

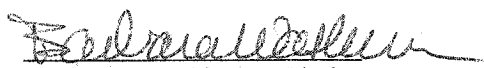
PREVIEW

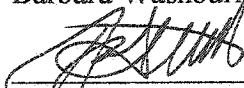
HEAT SHOCK PROTEINS 60 & 70 AS BIOMARKERS: EFFECTS OF  
GENDER ON THE STRESS RESPONSE IN FISH


JOHN JASON MORELAND

Department Of Biological Sciences

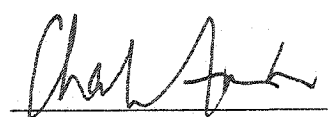
Approved:

  
Barbara Washburn, Chair

  
Robert Webb

  
Louis Irwin

  
Kenneth Brinson, Jr.

  
Associate Vice President  
for Graduate Studies

HEAT SHOCK PROTEINS 60 & 70 AS BIOMARKERS: EFFECTS OF GENDER ON  
THE STRESS RESPONSE IN FISH

By

JOHN JASON MORELAND, B.S.

THESIS

Presented to the Faculty of the Graduate School of  
The University of Texas at El Paso  
in Partial Fulfillment  
of the Requirements  
for the Degree of  
MASTER OF SCIENCE

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

MAY 2001

UMI Number: ep05474

PREVIEW

UMI<sup>®</sup>

---

UMI Microform ep05474

Copyright 2003 by ProQuest information and Learning Company.

All rights reserved. This microform edition is protected against  
unauthorized copying under Title 17, United States Code.

---

ProQuest Information and Learning Company  
300 North Zeeb Road  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

## TABLE OF CONTENTS

	Page
LIST OF FIGURES .....	v
CHAPTER 1: INTRODUCTION.....	1
1.1 Stress Proteins.....	1
1.2 Heat Shock Proteins as Biomarkers.....	4
1.3 Literature Cited.....	9
CHAPTER 2: VALIDATION AND OPTIMIZATION OF METHODS .....	15
2.1 Animal Culture and Exposures .....	15
2.2 Cell Culture .....	15
2.3 Western blot .....	16
CHAPTER 3: PRELIMINARY EXPERIMENTS AND RESULTS .....	20
3.1 Exposure of PLHC-1 cells to copper and estrogen .....	20
3.2 Copper dose response in whole animals .....	21
3.3 Legend.....	29
CHAPTER 4: J.J. Moreland, D.E. Arrieta, B.S. Washburn. <i>Effects of Estrogen on the Stress Response</i> in <i>CuSO<sub>4</sub> Exposed PLHC-1 Cells</i> . Marine Environmental Research. 2000.....	31

CHAPTER 5: J.J. Moreland, B.S. Washburn. <i>Effects of gender and sexual maturity on Hsp60 and Hsp70 expression in copper expose Fathead Minnows, Pimephales promelas</i> . Manuscript submitted to Environmental Toxicology and Chemistry, 2000.....	41
CURRICULUM VITAE.....	67

PREVIEW

## LIST OF FIGURES

Figure	Page
3.1. Cytotoxicity assay for copper exposure.....	23
3.2. Cytotoxicity assay for 48 hour exposure to 17-B-Estradiol .....	24
3.3. Copper dose-response (hsp60) for PLHC-1 cells in 2 medias .....	25
3.4. Copper dose-response (hsp70) for PLHC-1 cells in 2 medias .....	26
3.5. Range finder Hsp60 induction .....	27
3.6. Range finder Hsp70 induction .....	28
4.1 Effects of 1 $\mu$ M E <sub>2</sub> on Hsp60 and 70 induction in PLHC-1 cells exposed to 16 ppm copper.....	39
5.1 Hsp60 Liver.....	60
5.2 Hsp70 Liver.....	61
5.3 Hsp60 Gill.....	62
5.4 Hsp70 Gill.....	63
5.5 Hsp60 Muscle.....	64
5.6 Hsp70 Muscle.....	65

