EXPRESSION OF INDUCIBLE AND CONSTITUTIVE HEAT SHOCK PROTEIN 70 IN TISSUES OF THE WESTERN PAINTED TURTLE, *CHRYSEMYS PICTA BELLI*

by

Michelle Anne Scott

A thesis submitted in conformity with the requirements
for the degree of Master of Science,
Graduate Department of Zoology,
University of Toronto

I ABSTRACT

Michelle Anne Scott, for the degree of Master of Science, 2001, Graduate Department of Zoology, University of Toronto, Toronto, Canada

EXPRESSION OF INDUCIBLE AND CONSTITUTIVE HSP70 IN THE ANOXIA-TOLERANT WESTERN PAINTED TURTLE

This study examines the expression of the inducible and constitutive form of the 70 kDa heat shock protein (Hsp72 and Hsp73, respectively) in tissues of the western painted turtle (*Chrysemys picta belli*) in response to heat shock and anoxia. Brain and liver displayed high expression of Hsp73 under control conditions as compared to low expression in heart and pectoral muscle. Upon stress (heat shock, anoxia and 1 h normoxic recovery) Hsp73 levels did not differ significantly with the exception of liver where levels of Hsp73 decreased significantly after heat shock and 1 h normoxic recovery. Expression of Hsp72 under control conditions was low in all four tissues. After 1 h 35°C heat shock exposure Hsp72 expression was elevated in all tissues examined. A 24 h period of anoxic submergence resulted in no significant induction of Hsp72 in brain or liver; however, Hsp72 expression increased in heart and pectoral muscle. Following 1 h normoxic recovery Hsp72 expression was elevated in all tissues examined. Data suggest that the expression of Hsp73 and Hsp72 is stress- and tissue-specific.

II TABLE OF CONTENTS

		Page number
(I)	Abstract	ii
(II)	Table of contents	iii
(III)	List of abbreviations	vi
(IV)	List of figures	x
(V)	Acknowledgements	xi

CHAPTER 1 INTRODUCTION

1.1	Oxygen deprivation in nature	1
1.2	Anoxia-sensitive vertebrates	2
1.3	The freshwater turtle: a rare anoxia-tolerant vertebrate	3
1.4	Heat shock proteins	6
	(i) Large Hsps	7
	(ii) Hsp90	7
	(iii) Hsp70	8
	(iv) Hsp60	8
	(v) Small Hsps	9
1.5	Hsp70: key molecular chaperone	9
	(i) Hsp70 inducers	10
	(ii) Hsp70 gene regulation	10
1.6	Hsp70 and stress tolerance	13
1.7	Hsp70 induction by hypoxia and reoxoygenation	16

CHAPTER 2 METHODS

2.1 Animals 19

2.2 Experimental setup 19

(i) Hyperthermic experiment 19

(ii) Submergence experiment 20

2.3 Anoxic dive and recovery experiment 20

(i) Blood pO_2 measurement 20

(ii) Deproteinization of blood samples 20

(iii) Lactate assay 21

2.4 Two-dimensional gel analysis 21

2.5 Isolation of protein and Western blot analysis 22

2.6 Statistical analysis 23

CHAPTER 3 RESULTS

3.1 Specificity of mammalian antibodies to turtle heat shock protein 70 24

3.2 Standard curves 24

3.3 Positive control heat shock experiment 29

3.4 Anoxic dive and recovery experiment 34

(i) Lactate measurement 34

(ii) Arterial blood oxygen measurement 34

(iii) Hsp73 expression after anoxia and reoxygenation 39

(iv) Hsp72 expression after anoxia and reoxygenation 39

(v) Levels of Hsp73 and Hsp72 expression after anoxic exposure and recovery 44

(a) Brain 44

(b) Heart 44

(c) Liver 44

(d) Muscle 45

CHAPTER 4 DISCUSSION

4.1 Pattern of Hsp70 expression after heat shock 54

4.2 Anoxic dive and recovery experiment 55

(i) Anaerobic status 55

(ii) Pattern of Hsp70 anoxia- and reoxygenation-induced expression 57

(iii) Correlation between Hsp72 and Hsp73 expression 60

4.3 Differential responses of other stress-related proteins 61

4.4 Protein denaturation caused by heat shock, anoxia and reoxygenation 62

4.5 A general model of Hsp70 induction in turtle tissues 64

REFERENCES 66

III LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ATPase	ATP hydrolysis ability
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
°C	Degree Celsius
Ca^{++}	Calcium
CA1	*Calcar avis* 1 (a defined region of hippocampus)
cm	Centimetre
D	Aspartate
ddH_2O	deionized distilled water
DNA	deoxyribonucleic acid
DnaK	*Escherchia coli* Hsp70
DTT	1, 4-Dithio-DL-threitol
E	Glutamate
EDTA	Ethylenediaminetetraacetic acid
E. coli	*Escherchia coli*
ER	Endoplasmic reticulum
g	Gravitational constant
g	Gram

GABA	gamma-aminobutyric acid
GPT	Glutamic pyruvic transaminase
h	Hour
HSBP1	Heat shock factor binding protein isoform
HSE	Heat shock element
HSF	Heat shock factor
HSF1	Heat shock factor isoform
HSF2	Heat shock factor isoform
HSPs	Heat shock proteins
Hsps	Heat shock proteins
Hsc70	Constitutive isoform of heat shock protein 70
Hsp60	Heat shock protein 60
Hsp70	Heat shock protein 70
Hsp72	Inducible isoform of heat shock protein 70
Hsp73	Constitutive isoform of heat shock protein 70
Hsp90	Heat shock protein 90
Hsp100	Heat shock protein 100
IEF	Isoelectric focusing
IGEPAL	(Octylphenoxy)polyethoxyethanol
K^+	Potassium
KCl	Potassium chloride
KOH	Potassium hydroxide
KDa	Kilodalton

LDH	L-Lactate dehydrogenase
m	Metre
mM	Millimolar
M	Molar (concentration)
mmHg	Milimetre mercury
min	Minute
mRNA	Messenger ribonucleic acid
n	Sample size
N_2	Nitrogen
Na^+	Sodium
Na^+/Ca^{++} exchanger	Sodium-calcium ion exchange pump
Na^+/K^+-ATPase	ATP-dependent sodium and potassium ion exchange pump
NaOH	Sodium hydroxide
NAD	β-Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
nm	Nanometre
NMDA	N-methyl-D-aspartate
pg	Picogram
pO_2	Oxygen tension
PKC	Protein kinase C
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second

TEMED	N, N, N, N'-Tetraethylethylenediamine
TBS	Tris buffer saline
TBS-T	Tris buffer saline with Tween-20
µg	Microgram
µl	Microlitre
V	Valine

IV LIST OF FIGURES AND TABLES

1.	Specificity of antibodies for Hsp72 and Hsp73 in turtle liver	25
2.	Representative Western blots of Hsp72 and Hsp73 standards and corresponding curves	27
3.	Expression of Hsp73 following heat shock	30
4.	Expression of Hsp72 following heat shock	32
5.	Evaluating metabolic status by lactate measurement	35
6.	Assessment of blood pO_2 level	37
7.	Expression of Hsp73 in response to normoxia, anoxia and reoxygenation	40
8.	Expression of Hsp72 in response to normoxia, anoxia and reoxygenation	42
9.	Quantification of brain Hsp73 and Hsp72 protein after normoxic and anoxic exposure and after recovery	46
10.	Quantification of Hsp73 and Hsp72 protein in heart tissue after normoxic and anoxic exposure and after recovery	48
11.	Quantification of liver Hsp73 and Hsp72 protein after normoxic and anoxic exposure and after recovery	50
12.	Quantification of skeletal muscle Hsp73 and Hsp72 protein after normoxic and anoxic exposure and after recovery	52
13.	Proposed mechanism of stress induced increase in HSP70 in turtle tissues	66

CHAPTER 1 INTRODUCTION

1.1 Oxygen deprivation in nature

Molecular oxygen is required for all eukaryotic life, acting as the terminal electron acceptor in the mitochondrial respiratory chain. In the absence of oxygen (anoxia) or with insufficient oxygen (hypoxia) the oxidative phosphorylation pathway is deprived of its main electron acceptor causing the less efficient anaerobic glycolytic pathway to replace oxidative phosphorylation as the principal source of ATP production. Glycolysis generates two moles of ATP compared to the approximately 36 moles of ATP produced by oxidative phoshorylation. Consequently during periods of hypoxia or anoxia there are insufficient amounts of high-energy phosphates for long-term cellular survival and cellular injury follows. When hypoxia is reversed the ensuing reoxygenation can also produce cell-damaging effects due to increased levels of reactive oxygen species. Oxygen radicals and reactive oxygen species form as a result of the partial reduction of oxygen to water and can be highly destructive causing oxidative damage to proteins, nucleic acids, lipids and cell membranes (Czubryt *et al.*, 1996).

The ability to survive extended periods of anoxia is a relatively common adaptation among prokaryotes and invertebrates. For example, adult wild type *Drosophila melanogaster* flies are able to survive complete anoxia (0% oxygen) for up to 4 h and recover completely from the anoxic stress (Krishnan *et al.*, 1997). As well, cells of encysted embryos of *Artemia franciscana*, the brine shrimp, are capable of surviving continuous anoxia for periods of years (Clegg *et al.*, 2000).

In comparison the ability to survive for longer than a few minutes of anoxia is a rare adaptation among vertebrates. While neonate mammals have an increased resistance to cellular damage resulting from hyoxia, adult mammals are unable to survive for longer than a few minutes at physiological temperatures when exposed to very low levels of oxygen. However, there are a few vertebrates that have the ability to withstand extremely low levels of oxygen or a total lack of oxygen for extended periods.

1.2 Anoxia-sensitive vertebrates

Principally due to the lack of high-energy phosphates in an anoxic and/or hypoxic state most vertebrate species are unable to survive for longer than a few minutes at normal physiological temperatures. Thus hypoxia is considered a stress that depresses cell function and threatens cell viability while anoxia is considered to be lethal. The brain is the most sensitive organ to lowered levels of oxygen and will be used as an example to demonstrate the cellular events accompanying lowered oxygen availability in mammals.

During anoxia a rapid decrease in ATP production without a comparable reduction in ATP utilization quickly leads to an energy imbalance. This is particularly detrimental to the brain since most of the energy of brain cells is dedicated to crucial neuronal functions. In the anoxic mammalian brain ATP depletion occurs within one to two minutes (Lutz and Nilsson, 1997). When ATP levels start to decline the Na^+/K^+-ATPase pump begins to fail and Na^+ and K^+ ions flow down their concentration gradients through various ion channels in the neuronal membrane. The Na^+ gradient is crucial not only for maintaining the membrane potential but also for providing the driving force for Ca^{++} extrusion (via the Na^+/Ca^{++} exchanger), uptake of organic molecules such as amino

acid neurotransmitters and regulation of cell volume (Lutz and Nilsson, 1997). The inflow of Ca^{++} into neurons is considered to be the hallmark event in anoxic brain injury, in fact anoxic brain injury is considered reversible prior to the loss of Ca^{++} gradients.

The response of the mammalian brain to anoxia can be divided into 3 distinct phases. Initially (phase 1) there is a rapid decrease in electrical activity, a suppression of neurotransmission and a slow increase in extracellular K^+ concentration. Within a few minutes phase 2 begins which is characterized by rapid depolarization, a loss of ion gradients and a large outflow of neurotransmitters, some of which are highly excitotoxic at elevated extracellular concentrations. During phase 3 the neuronal membrane is fully depolarized and leads to irreversible cell damage and death if prolonged for longer than a few minutes (Lutz and Nilsson, 1997).

1.3 The freshwater turtle: a rare anoxia-tolerant vertebrate

Although vertebrates are generally very sensitive to oxygen deprivation there are a few non-mammalian vertebrates known to survive anoxic conditions for extended periods of time, the length of survival dependent on the species and the ambient temperature. Such anoxia-tolerant species include the crucian carp, the goldfish and some freshwater turtles. These anoxia-tolerant organisms have evolved numerous survival mechanisms to withstand prolonged anoxic periods. One of these anoxia-tolerant animals is considered a prime example of a species adapted to survive extended periods of oxygen deprivation. This species, the western painted turtle *Chrysemys picta belli*, has been shown to fully recover from a 24 h anoxic dive at 25°C as well as a 3 month

submergence at 3°C (Jackson and Ultsch, 1982). The western painted turtle has even been referred to as a "vertebrate facultative anaerobe" (Storey and Storey, 1990).

The normal range of the western painted turtle extends into north-central United States and southern Canada. This animal therefore experiences severe winters during which the ponds it inhabits become ice-covered for many months (Jackson, 2000). Gatten (1981) observed that during the winter months painted turtles are buried (up to 45 cm) in mud, sand or muskrat burrows. Consequently during the winter season the western painted turtle faces extremely hypoxic or anoxic conditions (Ultsh, 1989).

Various investigations have provided much insight into the unique physiological adaptations that allow this vertebrate species to survive such severe anoxic conditions. It has been shown that the main anoxic survival strategy is a large suppression of metabolic rate. Direct studies by calorimetry (Jackson, 1968) and indirect studies by measuring lactate accumulation in blood (Herbert and Jackson, 1985) have indicated that the transition from aerobic to anaerobic metabolism is associated with a decrease in energy metabolism to approximately 10 to 15% of the aerobic level. Studies at the organ level have also demonstrated a metabolic depression of similar magnitude. Lutz and colleagues (1985) estimated from blood lactate levels that brain metabolism fell by 90 to 95% and Buck and coworkers (1993) observed a 90% reduction in metabolic rate based on oxygen consumption and lactate production in isolated liver cells.

Since the anoxic cell is working at approximately 10% of the aerobic level there must be a coordinated reduction in key energy utilizing cellular processes. Reductions in ATP utilizing processes such as Na^+/K^+-ATPase activity (Buck *et al.*, 1993), ion channel activity (Buck and Bickler, 1998), electrical activity (Lutz *et al.*, 1984) and protein

synthesis (Land *et al.*, 1993) have been observed in anoxic turtle brain and liver cells and will be discussed below.

A general reduction in ion channel activity in response to anoxic stress is most likely the means by which the profound reduction in metabolism is achieved. In fact this kind of mechanism has been termed the channel arrest hypothesis by Hochachka (1986) and makes two predictions regarding anoxia-tolerance: 1) anoxia-tolerant species have fewer ion channels per area plasma membrane, and 2) anoxia-tolerant species have mechanisms by which ion channels can be acutely regulated. Indeed several studies show indirect evidence for channel arrest: downregulation of Na^+ channels in brain (Pérez-Pinzón *et al.*, 1992) and liver cells (Buck *et al.*, 1993) and of K^+ channels in brain (Chih *et al.*, 1989). Buck and Bickler (1998) provide direct evidence for the channel arrest hypothesis – in response to an anoxic insult, they show a reduction in Ca^{++} channel open probablility using single channel patch clamp methods in anoxic turtle brain. The reduction in channel activity in the brain contributes to an overall reduction in brain electrical activity termed spike arrest by Sick *et al.* (1998). Another factor that contributes to spike arrest is increased levels of the inhibitory neurotransmitter GABA (Nilsson and Lutz, 1991).

Protein synthesis, another energy-requiring process, requires the hydrolysis of four ATP equivalents per peptide bond and one additional ATP for amino acid transport. A reduction in protein synthesis termed translational arrest (Jackson, 2000) has been observed in anoxic turtle hepatocytes (Land *et al.*, 1993) and in anoxic turtle heart (Bailey and Driedzic, 1996). While a general down-regulation of protein synthesis occurs during anoxia a number of studies have shown the upregulation of specific proteins. In

anoxic or heat shocked turtle hepatocytes, the expression of 5 types of proteins was shown to increase (Land and Hochachka, 1994; Hochachka *et al.*, 1996). More specifically Chang and colleagues (2000) have shown an induction of heat shock proteins in response to anoxia in turtle heart.

Since protein synthesis is a costly process in terms of energetics the upregulation of heat shock proteins in anoxic tissues indicates that they may play a role in promoting anoxia tolerance. Heat shock proteins have been shown to be evolutionarily conserved and present in all organisms studied (Morimoto, 1993). While many studies have focused on the mechanisms of heat shock protein induction in mammalian tissues, little is known of their role in anoxia-tolerant species.

1.4 Heat shock proteins

In 1962 Ritossa discovered that in response to a 30 minute 37°C exposure and subsequent recovery to room temperature (25°C) fruit fly larvae salivary glands demonstrated chromosomal "puffing". The heat-induced puffing of the chromosomes, indicative of specific gene activity, was later found to be accompanied by an increase in the expression of proteins with molecular masses 70 and 26 kDa (Tissieres *et al.*, 1974) which were named heat shock proteins (Hsps). Subsequent studies demonstrated that Hsps belong to various families based on molecular mass and that stresses other than hyperthermia including ischemia, radiation, heavy metals, anoxia and reoxygenation (Lindquist and Craig, 1988) could induce the expression of Hsps. As a result the name "heat shock protein" is no longer considered to be the correct terminology and the more general term "stress protein" is currently being adopted.

All heat shock proteins are considered ATP-dependent molecular chaperones and thus recognize unfolded, misfolded and aggregated proteins (Welch, 1992; Xu and Lindquist, 1993). As molecular chaperones, all Hsps share an ability to modulate the folding and unfolding of other proteins and facilitate assembly and disassembly of multisubunit complexes (Parsell and Lindquist, 1993).

