.299 (0.036)	1.14 (0.11)	0.0031 (0.00034)	3.83 (0.41)
Control	1.04 (0.24)	0.320 (0.060)	0.332 (0.049)	0.99 (0.081)	0.0030 (0.00034)	2.97 (0.34)
Adrenaline after Atropine	1.08 (0.25)	0.313 (0.060)	0.336 (0.051)	1.00 (0.079)	0.0031 (0.00036)	2.97 (0.34)
Control	0.700 (0.097)	0.271 (0.036)	0.189 (0.025)	0.92 (0.14)	0.0016 (0.00023)	4.88 (0.69)
Adrenaline after Nadolol & Atropine	0.750 (0.093)	0.212 (0.030)	0.159 (0.025)	0.87 (0.13)	0.0013 (0.00020)	5.51 (0.86)

Discussion

Cardiac Performance under Normoxia

In the present study, *Trachemys scripta* acclimated to either 22 or 5°C for at least 5 weeks under normoxic conditions had *in vivo* heart rates of 25 and 5.5 beats min^{-1}, respectively. These values compare very well with those of Herbert and Jackson (1985b), who reported normoxic values of 30 beats min^{-1} at 20°C and 1.8 beats min^{-1} after 3 months at 3°C for *Chrysemys scripta.* However, this is lower than control values given for *T. scripta* at 22°C by Hicks (1994) and Comeau and Hicks (1994) which were 38 and 43 beats min^{-1}, respectively. These values probably represent turtles in an active ventilatory state, as turtles experiencing apnoea at 19°C have heart rates of 11 beats min^{-1} which increase sharply to 23-30 beats min^{-1} with ventilation (Shelton and Burggren, 1976).

In turtles, heart rate is under powerful inhibitory cholinergic control and a weaker adrenergic stimulatory control (White, 1976). This mechanism is demonstrated by the rapid elimination of sympathetic-induced tachycardia by vagal tone (Akselrod *et al.*, 1985). Intrinsic heart rates obtained from an *in situ* preparation for *C. scripta* acutely exposed to 15 and 5°C are 23.4 and 8.1 beats min^{-1} (Farrell *et al.*, 1994). This indicates that at 5°C heart rate could be under vagal inhibitory tone since *in situ* values are higher than *in vivo*. An alternative explanation is that acclimation to 5°C permits the expression of physiological changes that depress heart rate. The hearts of frogs *(Rana temporaria)* when acclimated to low temperature have lower contraction frequencies when tested over a wide range of test frequencies when compared to warm-acclimated hearts. Possible

mechanisms for these changes include the expression of myosin isoforms with lower ATPase activities (Voranen, 1994) or a lengthing of the activiation and relaxation phases of cardiac contraction. In fact, atropine infusion in the 5°C normoxic group did not significantly increase heart rate indicating little vagal tone was present (see Table 2.4).

Systemic stroke volumes at 22 and 5°C in the present study were 1.33 ml kg^{-1} and 0.75, respectively. Values reported by Hicks (1994), 1.8 ml kg^{-1} and Shelton and Burggren (1976), 1.34 ml kg^{-1} at 20°C are very similar. Since *in vivo* stroke volumes of 2.3-2.5 ml kg^{-1} (Shelton and Burggren, 1976) closely approximates the maximum stroke volume *in situ* of 2.5 ml kg^{-1} there could be little room for increase (Farrell *et al.* 1994). Increases in cardiac output would then primarily be modulated by heart rate which would reduce undersirable intracardiac mixing of venous and arterial blood caused by stroke volume changes (Satchell, 1991).

A comparison of mean systemic arterial pressure reported in several studies are in good agreement with the values in this study. Comeau and Hicks (1994) and Hicks (1994) found systemic blood pressure of 4.02 and 2.01 kPa, respectively at 20°C which compares favorably with 2.9 kPa in this study. Herbert and Jackson (1985b) reported mean arterial pressure of 3.4 kPa at 20°C , while at 3°C a value of 1.9 kPa was seen. At 5°C mean systemic arterial pressure was 1.54 kPa. Adrenaline infusion initiated a pressor response independent of temperature (see Tables 2.8 and 2.10). Turtles showed only slight tachycardia responses at 22°C, probably as a result of high vascular resistance due to the pressor response. In addition, the turtle heart has been shown to be a poor homeometric regulator *in situ* (Farrell *et al.*, 1994). Adrenaline infusion only initated a

significant pressor response at 22°C following atropine injection and following atropine and nadolol injection. At 5°C a slight increase in mean arterial pressure was seen.

Systemic cardiac output varies significantly between ventilating and apnoea (Wang and Hicks, 1996) which introduces variation in comparisons with other studies. In the present study systemic cardiac output and systemic cardiac power output were 32.0 ml min^{-1} kg^{-1} and 0.81 mW g^{-1} at 22°C. Comeau and Hicks (1994) and Hicks (1994) reported systemic cardiac outputs of 29.2 and 24 ml min^{-1} kg^{-1} and systemic cardiac power outputs of 1.4 and 0.44 mW g^{-1}, respectively at 20°C. Assuming systemic cardiac output is 1.8-times pulmonary cardiac output (Wang and Hicks, 1996) then total cardiac power output would be 2.1 mW g^{-1}. *In situ* maximum cardiac power output at 15°C was 2.56 mW g^{-1} (Farrell *et al.* 1994) while *in vivo* values are 3.02 mW g^{-1} (Comeau and Hicks, 1996) and 2.46 mW g^{-1} (Shelton and Burggren, 1976) at 20°C.

