THE RELATIONSHIP BETWEEN THE ORGANISMAL AND CELLULAR STRESS RESPONSES IN FISH

by

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ABSTRACT

Stress in fish is ubiquitous in natural and anthropogenic environments, and has the potential to cause major environmental and economic losses. Due to this, there is a need to develop a broad range of tools in risk assessment that facilitate the objective detection and assessment of stressed states in fish. It is well known that the exposure of fish to stressful stimuli activates a highly integrated stress response system that encompasses multiple levels of biological organization. At the organismal level, stress hormones (e.g. cortisol) are released into the circulatory system to mobilize energy stores, and at the cellular level, heat shock proteins (e.g. hsp70) have functions related to preserving the integrity of proteins. Given the similarities between these two levels of stress response (i.e. maintenance of homeostasis), the purpose of my thesis was to determine if the organismal (cortisol) and cellular (hsp70) stress responses were functionally related. In order to characterize this relationship, I: (1) studied a temperate salmonid (rainbow trout, Oncorhynchus mykiss) and a tropical finfish (mossambique tilapia, Oreochromis mossambicus); (2) measured the stress responses in hepatic and gill tissues; (3) exogenously introduced cortisol into fish via intra-peritoneal injections or dietary routes; (4) artificially raised levels of cortisol for acute (24 h) and sub-chronic (28 d) periods; (5) applied different stressors, including heat stress (2 h, +12°C or 2 h, +14°C immediate change), stress hormones (cortisol), and toxicants (β-napthoflavone; bnf) to fish; (6) characterized the stress responses at the organismal (i.e. cortisol, glucose, and lactate) and cellular (i.e. hsp70 and glucocorticoid receptor) levels; and (7) applied novel laboratory techniques to describe the association between hsp70 and the glucocorticoid receptor. By
studying the stress responses under a variety of experimental conditions, I gained a comprehensive understanding regarding the relationship between the organismal and cellular stress responses in fish. The major findings included: (1) high levels of cortisol suppressed, or prevented, the heat stress-related increases of hsp70 in hepatic and gill tissues of rainbow trout and gill tissues of mossambique tilapia; (2) sub-chronically stressed fish (exposed to cortisol or β-naphthoflavone) could not mount an organismal or cellular stress response when challenged with a heat stress; and (3) heat stress and cortisol exposure can promote the binding of hsp70 to the glucocorticoid receptor in fish. Collectively, these findings demonstrate that a functional relationship exists between the organismal and cellular stress responses in fish, and raises questions regarding the existence of a highly interrelated and complex stress response system that spans all levels of biological organization within the whole animal.
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CHAPTER 1. Introduction

1.1 General introduction

The ideologies regarding stress have existed for a long period of time in human culture, and have provoked discussions by notable scholars including Hippocrates, Charles Darwin, and Hans Selye. A universally accepted definition of stress has yet to be established given that this phenomenon transcends diverse fields, such as molecular biology, geography, physiology, ecology, psychology, and medicine (Barton, 1997). For the purposes of my thesis, I will refer to stress as any environmental change that impairs homeostasis and acts to reduce the fitness of an organism, a definition adapted from Brett (1958): 'a state produced by an environmental or other factor which extends the adaptive responses of an animal beyond the normal range or which disturbs the normal functioning to such an extent that, in either case, the chances of survival are significantly reduced'.

All organisms, including bacteria, plants, and humans, are exposed to stress (Canon, 1929; Brett, 1958). Fish are no exception to this. Individuals and populations of fish are often faced with potentially stressful conditions in their natural and anthropogenic environments. Common stressors that fish may encounter include extreme or sudden changes in their physical environment (e.g. temperature, salinity, and turbidity), interactions

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with other animals (e.g. predation, competition for food, and parasites), and anthropogenic-based stressors, including various aspects of intensive aquaculture (e.g. netting, transport, and crowding) and industrial by-products (e.g. mill effluents, low water pH, and heavy metals). Details regarding the effects of stressors on fish can be found in extensive review papers (Mazeaud et al., 1977; Pickering, 1981; Adams, 1990; Barton and Iwama, 1991; Wendelaar Bonga, 1997).