Stress proteins can be divided into six subfamilies based on molecular mass and function – the large Hsps of approximately 100 kDa, the Hsp90 family, the Hsp70 family, the Hsp60 family, and the small Hsps of approximately 20 kDa (Lindquist and Craig, 1988). Members of each family of stress proteins have unique functions as outlined below.

(i) Large Hsps

The eukaryotic Hsp100 proteins are members of a highly conserved protein family (Parsell and Lindquist, 1993). Family members range in size from approximately 100 to 110 kDa and are found in the cytoplasm, nucleus and nucleolus (Parsell and Lindquist, 1993). Each family member possesses ATPase activity and contains at least two ATP-binding domains. Stress functions include extreme heat tolerance, ethanol tolerance and long-term spore viability, and regulation of protease activity (Parsell and Lindquist, 1993).

(ii) Hsp90

The members of this family range in size from 82 to 96 kDa and are found in the cytosolic/nuclear compartment of all eukaryotes examined to date and are also present in the ER of higher eukaryotes (Parsell and Lindquist, 1993). Hsp90 proteins bind ATP (Csermely and Kahn, 1991) and have peptide-stimulated ATPase activities (Nadeau *et*

al., 1993). Members of the Hsp90 family have been found to have a regulatory function associated with many other cellular proteins, including several steroid hormone receptors (Pratt *et al.*, 1993), several protein kinases (Welch, 1992), calmodulin (Minami *et al.*, 1993), actin (Miyata and Yahara, 1991) and tubulin (Fostinis *et al.*, 1992). *In vitro* Hsp90 acts as a general chaperone, preventing the aggregation of proteins, promoting disaggregation of previously aggregated proteins and enhancing the activity of enzymes (Parsell and Lindquist, 1993). While Hsp90 has been shown to play a key role in helping cells cope with the stress of growth at moderately high temperatures (Borkovich *et al.*, 1989), its importance in helping cells survive extreme temperatures is less clear (Parsell and Lindquist, 1993).

(iii) Hsp70

The Hsp70 family is highly conserved with 60 – 78 % identity among eukaryotic cells and 40 – 60% identity between eukaryotic and prokaryotic cells (Lindquist and Craig, 1988). The Hsp70 family of stress proteins function as ATP-dependent molecular chaperones that stabilize and refold proteins and polypeptides damaged by stress. Members of this family have been shown to be present in nearly all cellular compartments and organelles (Gething and Sambrook, 1992). More details of this family of heat shock proteins will be provided (see section 1.5).

(iv) Hsp60

Hsp60 proteins are found in the matrix compartment of mitochondria in eukaryotes (Hartl *et al.*, 1992). Hsp60 exhibits chaperone activity, has a weak K^+-dependent ATPase activity that increases with temperature and the binding of ATP induces a major conformational change in the oligometric structure of the protein (Trent

et al., 1991). In mammals Hsp60 is expressed both constitutively and induced upon stress and has been shown to be necessary for normal mitochondrial functioning (Agsteribbe *et al.*, 1993).

(v) Small Hsps

The small Hsps vary in size from 20 to 30 kDa, are the least conserved of the major Hsp families and the most equivocal in function (Parsell and Lindquist, 1993). The small Hsps are found in the cytosol of eukaryotes and show a complex and confusing pattern of relocalization in response to heat and other stimuli (Rossi and Lindquist, 1989). The small Hsps are phosphorylated in response to heat and a variety of other stimuli and preliminary studies suggest they function in signal transduction pathways (Mendelsohn *et al.*, 1991).

1.5 Hsp70: key molecular chaperone

The 70 kDa family of stress proteins is the most extensively studied and best described and therefore will be the focus of this study. As mentioned previously the Hsp70 family of proteins are highly conserved and demonstrate a 60 to 78 percent base homology between eukaryotic Hsp70 and *E. coli* DnaK, which is similar to Hsp70 (Caplan *et al.*, 1993). The Hsp70 proteins have two key domains: a highly conserved ATPase N-terminal domain and a relatively variable peptide binding C-terminal domain with a highly conserved EEVD terminal sequence (Hightower, 1991). The ATPase and peptide-binding domains are interdependent – ATPase activity is strongly stimulated by substrate binding and the release of substrates is dependent upon the nature of the bound nucleotide (Parsell and Lindquist, 1993).

The Hsp70 proteins belong to a mulitgene family and several isoforms exist. The constitutive form of Hsp70 (Hsc70 or more specifically Hsp73) is present in unstressed cells within the cytosol and nucleus and is moderately stress inducible under certain conditions (Creagh *et al.*, 2000). Under non-stress conditions Hsp73 proteins act as chaperones for other cellular proteins, binding to nascent polypeptides to prevent premature folding and translocating proteins to organelles. The stress inducible form of Hsp70 (Hsp72) is present in unstressed cells; however, upon exposure to stressful conditions Hsp72 is highly inducible. During conditions of stress both Hsp72 and Hsp73 bind to damaged and misfolded polypeptides and can either facilitate their repair or target irreparably damaged polypeptides for degradation by the ubiquitin/proteasome-dependent pathway (Lindquist and Craig, 1988).

(i) Hsp70 inducers: A variety of stress conditions will induce the transcription of heat-shock genes. Heat shock is the classical inducer; however, there are a number of other stresses that will induced hsps including, oxidizing agents, sulfhydryl agents, amino acid analogs, carcinogens, ischemia and recovery, UV irradiation and anoxia and recovery (Nover, 1991).

(ii) Hsp70 gene regulation: The rapid, stress-inducible transcription of heat-shock proteins is a characteristic feature of the heat-shock gene response. Some heat shock genes are known to lack introns thus allowing transcription to occur rapidly (Wu *et al.*, 1985; Jolly *et al.*, 1999). The inducible transcription of Hsp70 genes is mediated by a family of transcription factors known as heat shock factors (HSF) that function as the molecular links between stressful conditions and Hsp70 proteins. All stress-inducible agents capable of inducing Hsp70 proteins do so by activating HSF (Morimoto, 1993).

Immediately upon exposure of tissues to a stressful condition HSFs are activated which leads to the elevated transcription of genes encoding Hsp70 proteins (Wu, 1995). Upon activation, HSF binds in a sequence-specific manner to heat shock responsive elements (HSE) located in the promoter regions of heat-shock genes resulting in elevated transcription (Voellmy, 1996). HSF has been found to be essential for survival in both *Saccharomyces cerevisiae* and *Drosophila*. In the latter, those organisms that lack HSF a larval development phenotype results in an increased sensitivity to stress (Jedlicka *et al.*, 1997). Higher eukaryotes encode an HSF multi-gene family and three or more genes have been characterized in the human, mouse, chicken and tomato genomes (Wu, 1995).

The role of multiple forms of HSFs in the transcriptional regulation of heat-shock genes may possibly be to provide specialized responses to a variety of environmental and developmental stresses encountered by higher eukaryotes. HSF1 and HSF2 are structurally related and 40 percent identical in overall amino acid sequence but are distinct by regulatory and functional criteria (Morano and Thiele, 1999). For example HSF1 is activated upon stress whereas HSF2 is activated in response to cues during early development and differentiation and is involved in inhibition of the proteosome pathway (Shi *et al.*, 1995).

A proposed mechanism of the stress-induced increase in HSPs in human and *Drosophila* cells has been suggested (Kiang and Tsokos, 1998). Under periods of non-stress HSF1 is found in the cytosol, is inactive and bound to Hsp70 proteins. When cells are stressed non-native proteins accumulate and compete with HSF1 for Hsp70 binding. As a consequence of the competition the pool of unbound, monomeric HSF1 increases and the probability of HSF1 trimer formation thus increases. Once HSF1 trimerizes and

acquires DNA-binding capability it translocates to the nucleus and activates Hsp70 transcription. Activation of HSF1 may require a number of conditions, including: oligomerization from an inert monomer to an active trimer, acquisition of DNA-binding capability, stress-induced serine phosphorylation by protein kinases, such as PKC and nuclear localization (Wu, 1995). Once in the nucleus HSF1 binds to HSEs in the promoter region of the Hsp70 gene. HSF1s are then further phosphorylated and Hsp70 mRNA is transcribed and leaves the nucleus for the cytosol. In the cytosol new heat-shock protein is translated, HSF1 returns to the cytosol and in attenuation of the stress response reattaches itself to free Hsp70.

While the previous model for the activation of HSF1 and the induction of Hsp70 proteins is considered correct, there have been other studies that suggest that other proteins are involved in the feedback regulation of HSF. In addition to Hsp70, other molecular chaperones have been shown to regulate the activity of HSF1; for example, a study by Zou *et al.* (1998a) demonstrated that reduced levels of Hsp90 lead to activation *in vitro*. Similar kinds of results have been obtained in the oocytes of *Xenopus laevis*, where formation of a heterocomplex between components of the Hsp90 chaperone machinery and HSF1 were shown to play a key role in modulating different steps of the HSF1 activation-deactivation pathway (Ali *et al.*, 1998; Bharadwaj *et al*, 1999).

Besides the feedback regulation of HSF1 by Hsps, other proteins have been found to bind HSF1. For example, in a yeast two-hybrid screen, a novel 8.5 kDa nuclear protein termed heat shock factor binding protein 1 (HSBP1) was found to interact with the oligomerization domain of an active HSF1, thereby negatively affecting HSF1 DNA

binding activity (Satyal *et al.*, 1998). During inactivation of HSF1 to an inert monomer, HSBP1 is associated with Hsp70.

All of the HSF1 interacting proteins reported to date act as negative regulators of HSF1 activity, suggesting that a multicomplex might be required to keep HSF1 in a state that can readily be activated.

1.6 Hsp70 and stress tolerance

For an unstressed cell direct exposure to a harsh stress would most likely be a terminal event leading to cell death. However, exposure to a milder stress causes the cell to induce Hsp70 proteins which have been shown to play a role in cytoprotection under further exposure to extreme stress (Jäättelä, 1999). This is a general phenomenon for the induction of stress tolerance and is termed preconditioning.

Specifically in terms of hyperthermia the induction of Hsp70 proteins has been shown to correspond with the development of thermotolerance. Thermotolerance refers to the observed cytoprotective effect of a mild heat shock treatment in advance of a more severe hyperthermic treatment. The preliminary heat treatment has been shown to condition cells to resist the effects of a subsequent temperature increase which would otherwise be lethal (Widelitz *et al.*, 1986; Steels *et al.*, 1992). Thermotolerance was first shown in the yeast *Saccharomyces cervisiae* when the survival of cells exposed directly to an extreme temperature (50°C) was compared to the survival of those that were first pretreated at a milder temperature (37°C) for 30 minutes before 50°C exposure. It was found that pretreatment improved survival at 50°C more than 1000-fold and that pretreated groups also showed elevated levels of heat shock proteins (Sanchez and

Lindquist, 1990). The thermotolerance phenomenon has been shown to be highly conserved and is now recognized as a general protective strategy cells employ against many other stress-inducing agents including heavy metals, oxidative stress, radiation, nitric oxide and alcohols (Lindquist and Craig, 1988).

However, the observed thermotolerance is temporary. When the temperature is returned to physiological levels the cells continue to function normally and levels of Hsps drop back to pre-stress levels with time. During the intervening period, while Hsps levels are high, the cells are resistant to various cytotoxic agents. Furthermore, it has been demonstrated that constitutive overexpression of Hsps can render cells resistant to the toxicity of many agents (Li *et al.*, 1991). For example, overexpression of Hsp72 or its induction by heat shock renders cells more resistant to the effects of a number of cytotoxic agents (Jäättelä *et al.*, 1992).

The preconditioning phenomenon can also involve cellular stresses other than hyperthermia. Preconditioning involving Hsp70 proteins has been observed in ischemia-reperfusion experiments. For mammals, ischemia-reperfusion is known as the best characterized *in vivo* model for oxidative stress (Mallouk *et al.*, 1999). During ischemia decreased availability of oxygen leads to a drop in ATP production and alterations in ion distribution. The resulting increase in intracellular Ca^{++} concentrations activates Ca^{++}-dependent proteases, while ATP depletion results in increased concentrations of AMP, which is further catabolized to adenosine, inosine, and hypoxanthine. The Ca^{++}-activated proteases convert hypoxanthine dehydrogenase into a free radical-producing xanthine oxidase. Thus during reperfusion when molecular oxygen is again available to oxygen-

deprived tissues large amounts of reactive oxygen species cause reperfusion injury (McCord, 1985).

Ischemic preconditioning has perhaps been best described in heart. In 1991 Knowlton and colleagues demonstrated that repetitive periods of ischemia-reperfusion in the rabbit heart resulted in an increase of inducible Hsp70 (Hsp72) mRNA synthesis and Hsp72 expression. In the same animal model a preconditioning protocol involving several periods of ischemia and reperfusion induced myocardial expression of Hsp70 at 24 and 48 h after preconditioning (Tanaka *et al.*, 1994). Occlusion of the left anterior descending coronary artery of the dog heart causing myocardial ischemia has been associated with an increase in the level of Hsp70 mRNA (Dillmann *et al.*, 1986). Repeated episodes of myocardial ischemia followed by 30 minutes of reperfusion enhanced the expression of Hsp70 in the pig heart (Sharma *et al.*, 1992).

Ischemia-reperfusion has also been shown to increase Hsp70 mRNA levels in other organs including liver (Saad *et al.*, 1995; Kume *et al.*, 1996). Kume and colleagues (1996) demonstrated that 15 min of ischemia in rat liver increased Hsp70 expression after 48 h and afforded significant protection of hepatic function and survival following a longer ischemic insult. Furthermore Saad and colleagues (1995) have studied the effects of hyperthermic preconditioning on ischemic tolerance in rat liver. Rats were first exposed to a 15 min whole body heat shock (42°C) which was shown to induce Hsp72 expression. Two days later *in situ* livers were subjected to 30 min of warm ischemia with subsequent reperfusion for 40 min and little cellular damage was observed (Saad *et al.*, 1995). These results are similar to those found in experiments involving Hsp70 over-expression in the heart.

The role of Hsp70 in neuronal cytoprotection was first discovered in brain when Kitagawa and colleagues (1991) subjected gerbils to global ischemia. A 5 min occlusion of the bilateral common carotid arteries resulted in marked neuronal death of CA1 hippocampal slices after 7 days of recovery. However, a 2 min ischemic pretreatment one to two days prior to the 5 minute ischemic treatment resulted in a 10-fold increase in neuronal survival. In 1991 Kirino and co-workers extended this study and showed accumulation of Hsp70 in the gerbil CA1 region after a 2 min ischemic period. Further studies have also shown that Hsp70 is induced in the brain as a result of ischemia/hypoxia and that its expression might be necessary for preconditioning (Aoki *et al.*, 1993).

The Hsp70 family of heat shock proteins is clearly important in the preconditioning phenomenon observed for both the hyperthermic and ischemia-reperfusion models with Hsp70 providing cellular protection during subsequent exposures to stress.

1.7 Hsp70 induction by hypoxia and reoxygenation

The stresses associated with hypoxia and reoxygenation have been shown to induce the synthesis of stress proteins, in particular Hsp70, in numerous animals including invertebrates such as *Drosophila* (Arbona and De Frutos, 1987; Drummond and Steinhardt, 1987) and non-mammalian vertebrates such as notothenioid fish (Carpenter and Hofmann, 2000), trout (Airaksinen *et al.*,1998) and *Xenopus laevis* (Mifflin and Cohen, 1994).

While it is well established that denatured proteins accumulate as a result of an increase in physiological temperature, resulting in the subsequent increase in transcription of heat shock specific genes, it is less well understood what acts as the initiator of the stress response in cells exposed to oxidative stresses such as hypoxia and reoxygenation.

Voellmy (1996) identified a variety of conditions and agents (including recovery from anoxia, H_2O_2 and free radicals) that induced Hsp70 gene expression, all of which ultimately alter the native conformation of proteins through intracellular oxidative stress. During hypoxia and reoxygenation, changes in the cellular redox status occur altering the ratio of oxidized and reduced forms of glutathione which in turn impacts on the redox state of cysteine residues present on cellular proteins (Piacentini and Karliner, 1999). Furthermore, high intracellular levels of radical oxygen species will directly cause protein damage (Czubryt *et al.*, 1996).

While the majority of investigations of the role of stress proteins during hyperthermia, anoxia and/or reoxygenation involve mammals, little is known in non-mammalian vertebrates such as the freshwater turtle. The main focus of this project is to investigate the tissue-specific expression of both the constitutive and inducible isoforms of Hsp70 – Hsp73 and Hsp72 – in response to heat stress and anoxia in the western painted turtle. In an animal adapted for long-term breath-hold diving, it is unclear whether or not anoxia is even stressful to the animal. Very little is known about the expression of stress proteins in this species; however, based on previous studies (Manzerra *et al.*, 1997; Carpenter and Hofmann, 2000) it is possible to generate several hypotheses.