## Chapter 1: INTRODUCTION

### 1.1: Stress proteins

How cells and organisms respond to stress has been of interest for many years. Initial studies of the adaptive response to stress were described in 1864 by Julius Sachs who experimented with plants and their response to heat. The more recent trigger of interest began after reports by F. Ritossa described the pattern of gene activity in *Drosophilla* salivary gland in 1962 and the first description of heat stress-inducible proteins (Hsp) in 1974 by A. Tissieres [1]. The first description of Hsp in *Drosophilla* initiated a remarkable era of research on similar proteins in all types of organisms and their function in stress tolerance [1]. Research led to the characterization of a rapidly increasing number of isoforms and at present 11 Hsp families. These Hsp families are conserved, structurally and functionally, in prokaryotes and eukaryotes from bacteria to invertebrates and vertebrates.

The heat shock response, or cellular stress response, entails the synthesis of a suite of proteins upon exposure to stress. Stress factors, which induce the response, include elevated or decreased temperatures, organic compounds, trace metals, pesticides, infectious and inflammatory agents, many xenobiotics, and other stressors [2]. It has been speculated that the role of the Hsp proteins is to aid in protein folding and offer protective and adaptive responses to stress [3, 4]. Stress proteins are important in cellular recovery



and protection and are constitutively present at different stages of development and cell proliferation [5]. The heat-shock response and the resulting production of stress proteins is important both in protecting the cell against damage caused by heat or other stress and in facilitating in the recovery of the cells from the damage. Stress proteins may serve to protect other proteins from stress or aid in the routing of damaged proteins to lysosomes for degradation.

Recent studies suggest that the induced synthesis of stress proteins is related to protein damage [6]. Upon stimulation, heat-shock factor (HSF), an initiator for the transcription of the stress protein genes, binds to the regulatory heat shock element (HSE) present upstream of the gene activating the genes for stress proteins [7, 8]. In mammalian cells, HSF is bound to chaperone molecules (i.e. Hsp60 & 70), which are released upon the appearance of non-native proteins. The stress inducible binding of heat shock factor to DNA is controlled by a monomer-to-trimer transition of HSF protein [6], [9]. The trimerization of heat shock factor increases the affinity for heat shock elements several orders of magnitude. However, it is the inducible phosphorylation of the serine residues of HSF that cause the stress-induced transcription of the Hsp genes, without phosphorylation the trimeric HSF will bind to HSE but transcription will not occur [6]. Heat shock elements are alternating repeats of the 5-bp sequence (A/G)GAAN; three (A/G)GAAN repeats are required for the high affinity interaction with heat shock factor [10]. Through the assembly of three DNA-binding domains of HSF in one complex, concurrent interactions with all three NGAAN boxes of the HSE are possible. Of these heat-shock inducible proteins, interest in the Hsp60 and Hsp70 families has grown. These

two families are the major Hsp involved in the transport and folding of proteins in the cell. They are both ubiquitous and constitutively present in the tissues of organisms.

Members of the Hsp70 family were first noted as a family of stress-inducible and constitutive proteins of approximately 70 KDa. Homologues of these proteins occur throughout eukaryotic cells, in the cytoplasm, nucleus, ER, mitochondria, and chloroplast [11]. Upon heat-stress, Hsp70 is the major translation product and it accumulates in the nucleolus of the stressed cells. This accumulation of Hsp70 in the nucleus of stressed cells is thought to be the feedback regulation of Hsp gene initiation caused by the trimeric HSF. Once the translation of the genes has exceeded the need for the protein the excess Hsp70 translocates to the nucleus to release and bind to the monomeric HSF [6]. The Hsp70 also binds to nuclear structures and its release is dependent on addition of ATP [11]. Hsp70 aids in the folding of proteins by binding to the unfolded proteins and guiding the folding, using different areas of hydrophobic and hydrophilic nature. The Hsp70 proteins also function to aid in the translocation of proteins by forming stable structures of unfolded or “loosely” folded proteins. The Hsp70 binds to the proteins, prevent them from folding completely, and protect them from degradation, in order to facilitate their translocation across membranes. During times of cellular stress, Hsp70 can help to protect native proteins by binding to them and maintaining their structure. If native proteins are damaged during cellular stress, Hsp70 can bind to the denatured protein and initiate the degradation of the protein [11].