All stressors, if sufficiently intense or prolonged, have the ability to impair homeostasis at all levels of biological organization. As a result of this, nature has evolved generalized stress response systems that are capable of protecting the organism (Selye, 1975; Wendelaar Bonga, 1997) or cell (Welch, 1993; Goligorsky, 2001). Characteristic features of the generalized stress response system include: (1) an integrated response that involves all levels of biological organization, ranging from molecules to cells to whole animals; (2) a highly dynamic and self-regulating system (i.e. it contains negative-feedback mechanisms); and (3) the ability to neutralize the deleterious effects of stress by physiological and biochemical mechanisms, which may be energetically costly, in an attempt to re-establish homeostasis.

1.2 Organismal stress response

The organismal, or neuro-endocrine stress response is the most well characterized stress response system in animal physiology (reviewed by Selye, 1975; Donaldson, 1981;
This stress response has been broadly sub-divided into primary, secondary, and tertiary responses. The initial perception of stress by the nervous system activates a humoral set of reactions along the hypothalamo-pituitary-interrenal (HPI) axis, ultimately leading to the rapid release of stress hormones (primary stress response), corticosteroids and catecholamines, into the circulatory system (Figure 1.1). The secondary response is comprised of a series of physiological and biochemical reactions that are initiated by the stress hormones in an attempt to mobilize energy reserves and metabolic processes required by the organism to maintain homeostasis. The tertiary response represents whole animal and population level changes associated with stress. Changes at the tertiary level may include decreased growth, disease resistance, reproductive success, and swimming performance. Intensive research during the past 20 years has allowed investigators to characterize the organismal stress response in fish. Nonetheless, it must be noted that this data is heavily biased towards salmonid species, as many of the studies regarding stress in fish have been conducted in temperate parts of the world where these species prevail (Barton, 1997).

The primary corticosteroid stress hormone, cortisol, is often used as a bio-indicator of stress in fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Mommsen et al., 1999). Cortisol is synthesized by various microsomal enzymes located in the interrenal cells of the head kidney tissue (Sandor et al., 1984). The regulation (i.e. its synthesis and release into the circulatory system) of this stress hormone is under the strict control of the hypothalamic portion of the brain (Donaldson, 1981). It was originally thought that the uptake of cortisol by target cells occurred via passive diffusion, but recent data has provided evidence
suggesting that a carrier-mediated process may facilitate this uptake (Vijayan et al., 1997a). Once inside a cell, the physiological effects of cortisol are mediated by the glucocorticoid receptor, a ligand-inducible transcription factor (Mommsen et al., 1999; Adcock, 2000). Ligand-binding activates the glucocorticoid receptor heterocomplex and promotes its migration to the nucleus where it can bind to glucocorticoid responsive elements (GRE) in the upstream promoter regions of glucocorticoid responsive genes. This interaction with the GRE changes the rate of gene transcription, resulting in the induction or repression of glucocorticoid responsive genes. These genes can influence the mobilization of energy, primarily by stimulating hepatic gluconeogenesis and affecting the intermediary metabolism of carbohydrates, proteins, and lipids. In addition to its energy-mobilizing effects, cortisol-mediated gene activation can influence osmoregulation, growth, and reproduction (Mommsen et al., 1999).

1.3 Cellular stress response

Heat shock proteins (hsp) are a family of highly conserved cellular proteins that have been observed in all organisms (reviewed by Lindquist, 1986; Morimoto et al., 1990; Welch, 1993; Feder and Hofmann, 1999), including fish (reviewed by Iwama et al., 1998; Iwama et al., 1999). Extensive studies on model species have revealed three major families of hsps: hsp90 (85-90 kDa), hsp70 (68-73 kDa), and low molecular weight hsps (16-24 kDa). In the unstressed cell, there is a constitutive production of these proteins as they have important functions in various aspects of protein metabolism (reviewed by Morimoto et al., 1990;
Hightower, 1991; Nover, 1991; Hendrick and Hartl, 1993; Fink and Goto, 1998). Hsp70, the most widely studied hsp in fish, is known to assist the folding of nascent polypeptide chains, act as a molecular chaperone, and mediate the repair and degradation of altered or denatured proteins. Hsp90 is active in supporting various components of the cytoskeleton, enzymes, and steroid hormone receptors. The low molecular weight hsps have diverse functions that are species specific and, unlike other hsps, these proteins have no known constitutive function and are only induced during stress. Collectively, the apparent simplicity governing hsp function (i.e. keeping proteins functional) reinforces their importance in the most basic of cellular processes, such as cell division and growth.