Hypotheses:

The western painted turtle:

1) maintains chronically high levels of both Hsp73 and Hsp72, causing it to be chronically preconditioned to the stresses imposed by anoxia and reoxygenation
2) maintains persistently high levels of constitutive Hsp73 and undergoes a strong induction of Hsp72 upon anoxic exposure or reoxygenation
3) maintains chronically high levels of constitutive Hsp73 and upon anoxic exposure or reoxygenation there is no induction of Hsp72
4) maintains low levels of Hsp73 and Hsp72 and undergoes a strong induction of Hsp72 upon exposure to anoxia or reoxgenation.

Thus the following are the specific aims of this project:

1) To expose western painted turtles to a hyperthermic stress to determine if Hsp73 and/or Hsp72 levels in brain, heart, liver and skeletal muscle can increase above normoxic baseline levels
2) To examine Hsp73 and Hsp72 levels in these four tissues after anoxia and reoxygenation.
3) To quantify the relative expression of Hsp73 and Hsp72 in these tissues.

CHAPTER 2 METHODS

2.1 Animals

This study was approved by the University of Toronto Animal Care Committee and conforms to relevant guidelines for the care of experimental animals.

Western painted turtles, *Chrysemys picta belli*, weighing between 590–760 g were commercially obtained from Lemberger Co., Inc. (Oshkosh, WI). Animals were housed in an indoor pond with dimensions 2 m x 4 m x 1.5 m. The pond had a sloping floor, with one end containing water approximately 0.5 m deep while the other end had a shallow, rocky basking platform with heating lamp. The aquarium was equipped with a flow-through dechlorinated freshwater system with water temperature maintained at approximately 15 – 20°C. Turtles were given continuous access to food and kept at a room temperature of 20°C on a 12:12 h light-dark cycle.

2.2 Experimental setup

(i) Hyperthermic experiment A plexiglass container of dimensions 0.5 m x 0.5m x 0.25 m (with plexiglass cover) was filled with water heated to a temperature of 35°C by a heat-pump exchanger. A total of 4 turtles were used in this experiment. Each turtle was placed in the heated pool for 1 h after which time the container was drained and refilled with room temperature water. The turtle remained in the room temperature bath for 5 h at which point the turtle was killed by decapitation. Brain, heart, liver and skeletal muscle tissue were rapidly dissected and frozen in liquid N_2.

(ii) Submergence experiment Three metal cages were placed in the aquarium for several days to allow turtles to acclimate. On the day of the experiment, water temperature was measured to be 16.5°C and water pO_2 44 mmHg. Turtles that voluntary swam into the cages were used for the experiment. Each cage was first lifted out of the water for 20 min then each cage was submerged gradually until entirely underwater. Nine turtles were submerged for 24 h and following this period, five turtles were removed from their cage and neck-clamped underwater to prevent the possibility of aeration and killed by decapitation. The remaining cage was brought to the surface and the turtles (n = 4) were allowed access to air for 1 h after which time they were killed by decapitation. One (1) ml of aortic blood was collected via a glass syringe for pO_2 analysis. An additional 1 ml of aortic blood was quickly collected and frozen in liquid N_2 to be used for lactate analysis. Animals were cut open with scalpel and bone saw and brain, heart, liver and skeletal muscle tissue were dissected in less than 3 min, wrapped in aluminum and frozen in liquid N_2.

2.3 Anoxic dive and recovery experiment

To determine the degree of anoxia, blood lactate levels and partial pressure of oxgyen were measured.

(i) Blood pO_2 measurement Aortic blood was collected via a glass syringe for pO_2 analysis using an oxygen meter (OM2000, Cameron Instruments) previously calibrated with nitrogen-bubbled distilled water.

(ii) Deproteinization of blood samples To deproteinize the blood samples 450 µl of blood was mixed with 50 µl of ice-cold 70% perchloric acid and sonicated for

30 sec. The mixture was then centrifuged at 2°C at 10,000 x g for 10 min. A 100 µl volume of neutralizing buffer (KOH/Tris/KCl) was added to the supernatant and centrifuged at 2°C at 10 000 x g for 10 min. The mixture was allowed to stand for 15 min on ice at which time the supernatant was removed and stored at -20°C until used for lactate assay.

(iii) Lactate assay Samples were analyzed for lactate content by an enzymatic method using a standard enzyme coupled assay (Noll, 1974). In this reaction hydrogen ions were transferred from lactate to NAD by lactic dehydrogenase in a glutamate buffer solution. The assay medium contained the following: 0.12 M glutamate buffer solution, 17.3 units of GPT (Sigma), 0.8 mM NAD (Sigma) and 100 µl of sample. The solution was mixed and allowed to equilibrate for 10 min at which point 21.8 units of LDH (Sigma) was added. The total volume of the assay mixture was 1 mL. The solution was mixed again and the absorbance of NADH was then read at 340 nm on a Spectronic 1001 Plus (Milton Roy) spectrophotometer at 1 min intervals until a constant absorbance value was obtained (typically 10 – 15 min).

2.4 Two-dimensional gel analysis

Isoelectric focusing (IEF) followed the technique of O'Farrell (1975) with the modifications described by Rodenhiser *et al.* (1985). Glass tubes (15 cm x 4 mm) were rinsed with double distilled water, dried and one end covered with parafilm. An acrylamide solution consisting of 5.5 g urea, 1.98 ml H_2O, 2 ml of 10% IGEPAL, 300 µl ampholines pH 5.0-8.0 and 200 µl ampholines pH 3.5-10, 1.33 ml of 40% acrylamide, 11 µl of 10% ammonium persulfate, and 13 µl TEMED was quickly poured to a height of 10

cm and carefully overlaid with ddH_2O. After the gels were polymerized and parafilm removed, gels were prerun at 200 volts for 15 min, 300 volts for 30 min and 400 volts for 1 h. During the prerun the upper buffer consisted of 50 mM NaOH and the lower buffer 138 mM phosphoric acid. Following the prerun the upper buffer was replaced and samples suspended in 5 µl 10% IPEGAL, 3 µl ampholines pH 5.0-8.0 and 2 µl ampholines pH 3.5-10, 5 µl β-mercaptoethanol and made up to 100 µl with 9 M urea, were placed in the tubes and electrophoresed for 14 h at 400 volts and 4 h at 800 volts. Following IEF separation gels were either frozen at -20°C or equilibrated for 1 h in 20% glycerol, 5% β-mercaptoethanol, 2% SDS and made up to 100 ml with 80 mM Tris (pH 6.8) then separated in the second dimension (SDS-PAGE) as described below.

2.5 Isolation of protein and Western blot analysis

Western blot analysis was performed according to Locke and Tanguay (1996). For protein extraction, tissues were sonicated on ice in 50 mM Tris (pH 7.4), 0.5 M EDTA, 1.25 M DTT and 0.5% Tween-20 in approximately one minute intervals until tissue was completely homogenized and supernatant was transferred to a fresh tube. Protein concentrations were determined using a bicinchoninic acid kit (BCA protein assay kit, Pierce, U.S.A.) using BSA standards. Equal amounts of protein were loaded into each well of a SDS-polyacrylamide (10%) gel and separated electrophoretically. Prestained molecular weight markers (GibcoBRL, U.S.A.) were used to estimate the positions of various proteins on the gel. Proteins were electro-blotted on to nitrocellulose membrane (NitroBind, Westborough, MA, U.S.A.) using a Helixx mini trans-blot electrophoretic transfer unit. Proteins were blocked overnight at 4°C in 5% non-fat dry

skim milk in TBS-T containing 20 mM Tris, pH 7.5, 500 mM NaCl and 0.05% Tween-20 (Johnson and Cohen, 1984). The blots were then incubated for 4 h with rabbit polyclonal antibody against Hsp72 (1:5000) or rat monoclonal antibody against Hsp73 (1:3000) (StressGen, Canada) and washed 2 x 5 min with TBS-T. Following the washes, the blot was reacted for 1 h with affinity purified goat anti-rabbit or anti-rat conjugated with alkaline phosphatase (1:3000) (Stressgen, Canada), washed 1 x 5 min with TBS-T and subsequently 1 x 5 min with TBS. Immunoreactivity was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) western blotting detection reagents (GibcoBRL, U.S.A.). To quantify the amount of Hsp73 and Hsp72 expressed in the tissues, standard curves were generated using commercially obtained pure Hsp72 and Hsp73 standards (Stressgen, Canada). Also, commercial Hsp72 or Hsp73 standards were loaded on each experimental gel as an internal control. Membranes were dried overnight, scanned and densitometry was performed using the Kodak 1D imaging system. All bands on the membrane were detected by the program and net intensity was measured for each entire band.

2.6 Statistical analysis

For metabolic status assessment and protein analysis results are expressed as means with the corresponding standard errors of means. The statistical analysis software program SigmaStat version 1.0 (Jandel Corporation) was used to perform the one-way anova Student-Newman-Keuls test ($\alpha = 0.05$).

CHAPTER 3 RESULTS

3.1 Specificity of mammalian antibodies to turtle heat shock protein 70

To determine whether mammalian antibodies to Hsp73 and Hsp72 are specific to turtle isoforms two dimensional gel electrophoresis (IEF/SDS-PAGE) was performed on control liver tissue (Fig. 1). Liver tissue shown in row A (Fig. 1) was probed with Hsp72 antibody only and a single spot appeared. Tissue in row B was probed with Hsp73 antibody only and a single spot appeared. Tissue in row C was probed with both Hsp72 and Hsp73 antibody and two spots appeared.

3.2 Standard curves

To determine absolute concentrations of Hsp73 and Hsp72 in turtle tissues, protein standards for Hsp73 and Hsp72 were immunoblotted with the respective antibodies and densitometry performed using the Kodak 1D imaging system (Fig. 2). Standard curves were generated to determine the correlation between net intensity of protein bands and absolute concentration of protein. These standard curves were used to calculate the absolute concentration of Hsp73 and Hsp72 in each turtle tissue examined. The regression equations are $y = 134.97x - 60.09$ with an r^2 value of 0.99 and $y = 1922.57x + 895.38$ with an r^2 value of 0.99, for the Hsp73 and Hsp72 standard curves, respectively.

Fig. 1. Specificity of antibodies for Hsp72 and Hsp73 in turtle liver. Representative two-dimensional Western blots of control turtle liver tissue (200 μg protein) were immunoblotted with antibodies specific to Hsp72 (A), Hsp73 (B) or both Hsp73 and Hsp72 (C).

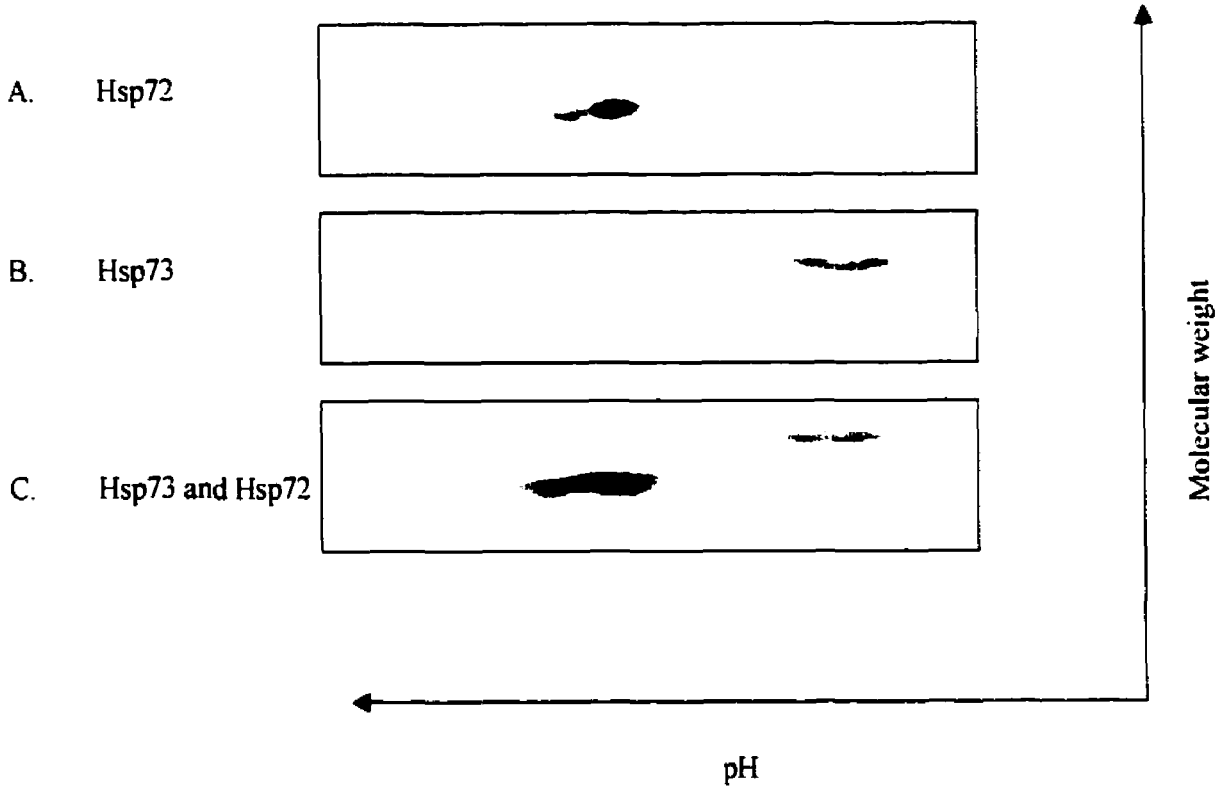
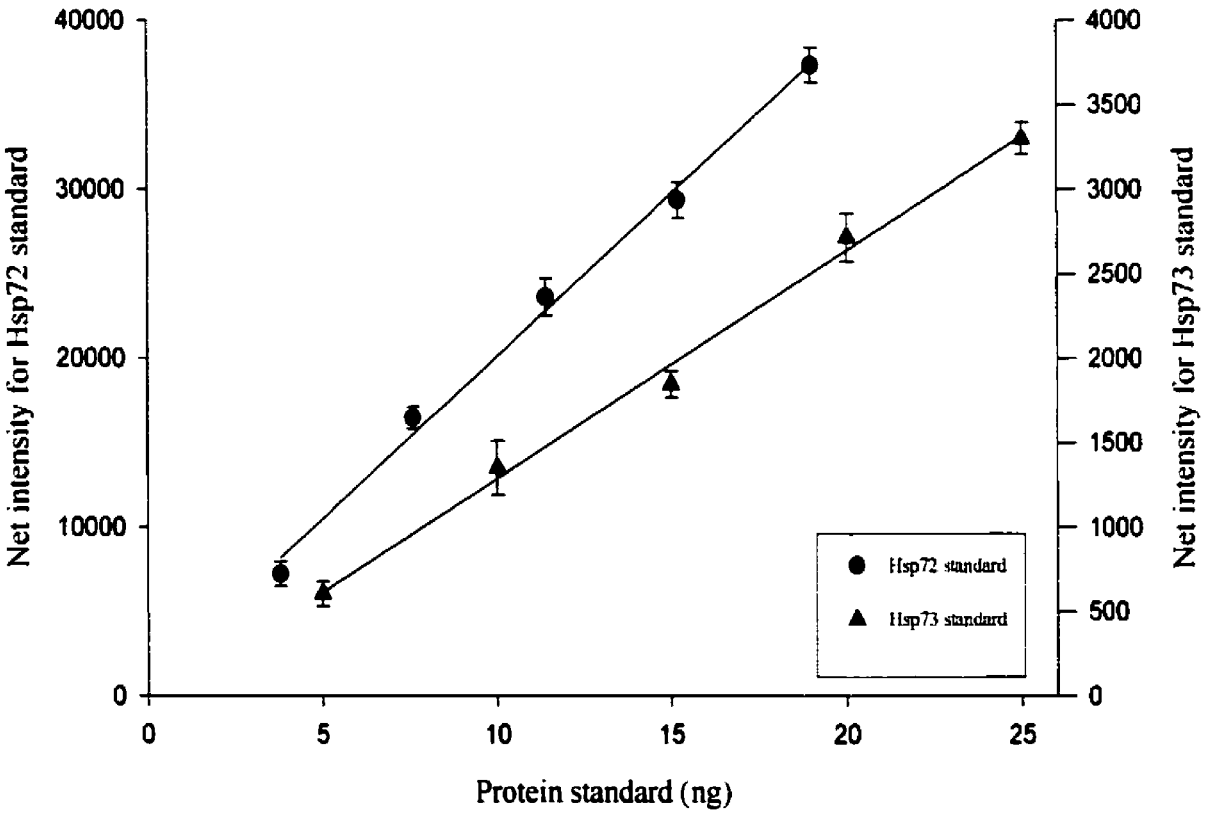

A. Hsp72
B. Hsp73
C. Hsp73 and Hsp72
Molecular weight
pH

Fig. 2. Representative Western blots of Hsp72 and Hsp73 standards and corresponding curves. Increasing amounts of commercially obtained Hsp72 and Hsp73 standards were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted with Hsp72- and Hsp73-specific antibodies (A and B, respectively). Several blots were scanned and net intensity of each band was determined using the Kodak 1D imaging program (C). Data is represented as means ± S.E.M. of 5 – 6 separate blots.