A similar family of heat-shock proteins is the Hsp60 family. Members of the Hsp60 family are stress inducible proteins that are also constitutively present in bacteria, mitochondria, and chloroplast. Hsp60 proteins serve as chaperones and aid in the folding

of proteins. Like the Hsp70 family, the Hsp60 binds to the unfolded proteins and cause conformational changes by way of hydrophobic and hydrophilic interactions. The Hsp60 has several different sub-units that form a chamber. The unfolded protein is brought into the center of this chamber and then, it is thought that, the top and bottom close like lids. This chamber that is formed contains the unfolded protein and undergoes conformational changes that result in changing the nature of the chamber from hydrophilic to hydrophobic. This change causes the protein to also undergo conformational changes. The Hsp60 chamber will then return to a hydrophilic nature and the target protein will change, resulting in the movement of the hydrophobic portions to the inside of the folded structure [11], [4]. The Hsp60 family also has members that aid in the transport and degradation of proteins, much like the Hsp70 family.

## **1.2: Heat shock proteins as biomarkers**

Both the Hsp60 and the Hsp70 families are constitutive proteins that can be induced under conditions of stress. The induction of the Hsp60 and 70 families by several different xenobiotics has been studied in several organisms. The results of many of these studies suggest that the induction of the Hsp60 and 70 families may be indicative of cellular stress. Many of the responses show a dose-response relationship between the xenobiotic and Hsp induction. Since the heat-shock response is induced by a variety of stressors, it has been suggested that heat-shock proteins may be useful as biomarkers for contaminant exposure [12]. Because stress proteins are part of the cell's protective strategy, their accumulation should be closely coupled with the organism's physiological state, giving them the potential to provide an early warning of impairment at the

organism level [12]. This potential to provide information about the physiological state of an organism may make stress proteins useful biomarkers. However, for Hsp60 and 70 to be useful as biomarkers of exposure they must not be readily inducible by normal physiologic or environmental factors such as sex, weight, hormones, age, reproductive status, diet, temperature, seasonal changes, and handling [13].

Research to validate the use of Hsp60 and 70 as biomarkers has addressed many of these issues in many species. In rat kidney, the basal levels of Hsp70 have been shown to increase with age compared to younger animals [14]. The effect of age in rats has also been shown to cause a decrease in the transcription for Hsp as well as attenuated protective capabilities [15], [16, 17]. These effects of age on the stress response have been found in liver, spleen, hypothalamic, pituitary, and adrenal tissues of rats, and muscle tissues of *Drosophila* [18-22].

Diet also has an effect on the stress response. Rats fed *ad libitum* have lower levels of Hsp70 than rats fed a caloric restricted diet [23, 24]. To further confound the affect of age and diet on the stress response, it has been shown that there are interactions between the two as well. In rat hepatocytes the response to heat shock was lowered in aged animals fed *ad libitum*, but this decrease was not as significant in rats of the same age fed a restricted diet [25]. Caloric level is not the only dietary factor that affects the stress response. Nutrient content of the diet is very important as well. A diet that is deficient in nutrients can alter the stress response. Copper deficiency has been shown to decrease the Hsp70 mRNA in the aorta of rats [26]. These effects of diet on the stress response may contribute to poor cell maintenance and early aging of the cell [26].

The understanding of the effects of gender, hormones, and reproductive state on the stress response is limited. Studies indicate that there are differences in the basal levels of Hsp in males and females, and that the induction of Hsp due to stress varies with sex as well [27]. Hormones can cause the induction or inhibition of Hsp in one sex and not the other [27]. Hormones affect Hsp70 during the menstrual cycle. At different stages of the cycle the levels of Hsp70 mRNA and protein vary in the human endometrium [28]. In rats there is an increase of Hsp in testis and ovaries during gametogenesis and hormone production.