While the term "heat shock" arose from early observations on *Drosophila* exposed to a severe heat stress, hsps can be induced when cells are exposed to a wide variety of stressors, particularly those that denature proteins (Hightower, 1991; Freeman *et al.*, 1999). This cellular stress response has been observed in nearly all organisms studied (Feder and Hofmann, 1999). In fish, the induction of various hsp families have been reported in cell lines, primary cultures of cells, as well as in tissues from whole animals (reviewed by Iwama *et al.*, 1998; Iwama *et al.*, 1999). While the majority of these studies have focused on the effects of heat shock, there is increasing interest in the physiological and protective role of hsps following exposure of fish to various environmental stressors. For example, elevated levels of various hsps have been measured in tissues of fish exposed to bacterial pathogens (Forsyth *et al.*, 1997; Ackerman and Iwama, 2001) and environmental contaminants, such as heavy metals (Williams *et al.*, 1996; Duffy *et al.*, 1999), industrial effluents (Janz *et al.*, 1997; Vijayan *et al.*, 1998), pesticides (Sanders, 1993; Hassanein *et al.*, 1999), and
polycyclic aromatic hydrocarbons (Vijayan et al., 1997b; Vijayan et al., 1998). It is noteworthy that while many indicators of fish stress (e.g. plasma cortisol concentrations) are altered by handling and sampling procedures, Vijayan et al. (1997b) demonstrated that handling stress does not alter levels of hepatic hsp70 in rainbow trout (O. mykiss).

A complete understanding of the mechanisms underlying the sensing of a stressor and the regulation of hsp70 are far from clear. Studies have demonstrated that the regulation of hsp70 gene expression occurs mainly at the transcriptional level (Morimoto et al., 1990; Nover, 1991; Fink and Goto, 1998). Analysis of hsp genes and a comparison of heat shock regulatory elements from a variety of organisms led to the identification of a palindromic heat shock element, CNNGAANNTTCNNG. The induction of hsps results from the binding of an activated heat shock transcription factor to heat shock elements upstream of hsp genes (Morimoto et al., 1992). Since most of the hsp genes do not contain introns, the mRNA is rapidly translated into nascent proteins within minutes following exposure to a stressor. Genomic sequences for hsp70 are slowly being elucidated in fish, including rainbow trout (O. mykiss; Kothary et al., 1984), medaka (Oryzias latipes; Arai et al., 1995), zebrafish (Danio rerio; Lele et al., 1997), and tilapia (O. mossambicus; Molina et al., 2000), and stress-related increases in mRNA expression have been documented in all these cases.
1.4 Relationship between the organismal and cellular stress response

The organismal and cellular stress responses share many common properties: (1) they are induced by a broad range of stressors; (2) they are self-regulated with negative-feedback mechanisms; (3) they are systems that enable the organism or cell to maintain homeostasis; (4) they are adaptive responses that have evolved over time; (5) they are integrated with other physiological processes; (6) they have been observed and characterized in a wide range of species; (7) their actions are remarkably similar in a wide range of organisms; (8) they have genetic components; and (9) they are influenced by environmental factors.

In addition to their functional similarities, evidence from the biomedical literature points to a relationship between steroid hormones and hsp7s. First, steroid hormones can alter hsp levels in HeLa cells (Kasambalides and Lanks, 1983), chicken oviduct cells (Baez et al., 1987), breast cancer cells (Ciocca and Luque, 1991), fungi (Brunt et al., 1990), tissues of male rats (Paroo et al., 1999), and rat myocytes (Sun et al., 2000). Second, the assembly, functionality, and transport of the glucocorticoid receptor is dependent upon the actions of hsp70, hsp90, and hsp56 (Pratt, 1993; Hutchinson et al., 1994; Pratt and Welsh, 1994). Third, studies on rats have demonstrated that a functional hypothalamo-pituitary-adrenal axis, equivalent to the piscine HPI axis, is necessary for the induction of hsp70 (Blake et al., 1991; Udelsman et al., 1993; Udelsman et al., 1994).
The interactions between cortisol and hsp70 in fish have recently been explored. Deane et al. (1999) found that daily injections of cortisol (4 μg/g body weight) into the silver sea bream (Sparus sarba) did not alter hepatic hsp70 levels. However, these authors did not explore the effects of physiological stress, or measure the levels of circulating plasma cortisol in their fish. Ackerman et al. (2000) demonstrated that high levels of cortisol (75 μg/g body weight) significantly reduced the heat stress-related increase of hsp30, but not hsp70, in the gills of cutthroat trout (O. clarki clarki). Analogous to these findings, Sathiyaa et al. (2001) observed that pharmacological levels of cortisol (1000 ng/mL) can suppress the heat stress-induced expression of hsp90 mRNA in cultured rainbow trout (O. mykiss) hepatocytes.