A

B

C


40000
30000
20000
10000
0
Net intensity for Hsp72 standard
4000
3500
3000
2500
2000
1500
1000
500
0
Net intensity for Hsp73 standard
0
5
10
15
20
25
Protein standard (ng)
Hsp72 standard
Hsp73 standard

3.3 Positive control heat shock experiment

In mammals the induction of stress proteins occurs upon exposure to a variety of stressors including heat shock. Therefore, as a positive control and to determine the degree to which the expression of Hsp73 and Hsp72 can increase, the effect of heat shock on the basal expression of Hsp73 and the inducible expression of Hsp72 was examined in turtle brain, heart, liver and skeletal muscle.

In heat shock experiments, turtles were placed in a warm water bath (35°C) for 1 h and then allowed to recover in a room temperature water bath (22°C) for 5 h. After the allotted time turtles were sacrificed and Hsp73 and Hsp72 protein expression was examined by Western blot analysis (Fig. 3A and Fig. 4A, respectively). Quantification of Hsp73 and Hsp72 protein level in each tissue was performed by densitometry (Fig. 3B and Fig. 4B, respectively). Levels of Hsp73 after heat shock were low in all tissues examined compared to controls; however, levels of Hsp72 were significantly elevated in all tissues, compared to control levels (one-way anova Student-Newman-Keuls test, $p < 0.05$).

Fig. 3. Expression of Hsp73 following heat shock. Representative Western blot (A) and densitometric analysis (B) of Hsp73 protein in turtle tissues following heat shock. Turtles were maintained at a water temperature of 35°C for 1 h and recovered at a water temperature of 22°C for 5 h ($n = 4$). Total protein from each tissue was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted with Hsp73-specific antibody. An equal amount of protein (50 μg) was loaded in each lane. Individual blots were scanned and net intensity of each band was determined using the Kodak 1D imaging program. Values are expressed as absolute concentrations calculated from standard curves. Statistical significance (one-way anova Student-Newman-Keuls test; $p < 0.05$) compared to control is indicated by an asterisk (*).

A

Brain Heart Liver Muscle

B

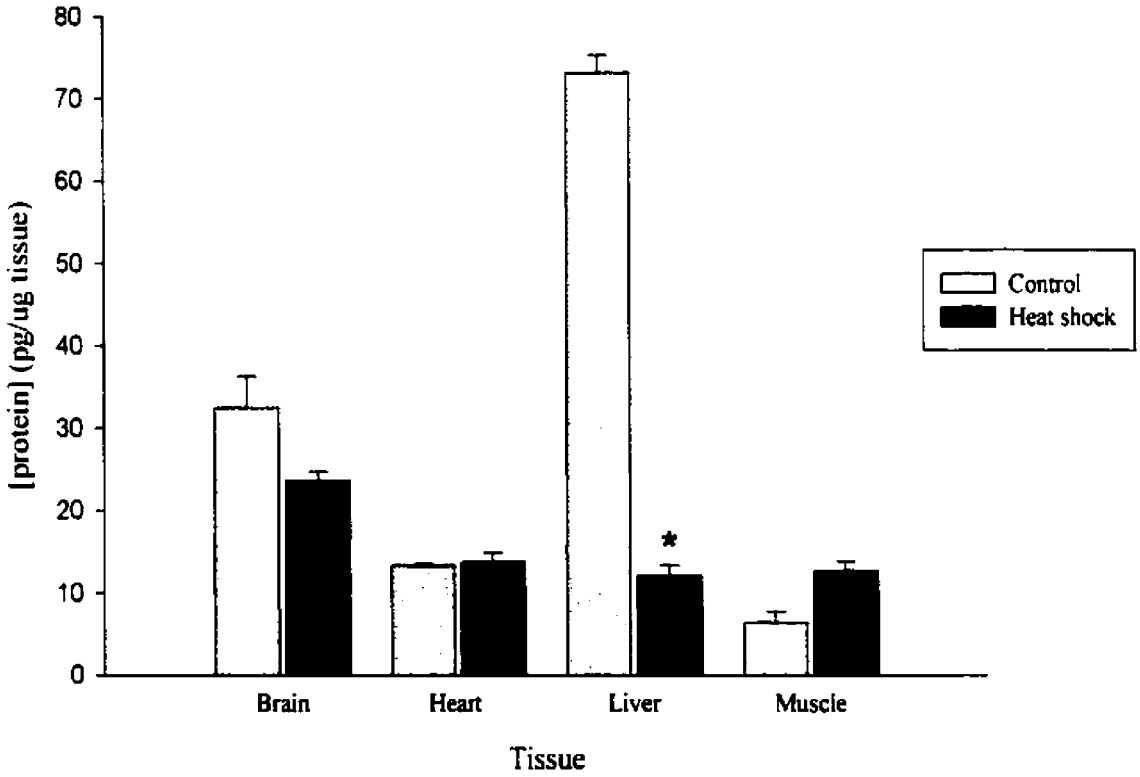
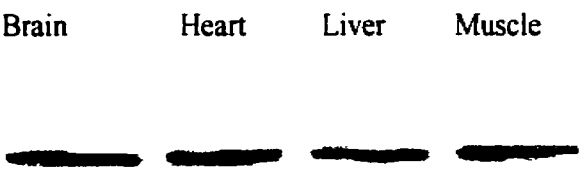
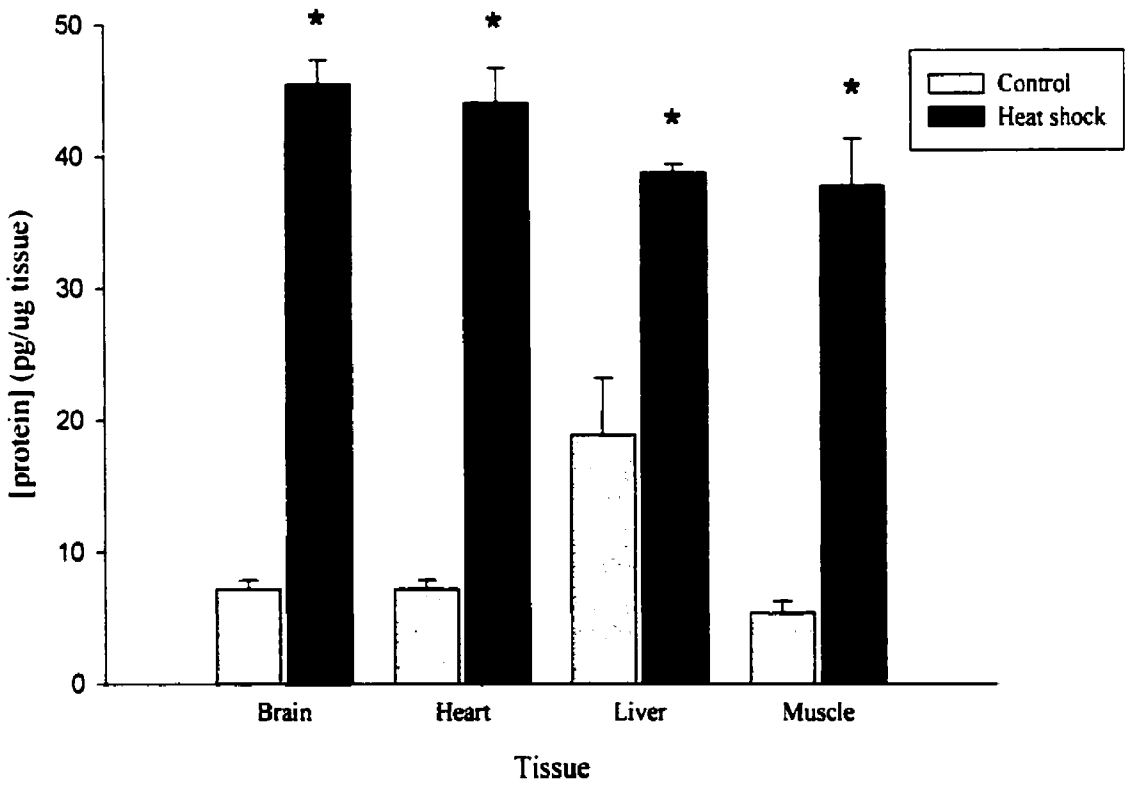


Fig. 4. Expression of Hsp72 following heat shock. Representative Western blot (A) and densitometric analysis (B) of Hsp72 protein in turtle tissues following heat shock. Turtles were maintained at a water temperature of 35°C for 1 h and recovered at a water temperature of 22°C for 5 h ($n = 4$). Total protein from each tissue was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted with Hsp72-specific antibody. An equal amount of protein (50 μg) was loaded in each lane. Individual blots were scanned and net intensity of each band was determined using the Kodak 1D imaging program. Values are expressed as absolute concentrations calculated from standard curves. Statistical significance (one-way anova Student-Newman-Keuls test; $p < 0.05$) compared to control is indicated by an asterisk (*).

A


Brain
Heart
Liver
Muscle

B


50
40
30
20
10
0
[protein] (pg/ug tissue)
*
*
*
*
Control
Heat shock
Brain
Heart
Liver
Muscle
Tissue

3.4 Anoxic dive and recovery experiment

(i) Lactate measurement

To determine whether turtles were relying on anaerobic metabolism during the dive and for comparison to other published values for this dive duration, blood lactate levels were measured in control animals, in animals dived for 24 h and in 1 h recovery animals (Fig. 5). Lactate concentrations increased after 24 h of submergence from a control value of 1.74 ± 0.34 mM to 53.14 ± 7.60 mM (one-way anova Student-Newman-Keuls test, $p < 0.05$). Lactate concentrations remained significantly raised after a recovery period of 1 h (53.74 ± 3.32 mM; one-way anova Student-Newman-Keuls test, $p < 0.05$).

(ii) Arterial blood oxygen measurement

To confirm the anoxic status of the animals and to ascertain whether animals were obtaining oxygen via extrapulmonary respiratory routes arterial blood oxygen (pO_2) levels were measured under control conditions and after 24 h dive and 1 h normoxic recovery (Fig. 6). Arterial blood oxygen level dropped significantly from 18.70 ± 4.0 mmHg during normoxia to 0.22 ± 0.16 mmHg after a 24 h anoxic period (one-way anova Student-Newman-Keuls test, $p < 0.05$). After a 1 h recovery period pO_2 levels rose significant above the normoxic and anoxic values to 59.95 ± 3.64 mmHg (one-way anova Student-Newman-Keuls test, $p < 0.05$).

Fig. 5. Evaluating metabolic status by lactate measurement. Blood lactate measurements in control, 24 h dived and 1 h recovery turtles. Values are means ± S.E.M. of 3 – 5 turtles. Blood lactate concentrations of turtles dived for 24 h and 1 h normoxic recovery were significantly higher than turtles housed under control conditions (one-way anova Student-Newman-Keuls test; $p < 0.05$). Statistical significance compared to control is indicated by an asterisk (*).

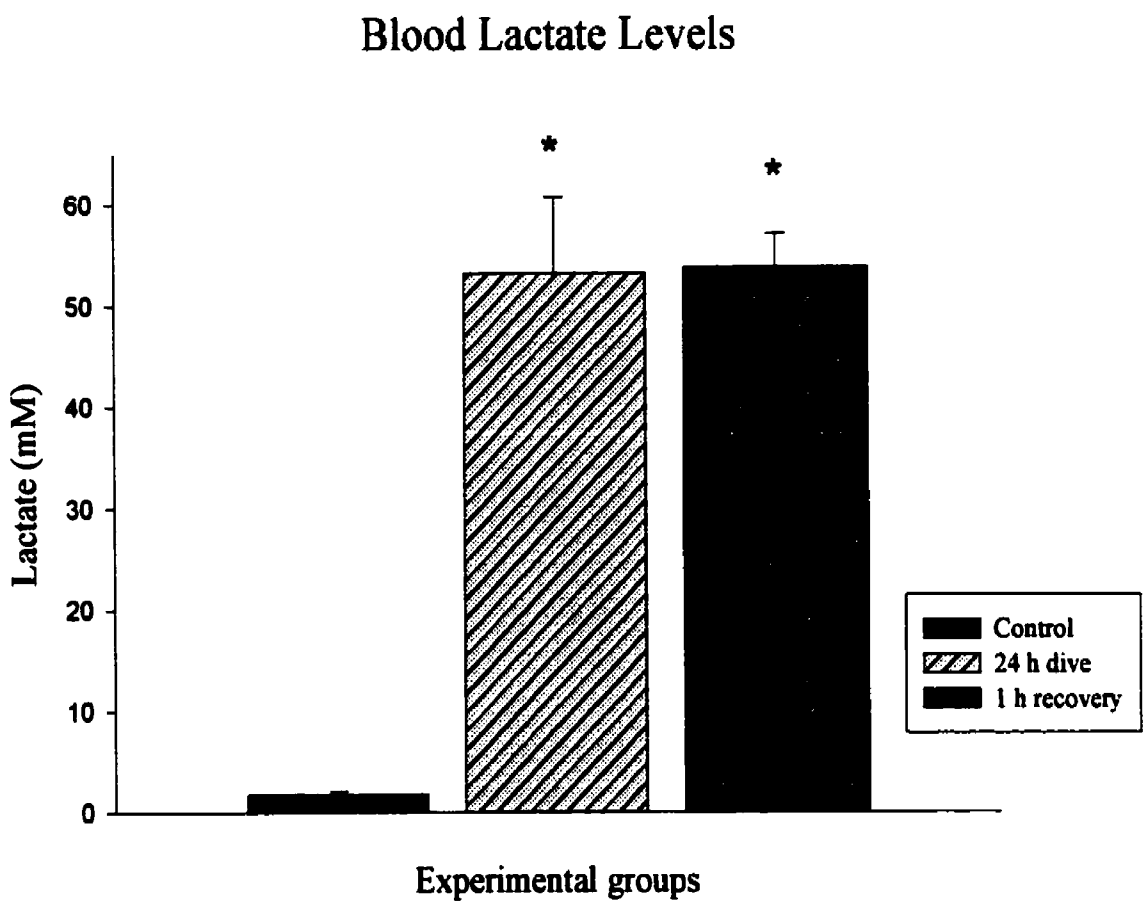
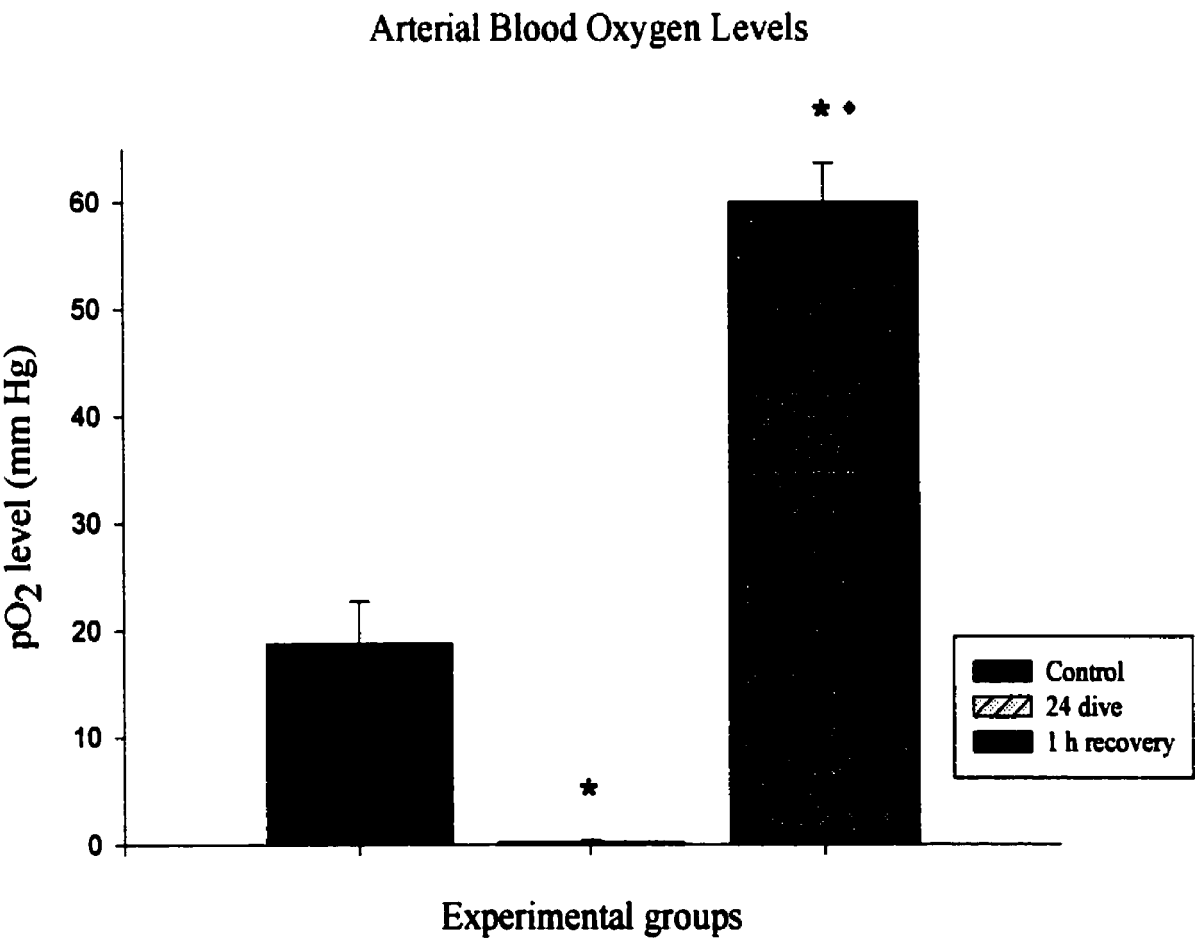

Blood Lactate Levels
Lactate (mM)
0
10
20
30
40
50
60
*
*
Control
24 h dive
1 h recovery
Experimental groups

Fig. 6. Assessment of blood pO_2 level. Arterial blood oxygen measurements in control, 24 h dived and 1 h recovery turtles. Values are means ± S.E.M. of 3 – 5 turtles. Statistical significance compared to control is indicated by an asterisk (*) and a comparison between 24 h dived and 1 h recovery animals is denoted by ♦ (one-way anova Student-Newman-Keuls test, $p<0.05$).