Cardiac Performance under Anoxia

Anoxic exposure significantly decreased heart rate and stroke volume and increased systemic resistance. At 22°C, a 2.5-fold decrease in heart rate and a 1.8-fold reduction in stroke volume was seen, while at 5°C the corresponding numbers are 5.5-fold and 2.8-fold. At 22°C, the reduction in chronotrophy appears to be caused by vagal inhibitory tone as heart rate increased after atropine infusion (see Table 2.4). Signore and Jones (1995) noted that nadolol had no effect on diving bradycardia in muskrats. They suggest that intense vagal activity would block sympathetic input and thereby take over cardiac control. This would be an efficient means of rapidly suppressing sympathetic

influences since sympathetic responses are slower than parasympathetic (Akselrod *et al.*, 1985; Furilla and Jones, 1987). Bailey and Driedzic (1995) found no significant difference in heart rate between isolated hearts perfused with normoxic and anoxic saline at 15°C, supporting vagal-induced bradycardia.

Vagal inhibiton of heart rate would be an effective design for short-term diving but at lower temperatures a different system appears to operate. Atropine infusion into 5°C-acclimated turtles under anoxia resulted in only minor increases in heart rate. As well, nadolol infusion resulted in only minor reductions in heart rate. This suggests that intrinsic factors such as anoxia, acidosis and temperature may have substantial roles in effecting the massive decrease in heart rate seen in cold anoxia. Anoxia has been shown to decreases the rate of spontaneous contractions of cardiac muscle strips (Bing *et al.*, 1972; Jackson, 1987). This intrinsic component was also seen as a 2-3 beat min^{-1} bradycardia without acidosis in *in situ* turtle hearts independent of temperature (Farrell *et al.* 1994). This could also be augmented by the acidosis associated with submersion in turtles. In fact, acidosis has been implicated as an important controlling factor of the down-regulation of metabolism during anoxia in anoxia-tolerant animals (Malan *et al.*, 1985). This is supported by observations that turtles with a more pronounced acidosis defend glycogen stores better indicating that energy demand is lower in these animals (Wasser *et al.*, 1991).

Under anoxia, the pressor response to adrenaline was blunted indicating that **adrenergic sensitivity was reduced independent of temperature.** A reduction of adrenergic sensitivity under these conditions would depress cardiac function at a time of

energy constraint. In addition, Wasser and Jackson (1991) have measured elevated catecholamine levels in turtles exposed to anoxia, yet heart rate and blood pressure fall under these conditions (Herbert and Jackson, 1985b). The mechanism of this response will be focused on in chapter 3.

The reduction of blood pressure by low temperature and anoxia could be an effective modulator of blood redistribution. When transmural pressure falls to a critical pressure so that the elastin fibers are unstrectch in vascular smooth muscle the vessel collaspses removing blood flow to the peripheral vascular beds (Burton, 1961). Due to their small diameter and large amount of smooth muscle the arterioles are the critical vessels which restrict blood flow (Burton, 1972). Peripheral vascular beds located in the brain will have lower critical pressures which will maintain blood flow. However, less critical organs, like the gut, have higher critical pressures and so will lose blood flow. This represents an efficent mechanism of peripheral circulation control which allows blood pressure to be the driving force behind blood redistribution.

The present study is the first to measure systemic cardiac power output in turtles acclimated to cold anoxia. A comparison of systemic cardiac power output of 22°C-acclimated turtles under normoxia with 5°C-acclimated turtles under anoxia reveal a 312-fold reduction from 0.81 to 0.0026 mW g^{-1}. It is interesting to note that this is an underestimation of the actual difference, since the cardiac power output is greater for the 22°C-acclimated group because the pulmonary component is not included. Cardiac power output is proportional to ATP demand which indicates that glycolytic rate is not depressed beyond the normoxic rate at 22°C and may slightly be depressed at 5°C. The

effect of acute anoxia at 22 and 5°C-acclimation decreased systemic cardiac power output by 7.4 and 15-fold, respectively. Therefore, acclimation to cold anoxia caused a 2.8-fold depression of PO_{sys} beyond that expected from the product of cold acclimation and short-term anoxia alone. This could represent the expression of physiological modifications which suppress cardiac function further upon acclimation to cold anoxia.

CHAPTER 3

Introduction

As detailed in Chapter 2, anoxic exposure blunts the pressor response of turtles infused with adrenaline independent of temperature. Following adrenaline injection, systemic resistance increased by 344% at 5°C and by 135% at 22°C under normoxia, however, under anoxia these increases were reduced to 14% and 32%, respectively. As well, a significant tachycardia was seen after adrenaline infusion in turtles acclimated to 22°C under normoxia but not in any other group. The mechanism of this effect is unknown but could be the result of a number of factors, including, a reduction in adrenergic sensitivity of cardiac muscle. Adrenergic sensitivity of cardiac muscle is modulated by cell-surface ß-adrenoreceptors as well as by levels of enzyme cascade intermediates, such as cyclic AMP.