1.5 Purpose of study

Potential stressors to fish are ubiquitous in natural and anthropogenic environments, and if not managed properly these may cause major environmental and economic losses. Thus, the objective assessment of stress in fish is an integral and necessary part of fish health management and environmental risk assessment. Established physiological bio-indicators of stress include the measurement of plasma stress hormones, such as cortisol and adenalin. However, there are shortcomings to these bio-indicators as they require specialized laboratory equipment, the sampling procedures may cause undue stress, large volumes of blood plasma are required, and stressed fish do not always show increased levels of stress hormones (Gamperl et al., 1994; Morgan and Iwama, 1997). Since stressed fish
can have detrimental impacts on ecosystem health, resource management, aquaculture industries, and recreational fisheries, there is a need to develop novel tools to detect, monitor, and assess stress in fish. Growing evidence suggests that hsp70s are good indicators of stressed states in fish as they are sensitive to a wide variety of stressors (Sanders, 1993; Iwama et al., 1998; Iwama et al., 1999) and are not affected by sampling procedures (Vijayan et al., 1997b). The current literature of hsp70s in fish is heavily biased towards their potential use as biomarkers, but before they are routinely applied as a tool in risk assessments, we need to resolve their physiological function within the whole animal.

1.6  General hypothesis and outline of project

While specific hypotheses were developed for individual projects that comprised my thesis (see enclosed chapters), the following statement is a general hypothesis that governed my project:

A functional relationship exists between the organismal and cellular stress responses in fish.

To characterize the relationship between the organismal and cellular responses to stress, I chose an element of each stress response that could be easily controlled and measured in a laboratory environment. I chose the stress hormone, cortisol, to represent the organismal stress response because: (1) it is the major corticosteroid stress hormone; (2) its
regulation and physiological effects are well known; (3) it can be artificially introduced into fish; and (4) plasma concentrations can be easily measured in our laboratory. I chose hsp70 to represent the cellular stress response because: (1) it is the most well characterized hsp in fish; (2) it has important cellular functions; and (3) I have access to laboratory facilities to quantify cellular levels of hsp70.

To test my hypothesis, I studied the effects of cortisol and heat stress on hsp70 levels in hepatic and gill tissues from two commercially important species of fish: a temperate salmonid (trout, *O. mykiss*) and a tropical finfish (tilapia, *O. mossambicus*) (Chapter 2). I explored the ability of trout to mount a stress response (organismal and cellular) following a sub-chronic dietary exposure to cortisol, a reference toxicant (β-naphthoflavone), and a mixture of both (Chapter 3). To investigate the molecular mechanisms governing my findings, I characterized the association of hsp70 with the glucocorticoid receptor in hepatic tissues of trout (Chapter 4). This is a unique receptor heterocomplex that contains elements of both the organismal and cellular stress responses (Figure 1.2). I have presented my findings as independent, stand-alone chapters; each has been accepted, or submitted for publication in respected, peer-reviewed journals. A general discussion following these chapters will explore the significance and implications of my findings (Chapter 5).
Figure 1.1 A generalized schematic of the hypothalamo-pituitary-interrenal axis in fish.
Solid lines represent positive (stimulatory) actions, and broken lines represent negative (inhibitory) actions.
**STRESS**

- **BRAIN**
  - hypothalamus
  - CRH (Corticotropin-releasing hormone)

- **Pituitary Gland**
  - ACTH (Adrenocorticotropin hormone)

- **Head Kidney**
  - (interrenal cells)

- **Corticosteroids**
  - (e.g. cortisol)