Arterial Blood Oxygen Levels
pO_2 level (mm Hg)
0
10
20
30
40
50
60
* ♦
*
Control
24 dive
1 h recovery
Experimental groups

(iii) Hsp73 expression after anoxia and reoxygenation

Using a mammalian antibody specific for the constitutive isoform of Hsp70, Hsp73 expression was determined by Western blot analysis under control, 24 h anoxic exposure and 1 h normoxic recovery (Fig. 7). After 24 h anoxic exposure Hsp73 expression varied in a tissue-specific manner with brain and liver expressing higher levels and heart and muscle expressing lower levels.

(iv) Hsp72 expression after anoxia and reoxygenation

The level of Hsp72 expression after normoxic, 24 h anoxic and 1 h recovery conditions was examined by Western blot analysis (Fig. 8). Similarly to Hsp73 expression Hsp72 levels varied in a tissue specific pattern; however, basal expression of Hsp72 was low in all tissues examined. Hsp72 expression after anoxia was elevated in heart and skeletal muscle tissue only and after 1 h normoxic recovery was elevated in all tissues.

Fig. 7. Expression of Hsp73 in response to normoxia, anoxia and reoxygenation. Representative Western blots of Hsp73 protein expression in turtle tissues under control, anoxic and recovery conditions. Control turtles were maintained at a water temperature of 16.5°C and water pO_2 of 44 mmHg for 24 h (n = 3). For the anoxic treatment, turtles were dived for 24 h (n = 5) and for recovery, turtles were dived for 24 h and then given access to air for 1 h (n = 4). Total protein from each tissue was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted with Hsp73-specific antibody. An equal amount of protein (50 μg) was loaded in each lane.

Control Hsp73

Anoxic Hsp73

Recovery Hsp73

Brain Heart Liver Muscle

Fig. 8. Expression of Hsp72 in response to normoxia, anoxia and reoxygenation.

Representative Western blots of Hsp72 protein expression in turtle tissues under control, anoxic and recovery conditions. Control turtles were maintained at a water temperature of 16.5°C and water pO_2 of 44 mmHg for 24 h (n = 3). For anoxic conditions, turtles were dived for 24 h (n = 5) and for recovery, turtles were dived for 24 h and then given access to air for 1 h (n = 4). Total protein from each tissue was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and reacted with Hsp72-specific antibody. An equal amount of protein (50 μg) was loaded in each lane.

Control Hsp72

Anoxic Hsp72

Recovery Hsp72

Brain Heart Liver Muscle

(v) Levels of Hsp73 and Hsp72 expression after anoxic exposure and recovery

a) Brain

The level of Hsp73 expression in brain tissue was elevated after each treatment (Fig. 9); however, there were no significant changes (control: 32.50 ± 3.80; after 24 h anoxia 44.50 ± 3.50; 1 h normoxic recovery: 31.70 ± 3.60 pg/μg tissue, respectively).

In contrast, brain Hsp72 expression (Fig. 9) was induced from a level of 7.20 ± 0.70 pg/μg tissue under control conditions to 14.50 ±1.00 pg/μg tissue after 24 h dive and again significantly increased to a level of 30.50 ± 3.50 pg/μg tissue after 1 h normoxic recovery (one-way anova Student-Newman-Keuls test, $p < 0.05$).

b) Heart

Hsp73 expression in heart tissue (Fig. 10) was low and non-variable under control and experimental conditions – control: 13.30 ± 0.20; 24 h dive: 8.40 ± 0.90; 1 h recovery 10.70 ± 1.30 pg/μg tissue, respectively.

Basal Hsp72 expression (Fig. 10) was low in heart tissue – 7.20 ± 0.70 pg/μg tissue. However, upon anoxic exposure Hsp72 expression was significantly induced to a level of 32.00 ± 4.40 pg/μg tissue and following 1 h recovery to 37.80 ± 4.00 pg/μg tissue (one-way anova Student-Newman-Keuls test, $p < 0.05$).

c) Liver

Constitutive expression of Hsp73 in liver tissue (Fig. 11) was high – 73.20 ± 2.10 pg/μg tissue and upon a 24 h anoxic dive decreased to 45.70 ± 6.10 pg/μg tissue. Following 1 h normoxic recovery Hsp73 expression decreased significantly to 17.20 ± 3.90 pg/μg tissue (one-way anova Student-Newman-Keuls test, $p < 0.05$).

Basal Hsp72 expression (Fig. 11) was low – 18.90 ± 4.30 pg/μg tissue and remained low after a 24 h anoxic dive – 9.60 ± 1.20 pg/μg tissue. However, upon 1 h normoxic recovery Hsp72 was strongly induced to a level of 38.80 ± 7.40 pg/μg tissue (one-way anova Student-Newman-Keuls test, $p < 0.05$).

d) Skeletal muscle

Expression of Hsp73 in skeletal muscle tissue (Fig. 12) was low under control conditions as well as under both experimental conditions – control: 6.40 ± 1.30; 24 h dive 4.50 ± 0.70; 1 h normoxic recovery 4.30 ± 0.40 pg/μg tissue, respectively.

Basal Hsp72 expression (Fig. 12) was low – 5.40 ± 0.90 pg/μg tissue. However, upon 24 h anoxic exposure was induced significantly to a level of 20.20 ± 1.10 pg/μg tissue and was induced even further after 1 h normoxic recovery to 34.40 ± 3.50 pg/μg tissue (one-way anova Student-Newman-Keuls test, $p < 0.05$).

Fig. 9. Quantification of brain Hsp73 and Hsp72 protein after normoxic and anoxic exposure and after recovery. Blots from control, anoxic and recovery animals were scanned and net intensity of each band was determined using the Kodak 1D imaging program. Values are expressed as absolute concentrations. Statistical significance compared to control is indicated by an asterisk (*) and comparison between 24 h dived and 1 h recovery tissue is denoted by ● (one-way anova Student-Newman-Keuls test, $p<0.05$).

Brain Hsp73 and Hsp72 levels

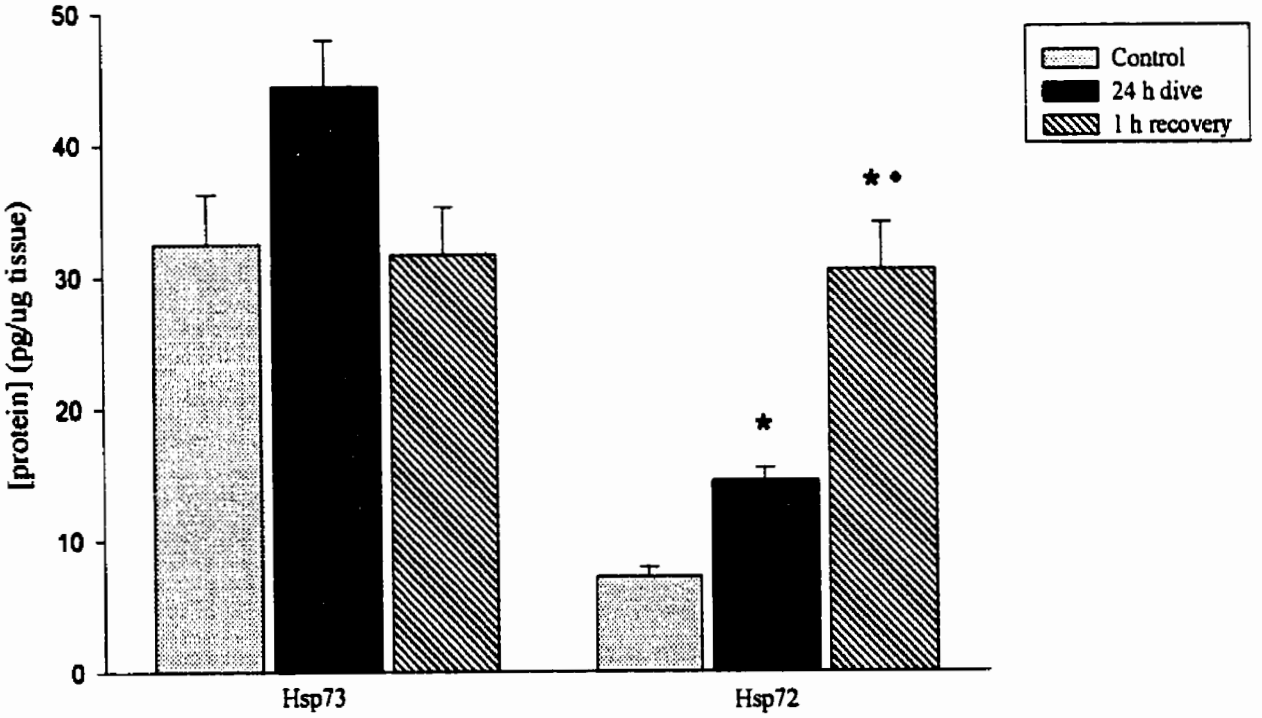


Fig. 10. Quantification of Hsp73 and Hsp72 protein in heart tissue after normoxic and anoxic exposure and after recovery. Blots from control, anoxic and recovery animals were scanned and net intensity of each band was determined using the Kodak 1D imaging program. Values are expressed as absolute concentrations. Statistical significance compared to control is indicated by an asterisk (*); one-way anova Student-Newman-Keuls test, $p<0.05$.

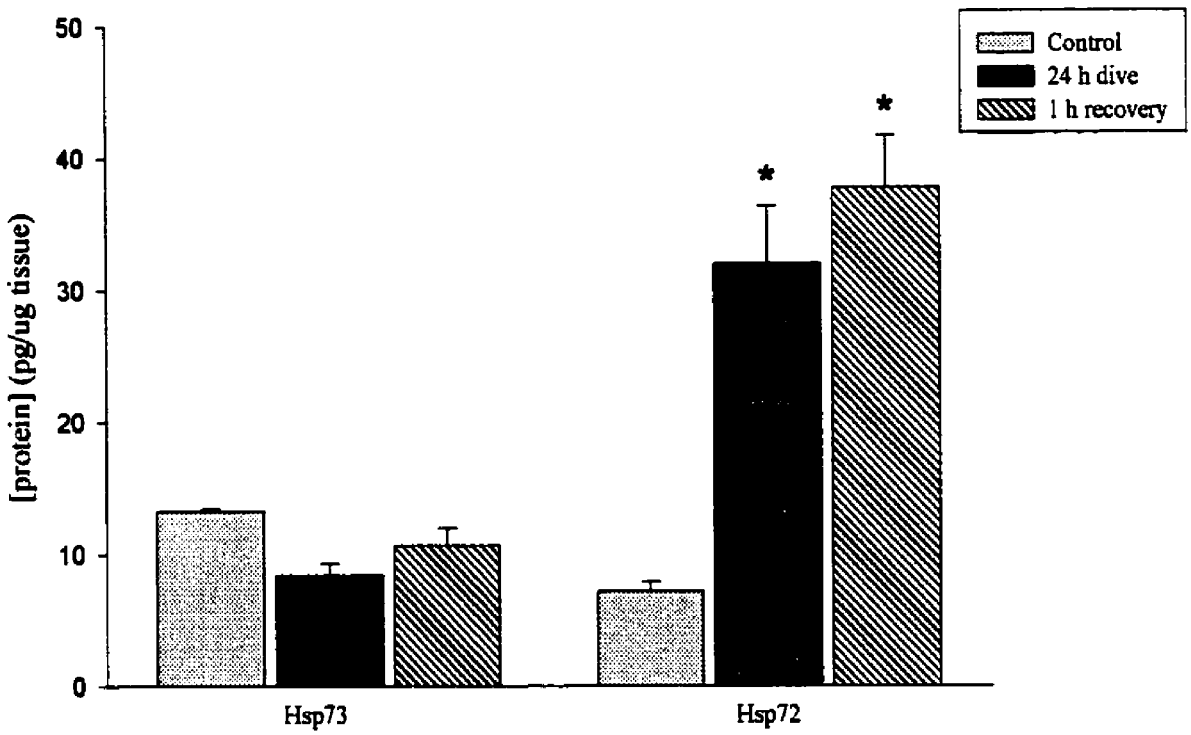

Heart Hsp73 and Hsp72 levels
[protein] (pg/ug tissue)
50
40
30
20
10
0
Hsp73
Hsp72
Control
24 h dive
1 h recovery
*
*

Fig. 11. Quantification of liver Hsp73 and Hsp72 protein after normoxic and anoxic exposure and after recovery. Blots from control, anoxic and recovery animals were scanned and net intensity of each band was determined using the Kodak 1D imaging program. Values are expressed as absolute concentrations. Statistical significance compared to control is indicated by an asterisk (*) and comparison between 24 h dived and 1 h recovery tissue is denoted by ● (one-way anova Student-Newman-Keuls test, $p<0.05$).

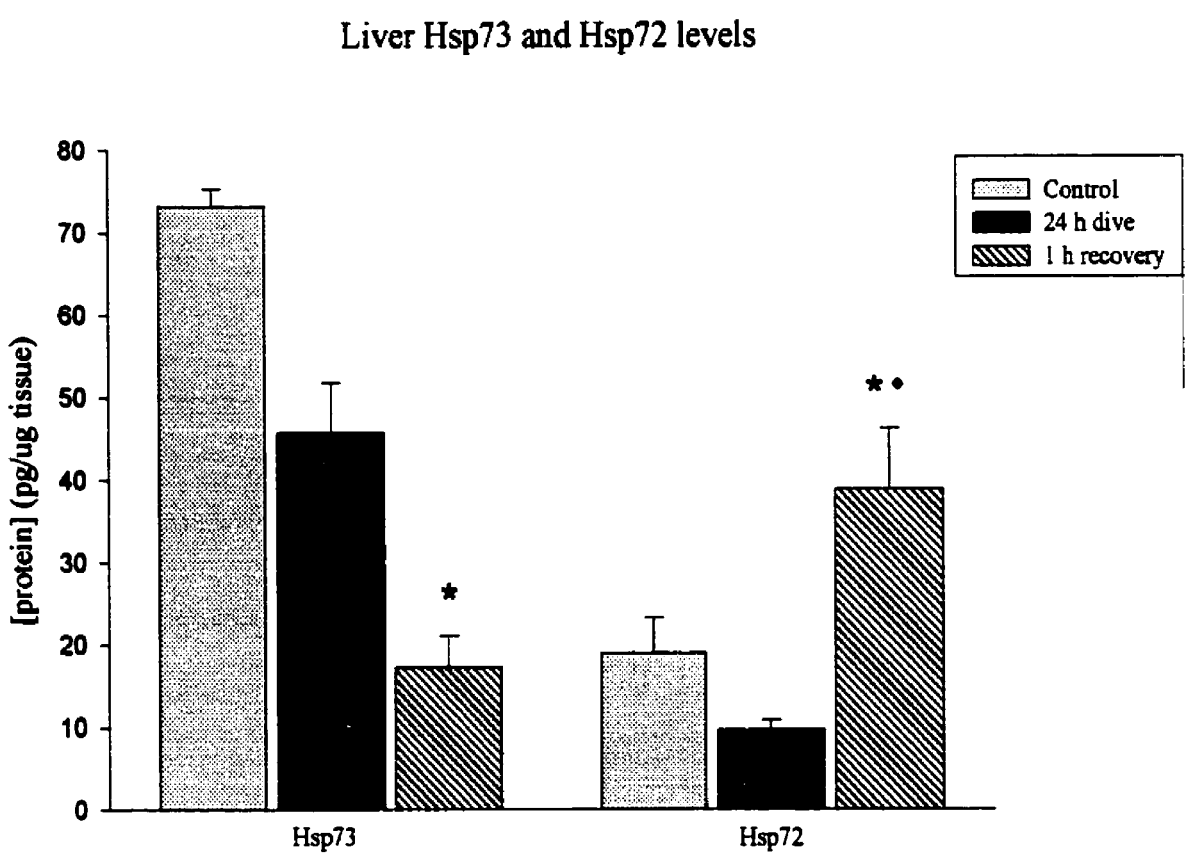
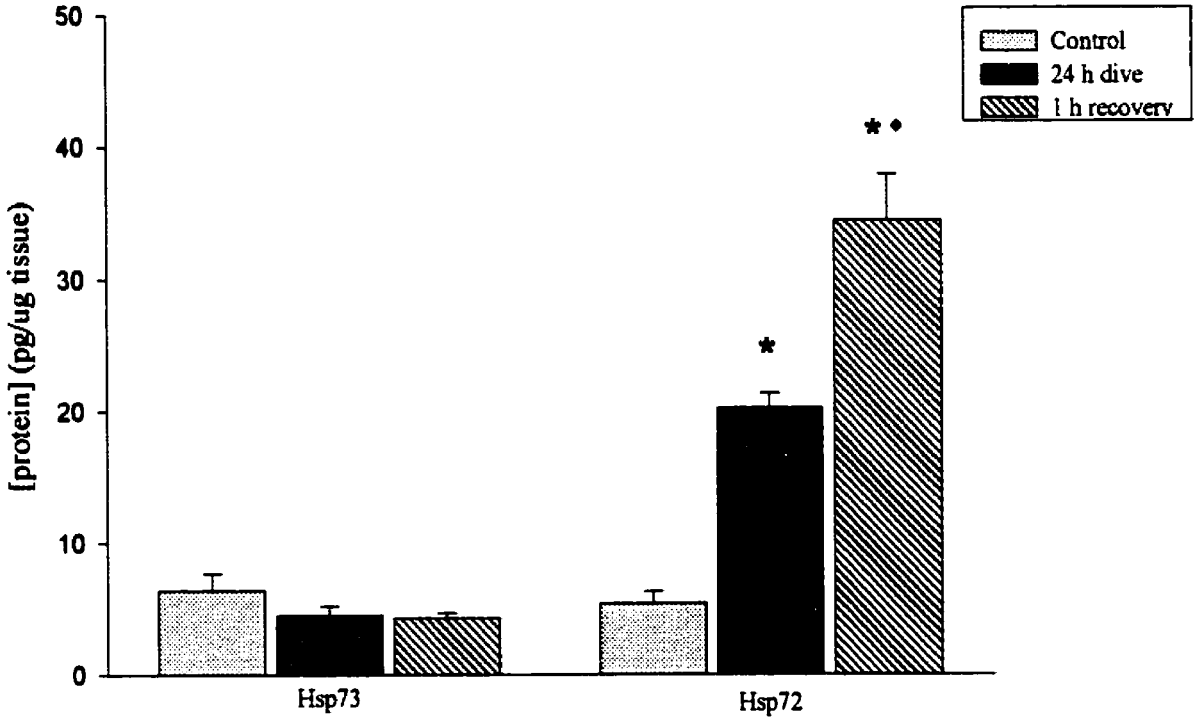