Exposure of mammalian cardiomyocytes to a hypoxic environment results in a 35-65% reduction in cell-surface ß-adrenoreceptor density (Rocha-sigh *et al.*, 1991; Bernstein *et al.*, 1990, 1992; Voelkel *et al.*, 1981). This loss of ß-adrenoreceptors diminishes the inotropic and chronotropic sensitivity of the myocardium to catecholamines, reducing cardiac performance. A reduction in the cell-surface ß-adrenoreceptor density and/or binding affinity could be an important mechanism for depressing cardiac function in freshwater turtles during anoxia exposure. Turtles seasonally experience anoxia when they over-winter in lake sediments. Moreover, catecholamine levels are known to increase during anoxic exposure in painted turtles (Wasser and Jackson, 1991) and this would tend to stimulate cardiac activity at a time

when it is known to be depressed. For example, heart rate fell by 3.5 times after anoxic exposure for 12 hours at 20°C (Herbert and Jackson, 1985b). In addition, heart rate decreased by 2.5 times after anoxic exposure for 12 hours at 22°C (see Table 2.1). Anoxic exposure reduced the isometric force of isolated cardiac strips from turtle ventricle to 53% of control normoxic tension (Jackson, 1987). Factors such as acidosis and a rise in inorganic phosphate, which are characteristic of anoxic exposure, certainly depress cardiac function (Orchard and Kentish, 1985; Kentish, 1986; Hibberd *et al.*, 1985; Jackson, 1987), but reduction of ß-adrenoreceptor density could represent a potent regulator of cardiac function. In fact, anoxic exposure has been shown to blunt the adrenergic response to drugs *in vivo* (T.Wang, pers. comm.). In this study, the pressor response initiated by adrenaline infusion was blunted by 4.3-times at 22°C and by 3.6-times at 5°C (see Chapter 2).

In this study, ß-adrenoreceptor densities in the ventricles of red-eared sliders (*Trachemys scripta*) acclimated for at least 3 weeks to either 22 or 5°C under normoxic or anoxic conditions were measured for the first time. The primary focus was to determine if the known loss of responsiveness to catecholamines in anoxic turtles was related to a reduction in cardiac ß-adrenoreceptor density.

Adrenergic sensitivity is known to vary regionally. Ball and Hicks (1996) found that muscle strips prepared from the dorsal half of the turtle ventricle were more sensitive to applied adrenaline than strips from the ventral half. The authors suggested that fiber orientation accounted for this difference since microscopic examination found that the majority of muscle fibers in the dorsal muscle strips were oriented longitudinally from

apex to base while ventral strips were arranged circumferentially. Thus, contraction of the longitudinal fibers would be more easily detected by force transducers. In the present study, dorsal and ventral halves of the ventricle were incubated independently to identify if the differences in adrenergic sensitivity were related to ß-adrenoreceptor densities. Haematological variables (pH, hematocrit and hemoglobin content) were also measured to assess the degree of anoxia.