- **Physiological Effects**
  - stimulatory effects
    - hydromineral balance
    - hepatic glycogen
    - plasma free fatty acids
  - inhibitory effects
    - muscle proteins
    - immune functions
    - growth
    - reproduction
Figure 1.2 A simplistic representation of the cellular link between the organismal and cellular stress responses. Stress causes (1) the release of cortisol into the circulatory system, and (2) induces various heat shock proteins (hsp). Once inside a cell, cortisol will bind to the glucocorticoid receptor. (3) The functionality of the glucocorticoid receptor depends on hsp56, hsp70, and hsp90 (Pratt, 1993). The binding of cortisol activates the glucocorticoid receptor heterocomplex, allowing this heterocomplex to translocate to the nucleus. (4) Hsp70 can disassociate from the heterocomplex after this receptor becomes activated (Pratt and Welsh, 1994). (5) Once in the nucleus, the activated heterocomplex can activate a variety of genes that comprise the organismal stress response (i.e. secondary and tertiary stress responses). (6) The hsp's induced by the initial stress comprise the cellular stress response. This diagram demonstrates that the glucocorticoid receptor interacts with components of both the cellular and organismal stress responses within a cell.
STRESS

KEY

○ cortisol

△ Hsp70

□ Hsp56

· Hsp56

□ glucocorticoid receptor

△ Hsp90

1. (1) 

2. (2) 

3. (3) 

4. (4) 

5. (5) 

6. Organismal stress response

7. Cellular stress response
CHAPTER 2. The effects of cortisol on heat shock protein 70 levels in two fish species

2.1 Abstract

The purpose of this chapter was to explore the effects of heat stress (2 h, +12°C) and increased levels of circulating cortisol (50 μg cortisol/g body weight) on hsp70 levels in hepatic and gill tissues of trout (O. mykiss) and tilapia (O. mossambicus). The administration of cortisol by intra-peritoneal injection (no heat stress) did not alter tissue levels of hsp70 compared to sham implanted (no heat stress) trout and tilapia. Heat stress significantly increased hepatic hsp70 in sham implanted trout. Cortisol significantly suppressed or inhibited the heat stress-induced levels of hepatic hsp70 in trout by 34.2% and 31.0%, 3 h and 24 h post-heat stress, respectively, compared to sham implanted trout. Additionally, cortisol significantly suppressed the heat stress-induced levels of gill hsp70 by 66.2% in trout (3 h post-stress) and 26.7% in tilapia (4 h post-stress), compared to sham implanted fish. The results from this chapter suggest that cortisol can mediate hsp70 levels in fish tissues following times of physiological stress, and that the organismal and cellular stress responses may be functionally related in these two different species of fish.

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2.2 Introduction

Exposure of fish to biological and abiotic stressors can invoke a series of common biochemical and physiological changes (reviewed by Sumpter, 1997; Wendelaar Bonga, 1997). At the organismal level, these changes are mediated by the neuro-endocrine system and are commonly characterized by increased concentrations of circulating stress hormones, such as cortisol (Gamperl, 1994; Sumpter, 1997; Mommsen, 1999). In addition to this organismal stress response, a generalized stress response at the cellular level has been observed in nearly all organisms studied to date following exposure to stressful stimuli (Lindquist, 1986; Hightower, 1991; Feder, 1999). The characteristic feature of this cellular stress response is the rapid induction of hsp70 (reviewed by Morimoto et al., 1990; Welch, 1993; Fink and Goto, 1998). Constitutive hsp70 have "housekeeping" functions within a cell related to protein assembly, folding, translocation, and denaturation. Additionally, hsp70 have been induced in fish that were exposed to various stressors, indicating a protective role for hsp70 during times of stress (reviewed by Iwama et al., 1998; Iwama et al., 1999). The underlying functions of these two levels of stress response (i.e. they maintain health) are highly conserved throughout evolution across a wide variety of organisms, ubiquitous in nature, and predictable during times of rest and stress (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Feder and Hofmann, 1999; Iwama et al., 1999).

There is increasing evidence from the mammalian and biomedical literature that steroid hormones may have a direct influence on the cellular stress response in cell lines and whole organisms (Baez et al., 1987; Brunt et al., 1990; Ciocca and Luque, 1991; Cviovo et
Recently, investigations into the possible interplay between cortisol and hsps in fish have begun. Deane et al. (1999) found that daily injections of cortisol into the silver sea bream (*Sparus sarba*) did not alter hepatic hsp70 levels. However, they did not explore the physiological relationship between cortisol and hsp70, or measure plasma cortisol concentrations following their injections. Sathiyaa et al. (2001) reported that pharmacological doses of cortisol (1000 ng/mL) suppressed the temperature-induced expression of hsp90 mRNA in primary cultures of rainbow trout (*O. mykiss*) hepatocytes. *In vivo* studies on cutthroat trout (*O. clarki clarki*) determined that elevated levels of circulating cortisol significantly attenuated the heat stress-induced levels of gill hsp30 (Ackerman et al., 2000). Collectively, these studies indicate that there seems to exist some interaction between cortisol and hsps in fish.