Liver Hsp73 and Hsp72 levels
[protein] (pg/ug tissue)
80
70
60
50
40
30
20
10
0
Control
24 h dive
1 h recovery
*
* •
Hsp73
Hsp72

Fig. 12. Quantification of skeletal muscle Hsp73 and Hsp72 protein after normoxic and anoxic exposure and after recovery. Blots from control, anoxic and recovery animals were scanned and net intensity of each band was determined using the Kodak 1D imaging program. Values are expressed as absolute concentrations. Statistical significance compared to control is indicated by an asterisk (*) and comparison between 24 h dived and 1 h recovery tissue is denoted by ♦ (one-way anova Student-Newman-Keuls test, $p<0.05$).

Skeletal muscle Hsp73 and Hsp72 levels



CHAPTER 4 DISCUSSION

While the physiological mechanisms underlying the remarkable anoxia tolerance of the freshwater turtle *Chrysemys picta belli* have been widely investigated (Lutz *et al.*, 1984; Hochachka *et al.*, 1996; Buck *et al.* 1993), the molecular mechanisms are less well understood. In particular, the role stress proteins play in anoxia tolerance has received little attention. Heat shock protein 70 has been investigated in mammals exposed to lowered oxygen levels and seems to be involved in protection against hypoxia- and reoxygenation-induced cellular damage in various animals and tissues (Hutter *et al.*, 1994; Nakano *et al.*, 1997; Donnelly *et al.*, 1992). Therefore, this study was undertaken to examine the changes in the expression levels of Hsp73 and Hsp72 after heat shock, anoxic exposure and normoxic recovery.

The results presented in this study show that expression of the inducible isoform of the Hsp70 family (Hsp72) increases in all but one of the four turtle tissues studied in response to anoxia, recovery and heat shock. Liver tissue showed a nonsignificant decrease in Hsp72 expression after 24 h anoxic exposure. The results also indicate a stress- and tissue-specific expression pattern for both isoforms, Hsp73 and Hsp72.

4.1 Pattern of Hsp70 expression after heat shock

Exposing tissues to elevated temperatures has been shown to induce the heat shock response in all organisms in which it was sought (Morimoto *et al.*, 1997). In this study it was shown that an ambient temperature increase from 17°C to 35°C induced Hsp72 expression in all four tissues of the western painted turtle examined (Fig. 4).

Hsp73 expression after heat shock was low in all tissues (Fig. 3). Similar increases in Hsp70 expression have been shown in non-mammalian species including the rainbow trout, the frog *Xenopus laevis* and the toad-headed agamid lizard. Airaksinen *et al.* (1998) demonstrated that a temperature increase from 18°C to 26°C induced the synthesis of 67 and 69 kDa proteins (members of the Hsp70 family of stress proteins) in cultured rainbow trout hepatocytes, gill epithelial cells and gonadal fibroblasts. Airaksinen and colleagues (1998) also demonstrated an increase in Hsp70 mRNA after heat shock. In cultured *Xenopus laevis* kidney epithelial cells Phang *et al.* (1999) showed that a temperature increase from 22°C to 35°C induced the synthesis of Hsp70 family members. Additionally, Ulmasov *et al.* (1999) demonstrated that induction of Hsp70 family members occurs at 39°C and proceeds up to 47-50°C in the lizard *Phrynocephalus interscapularis*. In mammalian species, heat shock also results in induction of Hsp70 proteins. Vogt and colleagues (2000) demonstrated that a whole-body heat shock of 42°C for 15 min. resulted in an increased expression of both Hsp72 and Hsp73 in myocardial rabbit tissue. Furthermore, a study by Bechtold *et al.* (2000) showed a marked increase in Hsp72 expression in brain regions of 5 h posthyperthermic rats while constitutive Hsp73 expression remained constant during control and experimental conditions.

4.2 Anoxic dive and recovery experiment

(i) Anaerobic status

In this study it was shown that turtles exposed to a 24 h anoxic period were relying on anaerobic metabolism (glycolysis) as indicated by increased lactate levels (Fig. 5). Blood lactate levels of this magnitude are associated with severe anoxia in the turtle

(Jackson *et al.*, 1996) and are indicative of severe stress. These data are supported by previous studies demonstrating lactate levels in the painted turtle increasing from low control levels (< 2 mM) to 25 mM after a 12 h dive in anoxic water at 22°C (Chang *et al.*, 2000), to 20 mM after a 12 h dive in anoxic water at 20°C (Herbert and Jackson, 1985) and to 14 mM after a 6 h dive in normoxic water at 20°C (Crocker *et al.*, 1999). Studies at lower temperatures (3°C) indicate that lactate concentrations can increase to approximately 50 mM after 2 months of forced submergence (Ultsch *et al.*, 1999).

As well, blood pO_2 levels indicate that turtles were anoxic during the 24 h anoxic period and had returned to normoxic levels after a 1 h recovery period (Fig. 6). Data is supported by a study by Crocker *et al.* (1999) demonstrating that blood pO_2 dropped from control levels of 88 mm Hg to 1.4 mmHg after 6 h dive at 20°C. Also Herbert and Jackson (1985) demonstrate that by 1.5 h of submergence blood pO_2 was minimal, O_2 stores were essentially exhausted and all turtles were operating largely anaerobically.

As can be seen in Figure 6, the blood oxygen level of control animals is fairly low (18.70 ± 4.0 mmHg). A possible reason for the lowered pO_2 level of control animals examined in this study may be that turtles were voluntarily diving prior to capture and sacrifice. Another explanation may be that since turtles breathe at intermittent intervals, blood resting pO_2 levels can vary greatly depending upon at which point of the animal's breathing cycle it is captured and sacrificed. This observation is supported by Ultsch *et al.* (1999) where turtles that had access to air had variable blood O_2 levels, ranging from approximately 5 mmHg to 90 mmHg.

The blood oxygen data also suggests that turtles dived for 24 h in aerated water were not utilizing extrapulmonary respiratory routes. While a study by Franklin (2000)

found that up to 69% of the oxygen requirements of an Australian freshwater turtle (*Rheodytes leukops*) submerged in normoxic water was obtained via aquatic respiration, this is not true of all freshwater turtles. *R. leukops* has two large cloacal bursae that are lined with multi-branching well-vascularised papillae which provide an excellent exchange surface for extrapulmonary respiration. In contrast, *C. picta* has a small area of exposed skin surface and it has been suggested this turtle is not particularly well adapted to extrapulmonary respiration (Ultsch *et al.*, 1984).

By repetitive blood sampling of cannulated animals during forced submergence, Ultsch and Jackson (1995) showed that the metobolic and acid-base responses to submergence at 20°C were not discernibly different whether the water was aerated or non-aerated and it has been suggested that aquatic oxygen uptake is of negligible significance in this species.

Additionally Ultsch *et al.* (1984) found that while *C. picta* survived significantly longer in normoxic water than in anoxic water at 10°C, cutaneous gas exchange was found to make a larger contribution at lower temperatures. In fact, Herbert and Jackson (1985) found that the contribution of cutaneous gas exchange was highest at 3°C and decreased at higher temperatures. The authors suggest that oxygen uptake from the water may be of greater importance at the low temperatures characteristic of overwintering.

(ii) Pattern of Hsp70 anoxia- and reoxygenation-induced expression

The pattern of Hsp73 and Hsp72 expression in response to anoxic exposure and recovery showed tissue- and stress-specificity, an observation that is common for stress protein expression in both mammalian and non-mammalian species. In this study, brain and liver tissue of the painted turtle expressed high levels of the constitutive isoform

Hsp73 under control conditions as well as after anoxia and reoxygenation (Fig. 9 and Fig. 11). In contrast heart and skeletal muscle tissues expressed low levels of Hsp73 under control as well as experimental conditions (Fig. 10 and Fig. 12).

In contrast to the expression pattern for Hsp73, brain and liver tissue showed very little or no expression of Hsp72 after a 24 h anoxic period (Fig. 9 and Fig. 11) whereas heart and skeletal muscle tissue showed significant increases in expression of Hsp72 during anoxia (Fig. 10 and Fig. 12). As well, the pattern of expression of Hsp72 after reoxygenation also differed from the expression after anoxia in that all tissues expressed high levels of Hsp72 (Fig. 9, Fig. 10, Fig. 11 and Fig. 12).

These results correlate well with studies examining stress protein expression in various species where tissue- and stress-specific responses have been demonstrated. In a study examining the effects of heat shock and hypoxia on protein synthesis in rainbow trout, Airaksinen and colleagues (1998) demonstrated that the induced synthesis of Hsps was cell-type- and tissue-specific; Hsp104 was induced only in gonadal fibroblasts and the intensity of one isoform of the Hsp70 family was greater in hepatocytes than in either gonadal fibroblasts or gill epithelial cells. As well they demonstrated that hypoxia induced a cell-type-specific response increasing the synthesis of 36, 39 and 51 kDa proteins in gill epithelial cells. While they did not demonstrate an increase in Hsp70 during hypoxia this may be explained by studies showing protein synthesis under anoxic conditions differing from that under hypoxic conditions (Wenger and Gassmann, 1996). Alternatively as suggested by Airaksinen *et al.* (1998) the differences between the freshwater turtle and the rainbow trout may be related to the evolutionary distance between the animals and/or to their differing anoxia-tolerances since the rainbow trout is

hypoxia-intolerant. In another study examining stress protein expression in four different marine teleost species, Dietz and Somero (1993) demonstrated that the synthesis of Hsp70 and Hsp90 was tissue- and species-specific in brain, gill and liver. Furthermore, in another study examining the stress- and tissue-specificity of Hsp70 expression in a non-mammalian species Lele *et al.* (1997) showed that neither heat shock nor exposure to ethanol induced Hsp70 mRNA accumulation in precartilagenous zebrafish embyronic cells. However, it was demonstrated that a substantial accumulation of Hsp70 mRNA was detectable in epithelial cells of the embryo following exposure to heat shock but not ethanol.

The tissue- and stress-specificity of Hsp70 expression is extended to other animals, including mammals and birds. For example, in a comparative study of Hsp70 expression in chicken and mouse testicular cells following heat shock, Mezquita *et al.* (1998) demonstrated considerable differences in the expression of Hsp70. Cells from adult chicken testis responded to a 2 h heat shock at 46°C with a marked increase in transcription of Hsp70, whereas no major changes in the amount of Hsp70 transcripts were observed in adult mouse testicular cells heat shocked for 2 h at 42°C. Furthermore, a study by Krueger *et al.* (1999) demonstrated that the expression of Hsp70 is cell-type and stress-specific in rat hippocampus. The authors show that Hsp70 is detected at 1.5 and 3 h in neurons after hyperthermia whereas by 24 h Hsp70 is primarily localized in glial cells. They also demonstrate that Hsp70 is primarily detected in neurons after kainic acid-induced seizures.

(iii) Correlation between Hsp72 and Hsp73 expression

Another interesting observation is the correlation between the expression of the two isoforms of Hsp70. In this study, brain and liver expressed high levels of constitutive Hsp73 with little or no induction of Hsp72 after 24 h anoxic submergence (Fig. 9 and Fig. 11). In contrast, heart and skeletal muscle tissue expressed low levels of Hsp73 and upon anoxia these tissues expressed high levels of Hsp72 (Fig. 10 and Fig. 12). Previous studies have shown similar patterns of expression correlating a high constitutive Hsp73 expression to low inducible Hsp72 expression and vice versa. In 1997 Manzerra *et al.* demonstrated that rabbit cerebrum showed high expression of constitutive Hsp73 and corresponding low expression of inducible Hsp72 upon hyperthermic conditions. As well Manzerra and colleagues (1997) showed that other tissues such as liver, heart and muscle had an opposite pattern of expression with these tissues showing low expression of Hsp73 and high expression of inducible Hsp72. Carpenter and Hofmann (2000) demonstrated a similar result showing a higher constitutive expression of Hsp70 in brain tissue as compared to white muscle tissue in several different notothenid fish species. There exist several other studies showing tissue-specific differences in Hsp72 induction following hyperthermia; however, these studies did not examine the constitutive levels of Hsp73 and basal levels of Hsp72 in relation to the induction response (Hotchkiss *et al.*, 1993; Lu and Das, 1993; Flanagan *et al.*, 1995). Furthermore, several studies have shown that when levels of Hsp73 or Hsp72 are elevated the stress response is diminished (DiDomenico *et al.*, 1982; Mosser *et al.*, 1993). In addition, it was shown that when Hsp73 was directly injected into *Xenopus* oocytes prior to an increase in temperature that would normally result in the induction of Hsp72, an attenuation of the stress response

was observed (Mifflin and Cohen, 1994). Thus these studies suggest that the amount of preexisting constitutive Hsp73 and/or Hsp72 can influence the inducible expression of Hsp72 upon exposure to stressful conditions.

4.3 Differential responses of other stress-related proteins

Interestingly differential responses of other stress-related proteins, such as mitogen-activated protein kinases, have been observed in various tissues of the freshwater turtle when exposed to anoxia and freezing. The MAP kinase family of proteins consists of the ERK (extracellular regulated kinases), JNK/SAPK (Jun N-terminal kinases/stress activated protein kinases) and the p38 kinases. Each of these protein kinases can be activated by a variety of different stresses, including hypoxia and reperfusion following ischemia (Seko *et al.*, 1996; Pombo *et al.*, 1994). Greenway and Storey (1999) demonstrated that red-eared slider turtles respond to whole-body anoxia or freezing for 1 or 5 h by differential activation of individual MAPKs in a tissue- and stress-dependent manner. ERKs were not activated by either stress except in the brain of frozen turtles. JNKs were transiently activated by anoxic exposure in brain, heart, kidney and liver tissues but their activity was suppressed during freezing. Changes in the concentrations of the transcription factors c-Fos and c-Jun were also examined and found to be stress- and organ-specific. After 5 h anoxia there were increases in the amount of c-Fos in liver and heart and anoxia induced a strong early increase in the concentration of c-Myc in liver and kidney. This study showed that the ERK and JNK pathways as well as the induction of immediate early genes respond differently to whole-body anoxia or freezing and that each kinase is affected in both a stress-specific and tissue-specific

manner. "Each organ clearly seems to have its own pattern of metabolic and genetic responses that are initiated to cope with the stresses imposed by either anoxia or freezing" (Greenway and Storey, 1999).

4.4 Protein denaturation caused by heat shock, anoxia and reoxygenation

The three stressors employed to examine Hsp73 and Hsp72 expression in this study are clearly different; however, all three lead either directly or indirectly to protein denaturation. Senisterra *et al.* (1997) define protein denaturation as a first-order transition from the native state of low entropy to a more disordered state of higher entropy. Denatured proteins are a hypothesized signal for HSF activation with the exposed hydrophobic groups acting as the specific signal. Studies examining the molten globule intermediate states of proteins which are prone to aggregation suggest that they may represent the critical parameter of the heat shock response (Gosslau *et al.*, 2001). The molten globule state is relatively compact and displays significant native-like secondary structures; however, there is also significant exposure of hydrophobic groups compared to the native protein (Creighton, 1990). Increases in ambient temperature can result in the direct denaturation and misfolding of proteins caused by weakening of polar bonds and exposure of hydrophobic groups normally sequestered from the cytosol.