Methods and Materials

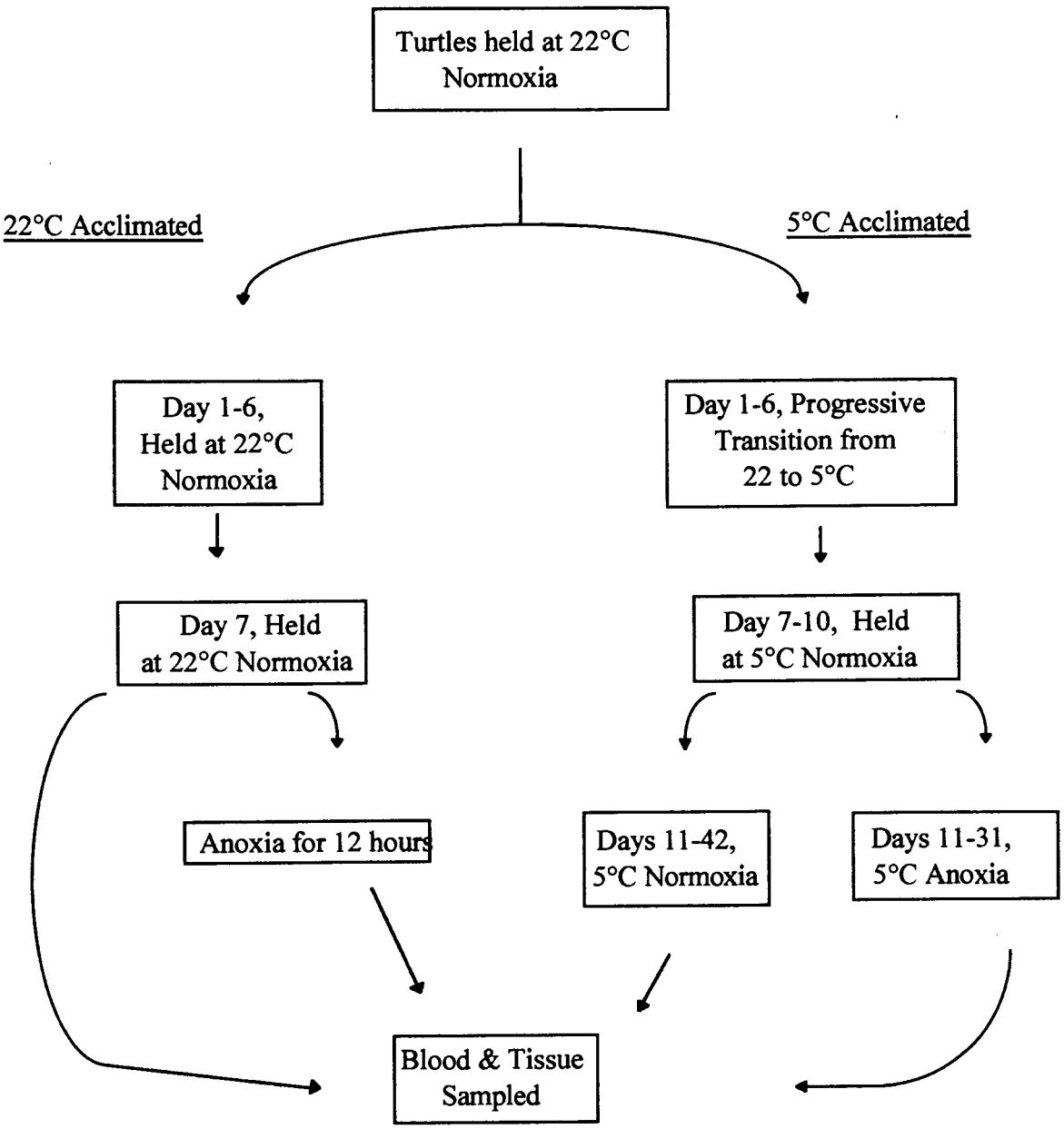
Experimental Animals

Red-eared sliders *(Trachemys scripta)* (mean body mass 761 ± 59g) were obtained from a commercial supplier (Carolina Biological Supply Co) and held indoors in standing water in polypropylene containers. The turtles were fed on commercial pellets (Wardley Co), small pieces of fish and lettuce at least three times per week. Turtles were given access to basking platforms as well as deep water. Room and basking lights were set for a 12L:12D photoperiod. There was no significant differences observed in mean body mass between the experimental groups (see Table 3.1).

Experimental Protocol

Turtles were acclimated for at least 3 weeks to either 22 or 5°C under normoxic or anoxic conditions as described previously in chapter 2 (see Figure 3.1). To control for possible differences between males and females, three males turtles and three female turtles were used in each test group. Turtles were removed from their holding tank, decapitated and a piece of umbilical tape was quickly secured around their neck. This

Figure 3.1. Experimental protocol for the ß-adrenoreceptor study.



prevented excessive blood loss prior to blood sampling. An electric bone saw (Mopec, Detroit, MI) was used to remove a 4 cm x 5 cm piece of the plastron exposing the heart and systemic output vessels. A syringe was used to withdraw 1.5 ml of blood from the left aorta which was analyzed for pH, hemoglobin and hematocrit. A blood pH analyzer (Radiometer, Copenhagen, DK) was used for pH measurement while hemoglobin content was determined spectrophotometrically using a Sigma total hemoglobin kit (Sigma Chemical Co., St.Louis, MO). Haematocrit was measured on 20 µl blood samples centrifuged at 10,000 g for 2 minutes. The ventricle was excised, washed with turtle saline, weighed and frozen in liquid nitrogen. Turtle saline consisted of the following (in mM): NaCl, 80; KCl, 2.7; $CaCl_2$, 2; $MgSO_4$, 1.4; $NaHCO_3$, 40; NaH_2PO_4, 2.2; Na_2HPO_4, 0.2. The tissue was stored at -70°C for five months prior to the ß-adrenoreceptor assay.

Cardiac ß-Adrenoreceptors

Cell-surface ß-adrenoreceptor density (B_{max}) and binding affinity (K_D) were determined using a tissue punch - tritiated ligand incubation technique. The technique originally used for mammalian hearts (Wilkinson *et al.*, 1991) has been modified for fish hearts Gamperl *et al.* (1994) and these modifications were applied here for turtle hearts. Dorsal and ventral ventricular tissue punches (2 mm diameter x 350 µm thick) were incubated with various concentrations of the hydrophilic ß-adrenoreceptor ligand [^{3}H] CGP-12177 (CGP) for two hours. Some of the tissue punches at each concentration were incubated with the competitive ß-adrenoreceptor antagonist timolol (10^{-5} M) to calculate non-specific binding. Following removal of the incubation medium, and two washes in turtle saline, tissue punches were added to into scintillation vials containing 4 ml of

Ecolite scintillation fluid (ICN biomedical, Costa Mesa, CA) and counted in a liquid scintillation counter (LS 6500, Beckmann). Specific binding was calculated by subtracting the radioactivity measured in punches incubated with CGP and timolol from the activity of punches incubated with CGP alone. Non specific binding was generally less than 23% of specific binding. Saturation binding curves were analyzed using the method of Zivin and Ward (1982) to determine B_{max} and K_D. Total protein content of representative punches was measured spectrophotometrically using a Bradford protein assay so that B_{max} could be expressed as fmol mg protein^{-1}. Dorsal and ventral punches were incubated separately to determine if the binding curves were different between these two portions of the heart.

Statistical Analysis

In all cases mean values (SEM) for six animals are presented. Differences between means of experimental groups were determined using one-way and two-way analyses of variance (ANOVA) for repeated measures, while multiple comparisons were performed using Student-Newman-Keuls tests. $P < 0.05$ was used as the level of significance.

Results

Blood variables are summarized in Table 3.1. Anoxic exposure significantly decreased blood pH from normoxic conditions independent of temperature. At 22°C, normoxic turtles had a mean blood pH of 7.86 while anoxic turtles had a value of 7.01. While at 5°C, the reduction in blood pH betweeen normoxic and anoxic groups was 7.76

to 7.17. The acidosis with 1 day of 22°C anoxia was significantly greater than that with 21 days of 5°C anoxia (pH 7.01 vs. 7.17).