Although most of the information on stress proteins is from mammalian models the use of Hsp60 and Hsp70 as biomarkers of exposure in fish has been proposed. Just as in mammals, the stress response in fish is readily induced by several contaminants and its regulation is closely linked to protein damage [5]. For stress proteins to be useful biomarkers in fish, they must not be readily inducible by normal physiologic and environmental factors. Studies of the stress response in aquatic organisms have revealed a great deal of information on the induction of Hsp and the interactions of normal physiological and environmental factors. The stress response and the accompanying increase in Hsp60 and Hsp70 has been demonstrated in several species in response to many different stressors.

Copper induces Hsp60 and Hsp70 in the mantle and gill tissues of *Mytilus* and this accumulation is directly related to a decrease in fitness [29]. The stress response has been induced by copper in fathead minnow epithelial cell line resulting in induction of Hsp70, but no increased synthesis of Hsp60 [12]. Dependent on the type of stress, the induction of Hsp60 and Hsp70 appears to be tissue specific. In the fathead minnow

epithelial cell line Hsp60 was induced by a 10°C heat shock but not by exposure to 0 to 750 µM copper [12]. The exposure to organic compounds also induces the stress response. Exposure to the herbicide oxyfluorfen has been found to induce Hsp70 in kidney tissue of fish [30].

As in the mammalian models, the stress response in fish is affected by normal physiological and environmental changes. Physiologic factors such as age, gender, reproductive status, diet, and weight affect the stress response in fish. The age of the fish affects the Hsp response; gills of rainbow trout and brook lamprey have reduced induction of stress proteins as a result of aging [31], [32]. Hormones also alter the stress response in fish, both Growth hormone and Prolactin were found to cause a 76% and 64% decrease in hepatic Hsp70 induced by heat shock, respectively [33]. The animal's health also has an impact on the stress response; the presence of an infection may alter the response. In Coho salmon, a bacterial kidney infection increased the levels of Hsp70 in both liver and kidney tissues compared to control animals [34]. Normal shifts in environmental factors such as seasonal variations in temperature, food supply or quality, and habitat have been suggested to alter the stress response. Seasonal changes seem to cause a change in the expression of Hsp70 in fish and mussels [35, 36]. However, the handling involved in the collection of feral fish for Hsp analysis has not been shown to alter protein levels in liver, muscle, or gill tissues of rainbow trout [37, 38].

Even though stress proteins are highly conserved, variations in the Hsp themselves also exist among different, closely related, species of fish. Among six species of *Poeciliopsis*, the heat inducible Hsp70 exhibited four different isoforms, and the Hsp60 was identical in five of the six species but a different isoform was found in one

species [39]. This variation in Hsp could lead to inaccurate analysis of the induction of stress proteins caused by exposure.

Since the successful use of Hsp60 and 70 as biomarkers of exposure and effect necessitates that they not be readily inducible by normal physiologic or environmental factors [13], their use as biomarkers requires a careful validation process. The effects of normal physiologic processes and environmental variations described above on the Hsp response seem to be limiting factors in the possible use of Hsp60 and 70 as biomarkers. Although there are known effects of these factors on the Hsp response in mammals, insects, and fish, the use of Hsp as biomarkers might be possible under very controlled conditions. Variations of age, sex, reproductive status, and feeding habits could cause variability in the Hsp response. While these factors as well as environmental variability can be controlled for in the laboratory, the use of Hsp induction in feral animals as a biomarker would become increasingly difficult and require consideration in the collection of specimens.

The purpose of this study was to further the knowledge of the effects that sexual maturity (i.e. gender and age) has on the stress response. The objectives of this study were: 1) to examine the affects of estrogen on the stress response in *Pocileopsis lucida* hepatoma cells (PLHC-1) exposed to copper, 2) to determine a exposure level of copper that elicits a stress response in fathead minnows (*Pimephales promelas*), and 3) evaluate the differences in the stress response elicited by copper in mature male, mature female, immature male, and estrogen fed immature males fathead minnows.