While it has been demonstrated that heat shock can cause a direct conversion to a molten globule state (Senisterra *et al.*, 1997), oxidative stresses such as anoxia and reoxygenation may cause a more indirect transition to molten globule intermediates through protein destabilization due to fragmentation and formation of non-native disulfide bonds (Creighton, 1990; Iwaki *et al.*, 1993). Disulfide bonds are formed by the

oxidation of thiol (-SH) groups in cysteine residues and they occur both intra- and intermolecularly. Intramolecular disulfide bonds stabilize the tertiary structures of proteins while those that occur intermolecularly are involved in stabilizing quartenary structure. Disulfide bonds are required for the formation of key secondary structures that lead to proper tertiary structure and conformation of proteins. During hypoxia and reoxygenation, reducing conditions occur in the cell which can lead to the loss of disulfide bonds which may result in partial loss of tertiary structure and cytosolic exposure of hydrophobic regions normally sequestered within the centre of the protein.

In addition, reactive oxygen species generated by oxidative stress can lead to direct damage to proteins. Free radicals act on proteins by oxidizing key thiol groups from cysteine residues or by breaking open disulfide bridges (Czubryt *et al.*, 1996). Hypoxia itself has been shown to increase intracellular levels of certain radical oxygen species. In a study by Vanden Hoek *et al.* (1997) levels of superoxide were shown to increase within chick embryo cardiomyocytes exposed to hypoxic conditions while levels of hydrogen peroxide and hydroxyl radicals were generated with reoxygenation. Reactive oxygen species have been shown to be a general trigger for HSF activation by redox modifications (Jacquier-Sarlin and Polla, 1996) and its activity in cardiac tissue is modified by hypoxia and reoxygenation.

Interestingly, a recent study by Zou and colleagues (1998b) hypothesize that all inducers of the heat shock response may generate abnormally folded (i.e., non-native) proteins and that these non-native proteins trigger the Hsp response. They also hypothesized that inducers of the Hsp response may be generally capable of triggering oxidation of non-protein thiols, particularly glutathione. Protein damage can occur when

changes in the cellular redox status, during hypoxia and reoxygenation after hypoxia, alter the ratio of oxidized and reduced forms of glutathione. Oxidized glutathione can exert deleterious effects through nonspecific reactions with free sulfhydryl groups of proteins to form mixed disulfides, reactions that can lead to protein destabilization (Halliwell and Gutteridge, 1989). Presumably, thiol adduction and cross-linking would affect the structure of proteins involved, resulting in unfolding of a fraction of these proteins, causing HSF activation. Zou *et al.* (1998b) tested thirteen different inducers of the Hsp response including H_2O_2 and heat shock and showed that all chemical inducers as well as heat shock caused drastic oxidation of glutathione under conditions under which they induce HSE DNA-binding activity. Under the same conditions all inducers also caused trimerization of HSF1. As well, Zou *et al.* (1998b) showed that several inducers (heat shock, cadmium and arsenite exposure) enhanced thiol oxidation of proteins.

Thus the three stressors investigated in this study, hyperthermia, anoxia and reoxygenation, can all lead to protein denaturation and thus the activation of HSF and the transcription of Hsps. An intriguing aspect of the regulation of induction of the stress proteins is that denatured proteins are both the signal that activates transcription of the stress protein genes and the substrate for the proteins themselves.

4.5 A general model of Hsp70 induction in turtle tissues

As discussed above, hyperthermia is well established as a cellular stress that results in protein denaturation; however, oxidative stress such as anoxic exposure and reoxygenation after anoxia can also lead to the denaturation of proteins. Although the exact nature of the trigger for Hsp70 induction is not established, it is thought that the

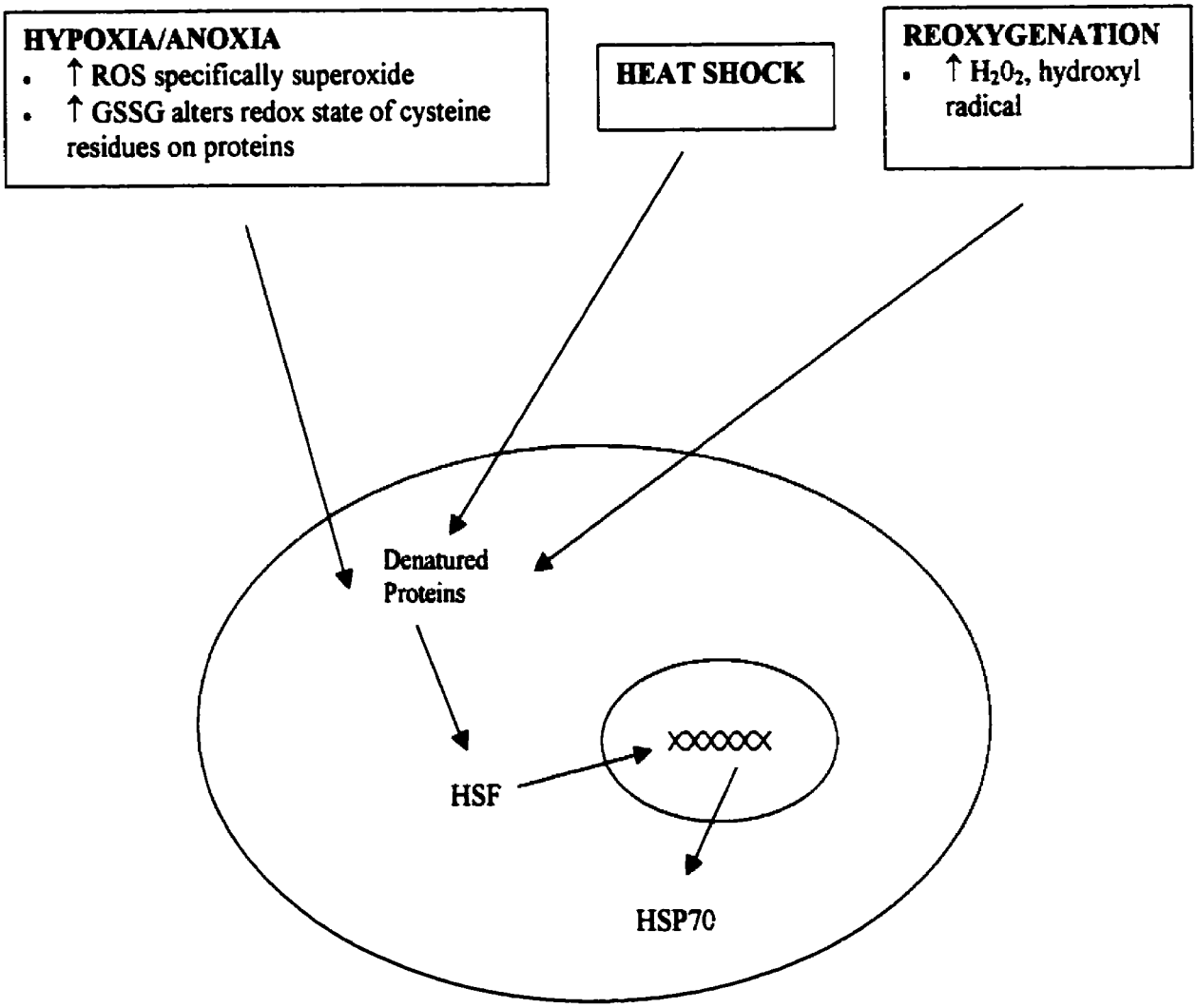
accumulation of denatured protein displaces HSF, which under unstressed conditions, binds to existing Hsp70 resulting in the activation of HSF and the transcription of Hsp70.

Although Hsps have not been extensively studied in the western painted turtle, it is presumed that the same mechanism of Hsp70 induction occurs as in mammalian species. Figure 13 provides a summary of the three conditions employed in this study and their possible link to HSF activation and Hsp70 expression.

As discussed above, this study shows that the expression of two stress proteins, Hsp73 and Hsp72, is both stress- and tissue specific. Also, while heat shock increases the expression of Hsp72 in all tissues, only heart and skeletal muscle tissue show comparable increases in Hsp72 after anoxic exposure. However, upon recovery from anoxic exposure, all tissues showed a significant increase in Hsp72 expression. It appears that the western painted turtle maintains chronically high levels of constitutive Hsp73 in brain and liver tissues and upon anoxic exposure there is no induction of Hsp72. In contrast, in heart and skeletal muscle tissues the western painted turtle maintains low levels of Hsp73 and Hsp72 and undergoes a strong induction of Hsp72 upon exposure to anoxia or reoxgenation.

A further examination of the precise molecular mechanisms underlying the remarkable anoxia tolerance of the western painted turtle and the possible role stress proteins play in this tolerance is no doubt required. Since protein synthesis is a costly process in terms of energetics, the upregulation of heat shock proteins in anoxic tissues indicates that they may play a role in promoting anoxia tolerance. It will be interesting to determine whether Hsp73 and Hsp72 do indeed play a role in the anoxia tolerance and if so, how large that role is.

Figure 13. Proposed mechanism of stress induced increase in HSP70 in turtle tissues. Summary of the three experimental conditions and the possible link to HSF activation and Hsp70 expression.


HYPOXIA/ANOXIA
• ↑ ROS specifically superoxide
• ↑ GSSG alters redox state of cysteine residues on proteins
HEAT SHOCK
REOXYGENATION
• ↑ H_2O_2, hydroxyl radical
Denatured Proteins
HSF
HSP70

REFERENCES

Agsteribbe, E., A. Huckriede, M. Veenhuis, M.H. Ruiters, K.E. Niezen-Koning, O.H. Skjeldal, K. Skullerud, R.S. Gupta, R. Hallberg and O.P. van Diggelen. (1993) A fatal, systemic mitochondrial disease with decreased mitochondrial enzyme activities, abnormal ultrastructure of the mitochondria and deficiency of heat shock protein 60. *Biochem Biophys Res Comm* 193(1): 146-54.

Airaksinen, S., C.M.I. Rabergh, L. Sistonen and M. Nikinmaa. (1998) Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. *J Exp Biol* 201: 2543-51.

Ali, A., S. Bharadwaj, R. O'Carroll and N. Ovsenek. (1998) HSP90 interacts with and regulates the activity of heat shock factor 1 in *Xenopus* oocytes. *Mol Cell Biol* 18: 4949-60.

Aoki, M., K. Abe, J. Kawagoe, S. Nakamura and K. Kogure. (1993) The preconditioned hippocampus accelerates Hsp70 heat shock gene expression following transient ischemia in the gerbil. *Neurosci Lett* 155: 7-10.

Bailey, J.R. and W.R. Driedzic. (1996) Decreased total ventricular and mitochondrial protein synthesis during extended anoxia in turtle heart. *Am J Physiol* 271(6 Pt 2): R1660-7.

Bharadwaj, S., A. Ali and N. Ovsenek. (1999) Multiple components of the HSP90 chaperone complex function in regulation of heat shock factor 1 *in vivo*. *Mol Cell Biol* 19: 8033-41.

Bechtold, D.A., S.J. Rush and I.R. Brown. (2000) Localization of the heat-shock protein Hsp70 to the synapse following hyperthermic stress in the brain. *J Neurochem* 74(2): 641-6.

Borkovich, K.A., F.W. Farrelly, D.B. Finkelstein, J. Taulien and S. Lindquist. (1989) hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. *Mol Cell Biol* 9(9): 3919-30.

Buck, L.T., P.W. Hochachka, A. Schon and E. Gnaiger. (1993) Microcalorimetric measurement of reversible metabolic suppression induced by anoxia in isolated hepatocytes. *Am J Physiol* 265(5 Pt 2): R1014-9.

Buck, L.T. and P.E. Bickler. (1998) Adenosine and anoxia reduce N-methyl-D-aspartate receptor open probability in turtle cerebrocortex. *J Exp Biol* 201 (Pt 2): 289-97.

Chang, J., A.A. Knowlton and J.S. Wasser. (2000) Expression of heat shock proteins in turtle and mammal hearts: relationship to anoxia tolerance. *Am J Physiol* 278: R209-14.

Caplan, A.J., D.M. Cyr, and M.G. Douglas. (1993) Eukaryotic homologues of Escherichia coli dnaJ: a diverse protein family that functions with hsp70 stress proteins. *Mol Biol Cell* 4(6): 555-63.

Carpenter, C.M. and G.E. Hofmann. (2000) Expression of 70 kDa heat shock proteins in antarctic and New Zealand notothenioid fish. *Comp Biochem Physiol* 125: A229-38.

Chih, C.P., M. Rosenthal M. and T.J. Sick. (1989) Ion leakage is reduced during anoxia in turtle brain: a potential survival strategy. *Am J Physiol* 257(6 Pt 2): R1562-4.

Clegg, J.S., S.A. Jackson and V.I. Popov. (2000) Long-term anoxia in encysted embryos of the crustacean, *Artemia franciscana*: viability, ultrastructure and stress proteins. *Cell Tis Res* 301: 433-46.

Creagh, E.M., D. Sheehan and T.G. Cotter. (2000) Heat shock proteins--modulators of apoptosis in tumour cells. *Leuk* 14(7): 1161-73.

Creighton, T.E. (1990) Protein folding. *Biochem J* 270(1): 1-16.

Crocker, C.E., G.R. Ultsch and D.C. Jackson. (1999) The physiology of diving in a north-temperate and three tropical turtle species. *J Comp Physiol* 169: B249-55.

Csermely, P. and C.R. Kahn. (1991) The 90-kDa heat shock protein (hsp-90) possesses an ATP binding site and autophosphorylating activity. *J Biol Chem* 266(8): 4943-50.

Czubryt, M.P., V. Panagia and G.N. Pierce. (1996) The roles of free radicals, peroxides and oxidized lipoproteins in second messenger system dysfunction. *EXS* 76: 57-69.

DiDomenico, B.J., G.E. Bugaisky and S. Lindquist. (1982) The heat shock response is self-regulated at both transcriptional and post transcriptional levels. *Cell* 31: 593-603.

Dietz, T.J. and G.N. Somero. (1993) Species and tissue-specific synthesis patterns for heat-shock proteins Hsp70 and Hsp90 in several marine teleost fishes. *Physiol Zool* 66: 863-80.

Dillmann, W.H., H.B. Mehta, A. Barrieux, B.D. Guth, W.E. Neeley and J. Ross. (1986) Ischemia of the dog heart induces the appearance of a cardiac mRNA coding for a protein with migration characteristics similar to heat-shock/stress protein 71. *Circ Res* 59(1): 110-4.

Donnelly, T.J., R.E. Sievers, F.L. Vissern, W.J. Welch and C.L. Wolfe. (1992) Heat

shock protein induction in rat hearts. A role for improved myocardial salvage after ischemia and reperfusion? *Circ* 85(2): 769-78.

Flanagan, S.W., A.J. Ryan, C.V. Gisolfi and P.L. Moseley. (1995) Tissue-specific hsp70 response in animals undergoing heat stress. *Am J Physiol* 268: R28-35.

Fostinis, Y., P.A. Theodoropoulos, A. Gravanis and C. Stournaras. (1992) Heat shock protein HSP90 and its association with the cytoskeleton: a morphological study. *Biochem Cell Biol* 70(9): 779-86.

Franklin, C.E. (2000) Breathing from both ends: respiratory feats of a freshwater turtle. *Comp Biochem Physiol* 126: S37.

Gatten, R.E. Jr. (1981) Anaerobic metabolism in freely diving painted turtles (*Chrysemys picta*). *J Exp Zool* 216: 377-85.

Gething, M.J. and J. Sambrook. (1992) Protein folding in the cell. *Nature* 355(6355): 33-45.

Gosslau, A., P. Ruoff, S. Mohsenzadeh, U. Hobohm and L. Rensing. (2001) Heat shock and oxidative stress-induced exposure of hydrophobic protein domains as common signal in the induction of hsp68. *J Biol Chem* 276(3): 1814-21.

Greenway, S.C. and K.B. Storey. (1999) Discordant responses of mitogen-activated protein kinases to anoxia and freezing exposures in hatchling turtles. *J Comp Physiol* 169: B521-7.

Halliwell, B. and J.M.C. Gutteridge. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 219: 1-14.

Hartl, F.U., J. Martin and W. Neupert. (1992) Protein folding in the cell: the role of

molecular chaperones Hsp70 and Hsp60. *Ann Rev Biophys Biomol Struct* 21: 293-322.

Herbert, C.V. and D.C. Jackson. (1985) Temperature effects on the responses to prolonged submergence in the turtle *Chrysemys picta bellii*. II. Metabolic rate, blood acid-base and ionic changes, and cardiovascular function in aerated and anoxic water. *Physiol Zool* 58(6): 670-81.

Hightower. L.E. (1991) Heat shock, stress proteins, chaperones, and proteotoxicity. *Cell* 66(2): 191-7.

Hochachka, P.W. (1986) Defense strategies against hypoxia and hypothermia. *Science* 231(4735): 234-41.

Hochachka, P.W., L.T. Buck, C.J. Doll and S.C Land. (1996) Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc Natl Acad Sci U.S.A.* 93(18) :9493-8.

Hotchkiss, R., I. Nunnally, S. Lindquist, J. Taulier, G. Perdrizet and I. Karl. (1993) Hyperthermia protects mice against the lethal effects of endotoxin. *Am J Physiol* 265: R1447-57.