Hematocrit also changed significantly with anoxic exposure, independent of temperature. Normoxic turtles (22.5%) had 25% larger hematocrit than anoxic turtles (17%). In contrast, mean cell hemoglobin content increased in 5°C- and 22°C-acclimated groups under anoxic exposure, by 11% (from 3.5 to 3.9 g dl^{-1} $\%^{-1}$) and by 22% (from 3.2 to 3.9 g dl^{-1} $\%^{-1}$), respectively (see Table 3.1).

Examples of saturation binding curves are shown in Figures 3.2 and 3.3. From these curves B_{max} and K_D were calculated (see Table 3.2). B_{max} and K_D were found to be similar in dorsal and ventral punches (Figures 3.2, 3.3). Although male turtles were found to have a larger relative ventricular mass, there was no difference found in either B_{max} or K_D between males and females (data not shown). Anoxic exposure significantly reduced ß-adrenoreceptor density independent of temperature. B_{max} decreased by 40% (from 80 to 48 fmol mg $protein^{-1}$) at 22°C and by 39% (from 62 to 38 fmol mg $protein^{-1}$) at 5°C. Although B_{max} values were numerically lower in 5°C- compared with 22°C-acclimated turtles, these differences were not significant. K_D values were not significantly different between any experimental group (Table 3.2).

Table 3.1: Body mass, blood pH, hematocrit, and hemoglobin content for each experimental group used for β-adrenoreceptor density determination. Mean values (SEM) are presented for N= 6 in each case. Dissimilar letters indicate significance between groups ($p<0.05$).

Test Group	Body mass (g)	Blood pH	Hematocrit (%)	Mean Cell Hemoglobin Content ($g\ dl^{-1}\ \%^{-1}$)
22°C Normoxic	666 (74.4)	7.86 (0.060) a	22.9 (0.81) a	3.18 (0.483)
22°C Anoxic	841 (39.4)	7.01 (0.032) b	16.6 (1.61) b	3.89 (0.556)
5°C Normoxic	707 (63.2)	7.76 (0.034) a	21.8 (0.99) a	3.46 (0.862)
5°C Anoxic	832 (60.7)	7.17 (0.034) c	17.2 (2.28) b	3.90 (0.454)

Figure 3.2 ß-adrenoreceptor binding curves for 22°C acclimated turtles. A. Turtles acclimated to normoxia for at least 4 weeks. B. Turtles exposed to anoxia for 12 hours. Significant differences between dorsal and ventral densities are indicted by asterisks.