Based on the possibility that the organismal and cellular stress responses are functionally related, the purpose of this chapter was to determine if elevated concentrations of plasma cortisol would mediate the cellular stress response in two fish species following physiological stress. I assessed the relationship between these two levels of stress response by comparing data from a temperate teleost (rainbow trout) and a tropical finfish (mossambique tilapia); both of significant commercial importance.
2.3 Materials and methods

*Fish*

Juvenile rainbow trout (mean weight ± SEM: 49.6 ± 0.4 g) were purchased from Spring Valley Trout Farm (Abbotsford, BC, Canada) and acclimated for two weeks at the University of British Columbia South Campus Aquaculture facility (Vancouver, BC, Canada) with dechlorinated city water (7 - 13°C) and constant aeration. Mossambique tilapia (mean weight ± SEM: 14.7 ± 0.7 g) were purchased from Okamura Fish Farms (Kapaau, HI, USA) and acclimated for eight weeks at the Hawaiian Institute of Marine Biology (Kaneohe, HI, USA) with dechlorinated city water (21 - 24°C) and constant aeration. All fish were held at a stocking density ranging from 16.5 to 19.5 g/L in oval tanks and fed to satiation once daily. Food was withheld for 48 h prior to all sampling periods.

*Elevated Cortisol and Heat Stress*

To determine the effects of elevated cortisol on levels of hsp70, fish were exposed to the following treatments: (1) control (no stress); (2) +12°C heat stress for 2 h; (3) vehicle control (sham implant) and no stress; (4) vehicle control (sham implant) with +12°C heat stress for 2 h; (5) cortisol implant and no stress; or (6) cortisol implant and +12°C heat stress for 2 h. For comparative purposes, an attempt was made to match sampling times as close as possible. Six trout were sampled 3 h, 24 h, and 72 h post-stress and six tilapia were sampled 4 h, 24 h, and 96 h post-stress for each treatment. Water temperatures during heat
stress increased from 10°C to 22°C for the trout study, and 22°C to 34°C for the tilapia study.

The heat stress was administered to fish around 9 am when ambient water temperatures had stabilized to 10°C for the trout study and 22°C for the tilapia study. Fish were quickly removed from their acclimation tanks and immediately placed in aerated tanks that were heated to achieve water temperatures that were sustained at +12°C above the ambient temperature. Following a 2 h heat stress, fish were immediately returned to their acclimation tanks at ambient temperatures. Fish not exposed to the heat stress were also moved for 2 h to simulate the handling stress experienced by the heat stressed fish.

Cortisol (hydrocortisone) was dissolved in coconut butter (15 mg cortisol/mL coconut butter) and injected intra-peritoneally into fish (50 µg cortisol/g body weight) as slow releasing implants (Specker et al., 1994). Control fish were implanted with only coconut butter (sham implants). Prior to all injections, fish were lightly anaesthetized with buffered tricaine methanesulfonate (TMS; 100 mg/L). Following injections, all fish were allowed to recover for 24 h prior to treatments.

Fish Sampling

At each sampling period, six fish per treatment were removed from their tanks, sacrificed by an overdose of buffered TMS (500 mg/L), and weighed. Approximately 0.5 mL of blood was obtained by caudal puncture using a heparinized syringe and spun at
10,000 rpm for 2 min to obtain plasma. Four gill arches and whole hepatic tissues were removed quickly from all fish. All plasma and tissues were immediately frozen at –80°C until analysis.

Measurements

Plasma cortisol (ng/mL) was measured using an enzyme-linked immunosorbent assay kit from Neogen Corporation (Lansing, MI, USA). Cortisol was measured directly from plasma samples on a 96-well microplate reader (λ = 450 nm).

Whole tissues were homogenized in ice-cold lysis buffer (containing 100 mM Tris-HCl (pH 7.5), 0.1% SDS, 1 mM ethylenediamine tetra acetic acid (EDTA), 1 μM pepstatin A, 1 mM α-toluenesulfonyl fluoride (PMSF), 1 μM leupeptin, and 0.01 μM aprotinin). The tissue lysates were cleared in a microcentrifuge at 16,500 g for 3 min at room temperature. Ten μL of supernatant were taken for protein determination with the bicinchoninic acid (BCA) assay using bovine serum albumin (BSA) as a reference. A 1:1 solution of supernatant and SDS-sample dilution buffer (Laemmli, 1970) was boiled for 3 min and then frozen at –80°C until hsp70 quantification.