Hutter, M.M., R.E. Sievers, V. Barbosa and C.L. Wolfe. (1994) Heat-shock protein induction in rat hearts. A direct correlation between the amount of heat-shock protein induced and the degree of myocardial protection. *Circ* 89(1): 355-60.

Iwaki, K., S.H. Chi, W.H. Dillmann and R. Mestril. (1993) Induction of HSP70 in cultured rat neonatal cardiomyocytes by hypoxia and metabolic stress. *Circ* 87(6): 2023-32.

Jaattela, M. (1999) Heat shock proteins as cellular lifeguards. *Ann Med* 31(4): 261-71.

Jaattela, M., D. Wissing, P.A. Bauer and G.C. Li. (1992) Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. *EMBO J* 11(10): 3507-12.

Jackson, D.C. (1968) Metabolic depression and oxygen depletion in the diving turtle. *J Appl Physiol* 24(4): 503-9.

Jackson, D.C. (2000) Living without oxygen: lessons from the freshwater turtle. *Comp Biochem Physiol* 125: 299-315.

Jackson, D.C., V.I. Toney and S. Okamoto. (1996) Lactate distribution and metabolism \ during and after anoxia in the turtle, Chrysemys picta bellii. *Am J Physiol* 271(2 Pt 2):R409-16.

Jackson, D.C. and G.R. Ultsch. (1982) Long-term submergence at 3°C of the turtle *Chrysemys picta belli*, in normoxic and severely hypoxic water. I. Survival, gas exchange and acid-base status. *J Exp Biol* 96: 11-28.

Jacquier-Sarlin, M.R. and B.S. Polla. (1996) Dual regulation of heat-shock transcription factor (HSF) activation and DNA-binding activity by H2O2: role of thioredoxin. *Biochem J* 318: 187-93.

Jedlicka, P., M.A. Mortin and C. Wu. (1997) Multiple functions of Drosophila heat shock transcription factor in vivo. *EMBO J* 16(9): 2452-62.

Jolly, C., C. Vourc'h, M. Robert-Nicoud and R.I. Morimoto. (1999) Intron-independent association of splicing factors with active genes. *J Cell Biol* 145(6): 1133-43.

Kiang, J.G. and G.C. Tsokos. (1998) Heat shock protein 70 kDa: molecular biology,

biochemistry, and physiology. Pharm *Ther 80(2): 183-201.*

Kirino, T., Y. Tsujita and A. Tamura. (1991) Induced tolerance to ischemia in gerbil hippocampal neurons. *J Cereb Blood Flow Metab* 11: 299-307.

Kitagawa, K., M. Matsumoto, M. Tagaya, R. Hata, H. Ueda, M. Niinobe, N. Handa, R. Fukunaga, K. Kimura, K. Mikoshiba et al. (1990). Ischemic tolerance phenomenon found in the brain. *Brain Res* 528: 21-4.

Knowlton, A.A., P. Brecher and C.S. Apstein. (1991) Rapid expression of heat shock protein in the rabbit after brief cardiac ischemia. *J Clin Invest* 87(1): 139-47.

Krishnan, S.N., Y.-A. Sun, A. Mohsenin, R.J. Wyman and G. Haddad. (1997) Behavioral and electrophysiologic responses of *Drosophila melanogaster* to prolonged period of anoxia. *J Insect Physiol* 43: 203-10.

Krueger A.M.R., J.N. Armstrong, J-C. Plumier, H.A. Robertson and R.W. Currie. (1999) Cell specific expression of Hsp70 in neurons and glia of the rat hippocampus after hyperthermia and kainic acid-induced seizure activity. *Mol Brain Res* 71: 265-78.

Kume, M., Y. Yamamoto, S. Saad, T. Gomi, S. Kimoto, T. Shimabukuro, T. Yagi, M. Nakagami, Y. Takada, T. Morimoto and Y. Yamaoka. (1996) Ischemic preconditioning of the liver in rats: implications of heat shock protein induction to increase tolerance of ischemia-reperfusion injury. *J Lab Clin Med* 128: 251-8.

Land, S.C., L.T. Buck and P.W. Hochachka. (1993) Response of protein synthesis to anoxia and recovery in anoxia-tolerant hepatocytes. *Am J Physiol* 265(1 Pt 2): R41-8.

Land, S.C. and P.W. Hochachka. (1994) Protein turnover during metabolic arrest in turtle

hepatocytes: role and energy dependence of proteolysis. *Am J Physiol* 266(4 Pt 1): C1028-36.

Lele, Z., S. Engel and P.H. Krone. (1997) hsp47 and hsp70 gene expression is differently regulated in a stress- and tissue-specific manner in zebrafish embryos. *Dev Gen* 21: 123-33.

Li, G.C., L.G. Li, Y.K. Liu, J.Y. Mak, L.L. Chen and W.M. Lee. (1991) Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene. *Proc Natl Acad Sci U.S.A.* 88(5): 1681-5.

Lindquist, S. and E.A. Craig. (1988) The heat-shock proteins. *Ann Rev Gen* 22 :631-77.

Locke, M. and R.M. Tanguay. (1996) Increased HSF activation in muscles with a high constitutive Hsp70 expression. *Cell Stress Chap* 1: 189-96.

Lu, D. and K. Das. (1993) Induction of differential heat shock gene expression in heart, lung, brain and kidney by a sympathomimetic drug, amphetamine. *Biochem Biophys Res Comm* 192: 808-12.

Lutz, P.L., P. McMahon, M. Rosenthal and T.J. Sick. (1984) Relationships between aerobic and anaerobic energy production in turtle brain in situ. *Am J Physiol* 247(4 Pt 2): R740-4.

Lutz, P.L., R. Edwards and P.M. McMahon. (1985) gamma-Aminobutyric acid concentrations are maintained in anoxic turtle brain. *Am J Physiol* 249(3 Pt 2): R372-4.

Lutz, P.L. and G.E. Nilsson. (1997) *The brain without oxygen.* Landes Bioscience. Austin, TX.

Mallouk, Y., M. Vayssier-Taussat, J.V. Bonventre and B.S. Polla. (1999) Heat shock protein 70 and ATP as partners in cell homeostasis. *Intl J Mol Med* 4(5): 463-74.

Manzerra, P., S.J. Rush and I.R. Brown. (1997) Tissue-specific differences in heat shock protein hsc70 and hsp70 in the control and hyperthermic rabbit. *J Cell Physiol* 170(2): 130-7.

McCord, J.M. (1985) Oxygen-derived free radicals in postischemic tissue injury. *New Eng J Med* 312(3): 159-63.

Mendelsohn, M.E., Y. Zhu and S. O'Neill. (1991) The 29-kDa proteins phosphorylated in thrombin-activated human platelets are forms of the estrogen receptor-related 27-kDa heat shock protein. *Proc Natl Acad Sci U.S.A.* 88(24): 11212-6.

Mezquita, B., C. Mezquita and J. Mezquita. (1998) Marked differences between avian and mammalian testicular cells in the heat shock induction and polyadenylation of Hsp70 and ubiquitin transcripts. *FEBS Let* 436(3): 382-6.

Mifflin, L.C. and R.E. Cohen. (1994) hsc70 moderates the heat shock (stress) response in Xenopus oocytes and binds to denatured protein inducers. *J Biol Chem* 269: 15718-23.

Minami, Y., H. Kawasaki, K. Suzuki and I. Yahara. (1993) The calmodulin-binding domain of the mouse 90-kDa heat shock protein. *J Biol Chem* 268(13): 9604-10.

Miyata, Y. and I. Yahara. (1991) Cytoplasmic 8 S glucocorticoid receptor binds to actin filaments through the 90-kDa heat shock protein moiety. *J Biol Chem* 266(14): 8779-83.

Moran, L.A., K.G. Scrimgeour, H.R. Horton, R.S. Ochs and J.D. Rawn. (1994) *Biochemistry.* Neil Patterson Publishers/Prentice-Hall, Inc., Englewood Cliffs, NJ.

Morano, K.A. and D.J. Thiele. (1999) The Sch9 protein kinase regulates Hsp90 chaperone complex signal transduction activity in vivo. *EMBO J* 18(21): 5953-62.

Morimoto, R.I. (1993) Cells in stress: transcriptional activation of heat shock genes. *Science* 259(5100): 1409-10.

Morimoto, R.I., M.P. Kline, D.N. Bimston and J.J. Cotto. (1997) The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. *Ess Biochem* 32: 17-29.

Mosser, D.D., J. Duchaine and B. Massie. (1993) The DNA binding activity of the human heat shock transcription factor is regulated in vivo by hsp70. *Mol Cell Biol* 13: 5427-38.

Nadeau, K., A. Das and C.T. Walsh. (1993) Hsp90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl prolyl isomerases. *J Biol Chem* 268(2): 1479-87.

Nakano, M., D.L. Mann and A.A. Knowlton. (1997) Blocking the endogenous increase in HSP 72 increases susceptibility to hypoxia and reoxygenation in isolated adult feline cardiocytes. *Circ* 95(6): 1523-31.

Noll, F. (1974) "Determination with LDH, GPT and NAD" in *Methods of enzymatic analysis*. Bergmeyer, H.U., ed., Academic, New York.

Nover, L. (1991) "Inducers of HSP synthesis: Heat shock and chemical stressors" in *Heat shock response*. Lutz, N., ed., CRC Press, Inc., Boca Raton, FL.

O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250(10): 4007-21.

Parsell, D.A. and S. Lindquist. (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Ann Rev Gen* 27: 437-96.

Perez-Pinzon, M.A., M. Rosenthal, T.J. Sick, P.L. Lutz, J. Pablo and D. Mash. (1992) Downregulation of sodium channels during anoxia: a putative survival strategy of turtle brain. *Am J Physiol* 262(4 Pt 2): R712-5.

Phang, D., E.M. Joyce and J.J. Heikkila. (1999) Heat shock-induced acquisition of thermotolerance at the levels of cell survival and translation in Xenopus A6 kidney epithelial cells. *Biochem Cell Biol* 77(2): 141-51.

Piacentini, L. and J.S. Karliner. (1999) Altered gene expression during hypoxia and reoxygenation of the heart. *Pharm Ther* 83: 21-37.

Pombo, C.M., J.V. Bonventre, J. Avruch, J.R. Woodgett, J.M. Kyriakis and T. Force. (1994) The stress-activated kinases are major c-jun amino-terminal kinases activated by ischemia and reperfusion. *J Biol Chem* 269: 26546-51.

Pratt, W.B., M.J. Czar, L.F. Stancato and J.K. Owens. (1993) The hsp56 immunophilin component of steroid receptor heterocomplexes: could this be the elusive nuclear localizatior signal-binding protein? *J Ster Biochem Mol Biol* 46(3): 269-79.

Ritossa, F. (1962) A new puffing pattern induced by heat shock and DNP in *Drosophila*. *Experientia* 18: 571-3.

Rodenhiser, D., J.H. Jung and B.G. Atkinson. (1985) Mammalian lymphocytes: stress-induced synthesis of heat-shock proteins in vitro and in vivo. *Cdn J Biochem Cell Biol* 63(7): 711-22.

Rossi, J.M. and S. Lindquist. (1989) The intracellular location of yeast heat-shock protein 26 varies with metabolism. *J Cell Biol* 108(2): 425-39.

Saad, S., M. Kanai, M. Awane, Y. Yamamoto, T. Morimoto, W. Isselhard, T. Minor, H. Troidl, K. Ozawa and Y. Yamaoka. (1995) Protective effect of heat shock pretreatment with heat shock protein induction before hepatic warm ischemic injury caused by Pringle's maneuver. *Surgery* 118: 510-6.

Sanchez, Y. and S.L. Lindquist. (1990) Hsp104 required for induced thermotolerance. *Science* 248: 1112-5.

Satyal, S.H., D. Chen, S.G. Fox, J.M. Kramer and R.I. Morimoto. (1998) Negative regulation of the heat shock transcriptional response by HSBP1. *Genes Dev* 12: 1962-74.

Seko, Y., K. Tobe, K. Ueki, T. Kadowaki and Y. Yazaki. (1996) Hypoxia and hypoxia/reoxygenation activate Raf-1, mitogen-activated protein kinase kinase, mitogen-activated protein kinases, and S6 kinase in cultured rat cardiac myocytes. *Circ Res* 78: 82-90.

Senisterra, G.A., S.A. Huntley, M. Escaravage, K.R. Sekhar, M.L. Freeman, M. Borrelli and J.R. Lepock. (1997) Destabilization of the Ca2+-ATPase of sarcoplasmic reticulum by thiol-specific, heat shock inducers results in thermal denaturation at 37 degrees C. *Biochem* 36: 11002-11.

Sharma, H.S., M. Wunsch, T. Brand, P.D. Verdouw and W. Schaper. (1992) Molecular biology of the coronary vascular and myocardial responses to ischemia. *J Cardiovas Pharm* 20 Suppl 1: S23-31.

Shi, Y., P.E. Kroeger and R.I. Morimoto. (1995) The carboxyl-terminal transactivation

domain of heat shock factor 1 is negatively regulated and stress responsive. *Mol Cell Biol* 15(8): 4309-18.

Steels, E.L., K. Watson and P.G. Parsons. (1992) Relationships between thermotolerance, oxidative stress responses and induction of stress proteins in human tumour cell lines. *Biochem Pharm* 44(11): 2123-9.

Storey, K.B. and J.M. Storey. (1990) Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Q Rev Biol* 65: 145-74.

Tanaka, M., H. Fujiwara, K. Yamasaki, M. Miyamae, R. Yokota, K. Hasegawa, T. Fujiwara and S. Sasayama. (1994) Ischemic preconditioning elevates cardiac stress protein but does not limit infarct size 24 or 48 h later in rabbits. *Am J Physiol* 267(4 Pt 2): H1476-82.

Tissieres, A., H.K. Mitchell and U.M. Tracy. Protein synthesis in salivary glands of *Drosophila melanogaster*: relation to chromosome puffs. *J Mol Biol* 84(3):389-98.

Trent, J.D., E. Nimmesgern, J.S. Wall, F.U. Hartl and A.L. Horwich. (1991) A molecular chaperone from a thermophilic archaebacterium is related to the eukaryotic protein t-complex polypeptide-1. *Nature* 354(6353): 490-3.

Ulmasov, K., O. Zatsepina, V. Molodstove and M. Evgen'ev. (1999) Natural body temperature and kinetics of heat-shock protein synthesis in the toad-headed agamid lizard *Phrynocephalus interscapularis*. *Soc Eur Herp* 20: 1-9.

Ultsch, G.R. (1989) Ecology and physiology of hibernation and overwintering among freshwater fishes, turtles, and snakes. *Biol Rev* 64: 435-516.

Ultsch, G.R., C.V. Herbert and D.C. Jackson. (1984) The comparative physiology of diving in North American freshwater turtles. I. Submergence tolerance, gas exchange and acid-base balance. *Physiol Zool* 57(6): 620-31.

Ultsch, G.R., M.E. Carwile, C.E. Crocker and D.C. Jackson. (1999) The physiology of hibernation among painted turtles: the eastern painted turtle *Chrysemys picta picta*. *Physiol Biochem Zool* 72(4): 493-501.

Vanden Hoek, T.L., Z. Shao, L. Changqing, P.T. Schumacker and L.B. Becker. (1997) Mitochondrial electron transport can become significant source of oxidative injury in cardiomyocytes. *J Mol Cell Cardiol* 29: 2441-50.

Voellmy, R. (1996) Sensising stress and responding to stress. *EXS* 77: 121-37.

Vogt, S., D. Troitzsch, H. Abdul-Khaliq, W. Bottcher, P.E. Lange and R. Moosdorf. (2000) Improved myocardial preservation with short hyperthermia prior to cold cardioplegic ischemia in immature rabbit hearts. *Eur J Cardio-Thor Sur* 18(2): 233-40.

Welch. W.J. (1992) Mammalian stress response: cell physiology, structure/function of s tress proteins, and implications for medicine and disease. *Physiol Rev* 72(4): 1063-81.

Wenger, R.H. and M. Gassmann. (1996) Little difference. *Nature* 380: 100.

Widelitz, R.B., B.E. Magun and E.W. Gerner. (1986) Effects of cycloheximide on thermotolerance expression, heat shock protein synthesis, and heat shock protein mRNA accumulation in rat fibroblasts. *Mol Cell Biol* 6(4): 1088-94.

Wu, B., C. Hunt and R. Morimoto. (1985) Structure and expression of the human gene

encoding major heat shock protein Hsp70. *Mol Cell Biol* 5(2): 330-41.

Wu, C. (1995) Heat shock transcription factors: structure and regulation. *Ann Rev Cell Dev Biol* 11: 441-469.

Xu, Y. and S. Lindquist. (1993) Heat-shock protein hsp90 governs the activity of pp60v-src kinase. *Proc Natl Acad Sci U.S.A.* 90(15): 7074-8.

Zou, J., Y. Guo, T. Guettouche, D.F. Smith and R. Voellmy. (1998a) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* 94: 471-80.

Zou, J., W.F. Salminen, S.M. Roberts and R. Voellmy. (1998b) Correlation between gluathione oxidation and trimerization of heat shock factor 1, an early step in stress induction of the Hsp response. *Cell Stress Chap* 3(2): 130-41.