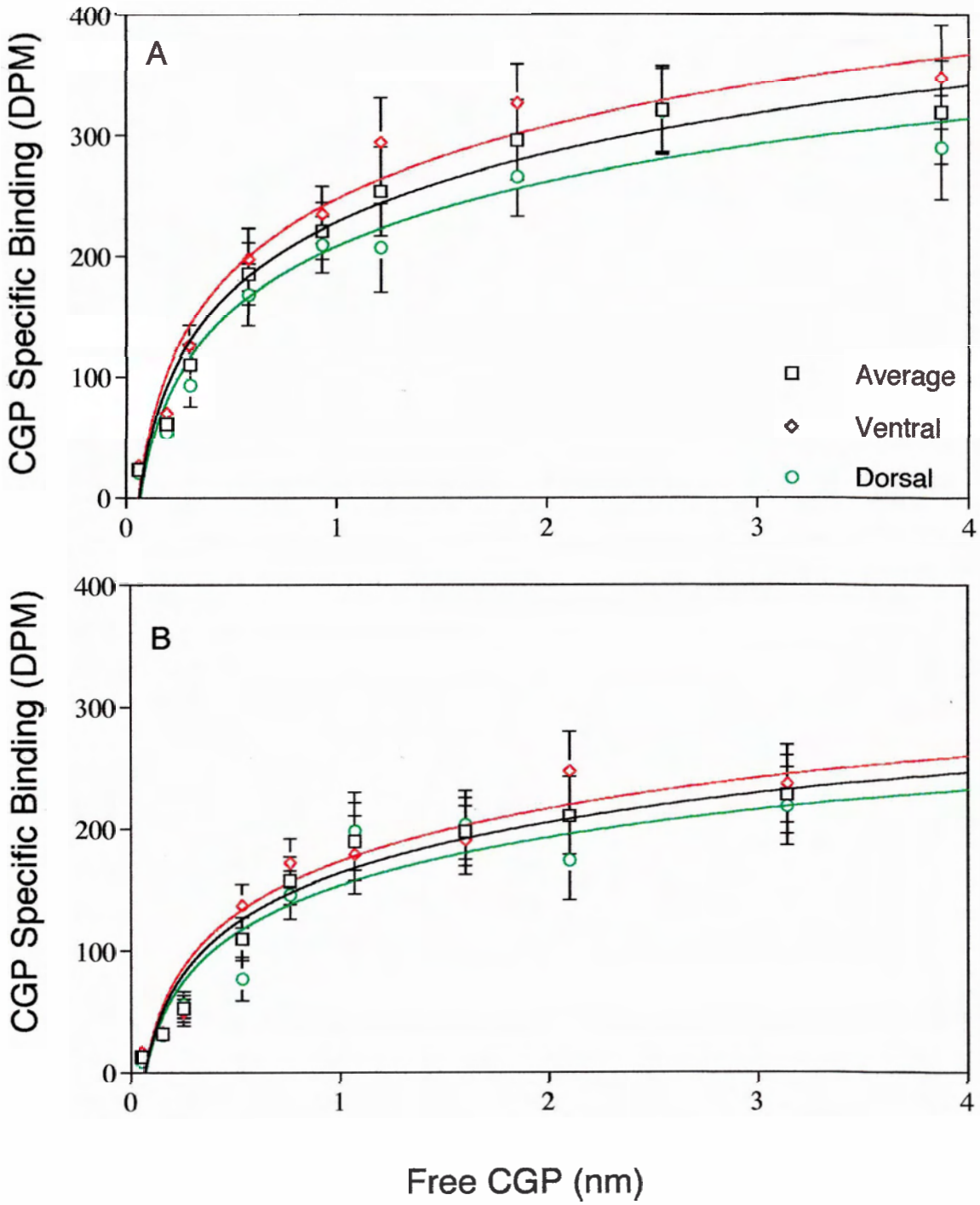

A
B
CGP Specific Binding (DPM)
CGP Specific Binding (DPM)
Average
Ventral
Dorsal
Free CGP (nm)
0
100
200
300
400
0
1
2
3
4

Figure 3.3 ß-adrenoreceptor binding curves for 5°C acclimated turtles. A. Turtles acclimated to normoxia for 5 weeks. B. Turtles exposed to anoxia for 3 weeks. Significant differences between dorsal and ventral densities are indicted by asterisks.

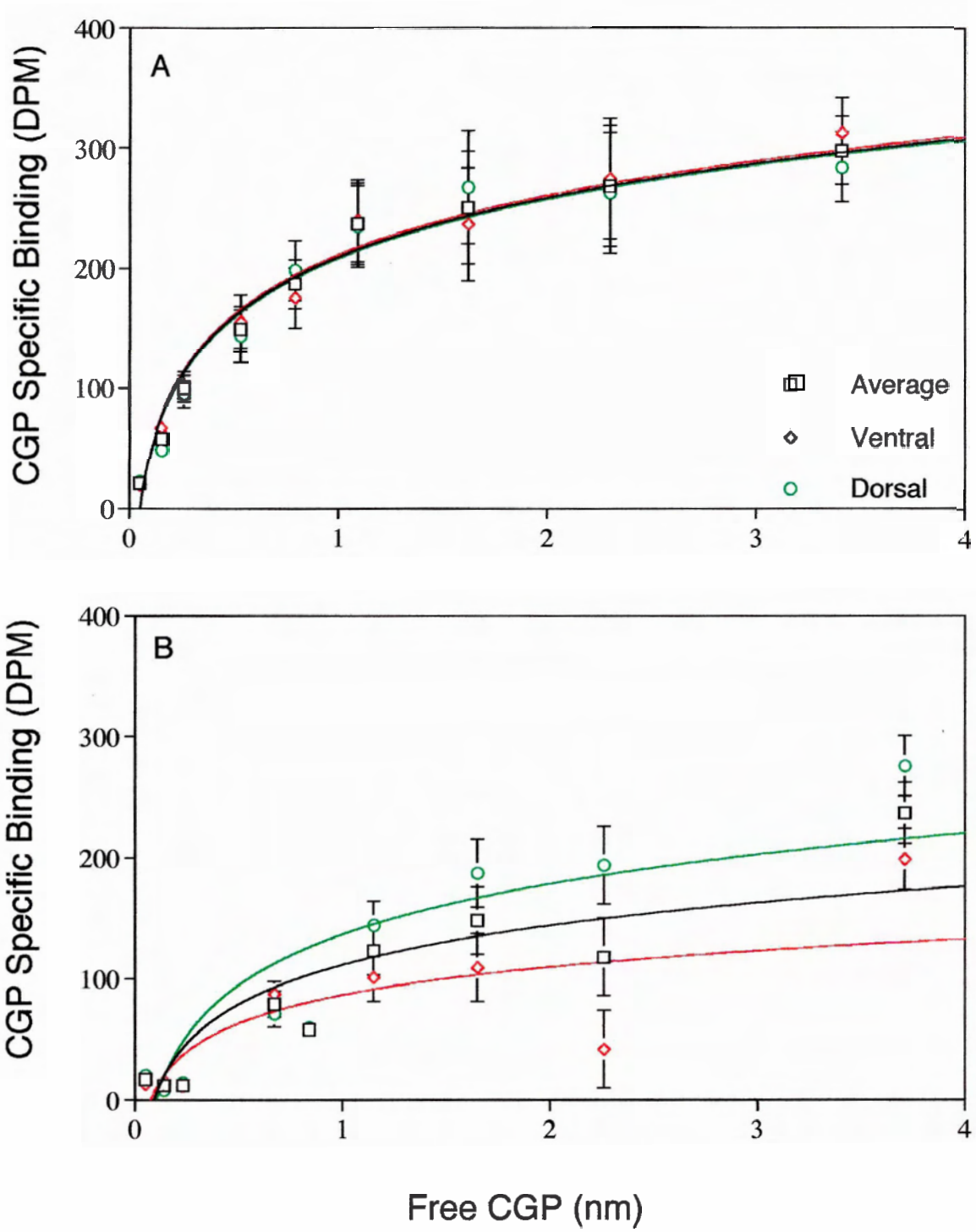

A
B
CGP Specific Binding (DPM)
CGP Specific Binding (DPM)
Average
Ventral
Dorsal
Free CGP (nm)
0
100
200
300
400
0
1
2
3
4

Table 3.2: Summary of ß-adrenoreceptor assay results for each experimental group. Mean values (SEM) are presented for N= 6 in each case. Dissimilar letters indicate significance between groups ($p<0.05$).

Test Group	B_{max} (fmol mg protein^{-1})	K_D (nM)
22°C Normoxic	79.6 (8.76) a	0.25 (0.038)
22°C Anoxic	48.1 (10.3) b,c	0.25 (0.06)
5°C Normoxic	61.7 (8.22) a,b	0.18 (0.047)
5°C Anoxic	38.2 (2.37) c	0.23 (0.034)

Discussion

Blood pH was found to decline significantly upon the onset of anoxia. Anaerobic metabolism is the primary means of energy production in anoxia and the resultant load of lactic acid decreases blood pH (Driedzic and Gesser, 1994). After only six hours of anoxia exposure at 22°C the decrease in blood pH exceeded that of turtles exposed to 5°C for three weeks. As seen in Chapter 2, acclimation to 5°C under anoxia is associated with a 25-fold reduction of heart rate and a 312-fold decrease in systemic cardiac power output which would slow the development of acidemia.

Ultsch and Jackson (1982) noted rapid drops of 8-10% in hematocrit over a 10-day period of anoxia which were reversed at a similar rate. This observation suggests that red blood cells can be trapped and then later released to the circulating blood. Sequestration of red blood cells would reduce blood viscosity and with it cardiac work. This would be an effective modification since the importance of oxygen carrying capacity of the blood is eliminated under anoxia, but the delivery of glucose and removal of lactate is still critical. Possible explanations for the decrease in hematocrit include an increase in plasma volume, removal of red blood cells into the spleen or a reduction of red blood cell volume. The rise in mean cell hemoglobin content and drop in hematocrit with anoxic exposure indicates that red blood cells may be shrinking.

Anoxia exposure reduced ventricular cell-surface ß-adrenoreceptor density to almost half of the normoxic value. This response is consistent with observations on avian and mammalian hearts. Cultured ventricular myocytes from rats and chicks decreased cell-surface ß-adrenoreceptor density by 29% and 62%, respectively, when exposed to

two hours of hypoxia (Rocha-Singh *et al.*, 1991; Marsh and Sweeny, 1989). Furthermore, exposure of rats to high-altitude hypoxia resulted in a 50% reduction in ventricular ß-adrenoreceptors (Voelkel, 1981). In mammals, this response may represent an effective mechanism for cardiac down-regulation that would guard against myocardial over stimulation at a time of energy constraint. Cardiac depression certainly occurs in turtles (Chapter 2) in the presence of elevated catecholamine levels (Wasser and Jackson, 1991). Whereas, Wasser and Jackson (1991) showed the elevation of catecholamines was more pronounced at higher temperatures (20 vs. 3°C), we found no significant difference in ß-adrenoreceptor density between 22 and 5°C groups. Therefore, the reduction in ß-adrenoreceptor density was an acute response independent of acclimation temperature.

The importance of circulating catecholamines in the diving response remains unclear but several possibilities exist. Catecholamines effect liver glycogen metabolism by increasing the rate of glycogenolysis and with it blood glucose levels (Hems and Whitton, 1980). Once endogenous supplies have been consumed tissues depend on glucose supplied from the liver for survival during anoxia (Reeves, 1963b). Blood glucose levels rise to 45 mM after 24 hours of anoxia in the turtle (Daw *et al.*, 1976). In fish, a rise in blood catecholamines stimulate red blood cell Na^+/H^+ exchange which regulates intracellular pH and oxygen transport properties (Nikinmaa, 1986). Whether this occurs in turtle erythrocytes is unknown. Elevated catecholamine levels combined with the restoration of ß-adrenoreceptor density could stimulate a rapid recovery of pre-dive conditions. Wasser and Jackson (1991) suggest that this role would have a narrow window of 30-45 minutes before plasma catecholamines are restored to pre-dive levels,

but this was measured in turtles recovering from anoxia at 20°C. In nature, turtles do not encounter anoxia at high temperatures (Gatten, 1987) so catecholamines would remain elevated for a longer period of time. The functional role of elevated catecholamines remains unclear but the universality of this diving response suggests it has an important role (Hance *et al.*, 1982).

An additional finding by Wasser and Jackson (1991) was that the degree of acidosis was correlated with catecholamine release. Turtles which respired N_2 had lower catecholamine levels than submerged turtles which encounter a greater acidosis. This remarkable synergism between hypoxemia and acidemia has also been noted in various mammals (Rose *et al.*, 1983; Lewis and Sadeghi, 1987) and amphibians (Boutilier and Lantz, 1989). Acidemia is a predictable consequence of submersion and as such would provide a strong cue to elevate catecholamine levels as a diving response. The anoxic groups at 5 and 22°C had similar blood pH 7.17 versus 7.01, respectively, and also similar levels of ß-adrenoreceptor density reduction 38% versus 40%, respectively. Whether a connection between these two events exists is uncertain, however, they both perform important roles in the loss of inotropy in anoxia.

As discussed in the previous chapter turtles exposed to anoxia at 22°C suppressed sympathetic responses by vagal inhibition that could be abolished by atropine infusion. In addition to this, ß-adrenoreceptor density was reduced by 40% over a 12-hour exposure period. In the 5°C-acclimated turtles exposed to anoxia little vagal tone was recorded so the reduction of ß-adrenoreceptors by 38% in this group could be particularly important. Anoxic exposure initiates a rapid down-regulation of metabolic rate (Herbert

and Jackson, 1985b; Jackson, 1968) so a reduction in the expression of receptors in this case would be beneficial until anoxia has ended and the threat of long-term energy shortage averted.

The reduction of ß-adrenoreceptor density does not exclude the possibility that portions of the transduction pathway are altered. Other intermediates in the extensive enzyme cascade could be modulated to produce changes in adrenergic sensitivity. For example, Keen (1992) found that thermal acclimation increased basal activity of adenylate cyclase as well as ß-adrenoreceptor density in the hearts of rainbow trout. This may account for the differences in adrenergic sensitivity of dorsal and ventral portions of the turtle ventricle (Ball and Hicks, 1996).

The B_{max} for the ventricle of turtles acclimated to 22°C normoxia in this study are higher than ß-adrenoreceptor density values reported for mammals, birds and fish.. Neonatal rat ventricle has a B_{max} of 44 fmol mg protein^{-1} (Rocha-Singh *et al.*, 1991) while turtles in this study had a value of 79.6 fmol mg protein^{-1}. Ventricluar micropunches for hamster , guinea pig (Watson-Wright *et al.*, 1989) and dog (Haddad *et al.*, 1987) were found to be 3.28, 5.00, and 10.3 fmol mg protein^{-1}respectively. Using the same method employed in this study values for Skipjack tuna, mahimahi, Sockeye salmon and Rainbow trout were found to be 27.6, 29.9, 33.4 and 18.4 fmol mg protein^{-1} (pers. com. H. Shiels). This evidence suggests that ectothermic vertebrates have a greater ability to modulate ß-adrenoreceptor density in response to changing environmental conditions than mammals and birds.

Male of turtles had a larger relative ventricular mass (RVM) but a similar ß-adrenoreceptor density as females. Various populations of rainbow trout have been shown to have a 19-35% sex-dependent difference in RVM (Graham and Farrell, 1992). The lack of an effect of sex on ß-adrenoreceptor density has also been shown in rainbow trout (Gamperl *et al.*, 1994) as well as spawning chinook salmon (Gamperl, pers. com.).

In conclusion, anoxic exposure was found to reduce the ß-adrenoreceptor density in turtle ventricles independent of temperature. The reduction in temperature from 22 to 5°C, however, was not associated with any change indicating that this is an acute response. The reduction of ß-adrenoreceptor density represents a potential physiological adaptation that assists the turtle in depressing activity during periods of energy limitation.

CHAPTER 4

Major Findings and Conclusions

The purpose of this thesis was to (a) measure cardiac power output in turtles acclimated to cold anoxia so that the degree of down-regulation could be estimated, (b) assess the control of cholinergic and adrenergic regulation in turtles acclimated to either 5 or 22°C under normoxic or anoxic conditions and (c) assay the β-adrenoreceptor density in the ventricles of turtles acclimated to each of these groups.

Objective 1.

To measure systemic cardiac power output in turtles acclimated to either 22 or 5°C under normoxic or anoxic conditions so that the degree of down-regulation can be estimated.

Findings:

Systemic cardiac power output (PO_{sys}) was 15-fold lower (from 0.81 to 0.053 mW g^{-1}) with 5- versus 22°C- acclimation. Anoxic exposure for 6 hours at 22°C resulted in a 7.4-fold drop in PO_{sys} from 0.81 to 0.11 mW g^{-1}. A comparison of turtles acclimated to 22°C normoxia with those acclimated to 5°C anoxia for five weeks, showed a 312-fold decrease in PO_{sys} (from 0.81 to 0.0026 mW g^{-1}). Therefore, acclimation to cold anoxia caused a 2.8-fold depression of PO_{sys} beyond that expected from the product of cold

acclimation and short-term anoxia alone. This suggests there is a benefit of acclimation to cold anoxia, possibly for the expression of physiological changes.

Objective 2.

Assess the control of cholinergic and adrenergic regulation in turtles acclimated to either 22 or 5°C under normoxic or anoxia conditions.

Findings:

Following adrenaline injection, systemic resistance increased by 344% at 5°C and by 135% at 22°C under normoxia, however, under anoxia these increases were reduced to 14% and 32%, respectively. This blunting of the adrenergic response could be an important mechanism for depressing cardiac function in freshwater turtles during anoxia exposure.

The infusion of atropine induced a significant tachycardia in 22°C-acclimated but not 5°C-acclimated turtles. As well, nadolol did not have any significant effect at 5°C anoxia. This indicates that intrinsic factors could be important at 5°C for controlling cardiovascular performance.

Objective 3.

To measure β-adrenoreceptor density (B_{max}) and binding affinity (K_D) in the ventricles of turtles acclimated to either 22 or 5°C under normoxic or anoxic conditions.

Findings:

B_{max} significantly decreased by 40% (from 80 to 48 fmol mg protein^{-1}) at 22°C and by 39% (from 62 to 38 fmol mg protein^{-1}) at 5°C. This could serve as a key mechanism for the reduction of cardiac inotropy in anoxia, particularly since catecholamines are elevated at this time. No significant difference was found between KD values of any group.

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