Levels of hsp70 protein were measured using the discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemmli (1970). Proteins (20 μg hepatic tissue and 75 μg gill) were resolved with a 4% stacking and 12.5% resolving gel on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). Prestained molecular
weight markers (Gibco-BRL, Burlington, ON, Canada) and tissue protein samples from six control (non-stressed) fish were added to every gel to normalize the data. Following electrophoretic separation, the proteins were transferred onto nitrocellulose membranes for immunoblotting as detailed by Forsyth et al. (1997). Briefly, membranes were incubated for 1 h in a polyclonal rabbit antibody raised against rainbow trout (RTG-2) hsp70. Following the incubation in primary antibody, membranes were incubated in an alkaline phosphatase conjugated goat anti-rabbit IgG (Gibco-BRL, Burlington, ON, Canada) secondary antibody for 1 h. Both antibodies were diluted with 2% w/v skim milk powder in TTBS (20 mM Tris, 500 mM NaCl, 0.05% v/v Tween-20, pH 7.5). Reactivity of the primary antibody with trout (Forsyth et al., 1997) and tilapia (G.K. Iwama, University of British Columbia, unpublished data) hsp70 have been previously demonstrated.

Statistical Analysis

Results are reported as mean ± SEM. Treatment comparisons were tested using one-way analysis of variance (ANOVA). A Student-Newman-Keuls test was applied for those treatments that had significant differences. A p-value of equal to, or less than 0.05 was considered significant for all statistical tests. For hsp70 quantification, band intensities were obtained using Sigma Gel software (Jandel Scientific, San Rafael, CA, USA) and values were normalized using band intensity values from resting (control) fish that were run concurrently on each gel.
2.4 Results

Plasma cortisol data

Trout subjected to heat stress (no implants) had plasma levels of cortisol that were significantly high, relative to control (non-stressed) trout (Table 2.1). A similar heat stress in tilapia consistently increased post-stress levels of plasma cortisol, relative to control (non-stressed) tilapia, but this increase was not statistically significant. Sham implants (with and without heat stress) significantly increased levels of plasma cortisol in trout, but did not alter plasma cortisol levels in tilapia, relative to control fish. In trout (all periods post-stress) and tilapia (4 h post-stress), cortisol implants significantly increased levels of plasma cortisol relative to all other treatments, including control fish.

Hepatic hsp70

Hsp70 was detected in hepatic tissues of all trout and tilapia sampled, including control fish (Figures 2.1A and 2.1B). All manipulated trout (ie. implanted or exposed to heat stress) had significantly increased levels of hsp70 relative to control trout, at all sampling periods (Figure 2.1A). Cortisol implants (no heat stress) did not alter hepatic levels of hsp70 compared to sham implanted (no heat stress) trout, at a given time. Trout subjected to heat stress (no implant and sham implant) had significantly increased levels of hepatic hsp70 compared all other treatments at 3 h and 24 h post-stress. However, trout that were implanted with cortisol and exposed to heat stress had mean hepatic hsp70 levels that were
significantly lower by 34.2% and 31.0%, 3 h and 24 h post-stress, respectively, when compared to sham implanted, heat stressed trout.

Relative to control tilapia, there were no significant changes in hepatic hsp70 in manipulated tilapia (Figure 2.1B).

**Gill hsp70**

Hsp70 was detected in gill tissues of all trout sampled, including controls (Figure 2.2A). Heat stress significantly increased levels of gill hsp70 in trout, relative to control trout. However, mean gill hsp70 levels in sham and cortisol implanted trout (no heat stress) ranged between 0.27 and 0.55 of values observed in control trout. Elevated levels of cortisol significantly decreased the heat stress-induced levels of gill hsp70 by 66.2%, 3 h post-stress, compared to sham implanted, heat stressed trout.

Hsp70 was detected in gill tissues of all tilapia sampled, including controls (Figure 2.2B). Heat stress alone (no implants) or implants (no heat stress) did not significantly alter gill hsp70 levels in tilapia, relative to control tilapia. Elevated levels of cortisol significantly decreased the heat stress-related levels of gill hsp70 by 26.7%, 4 h post-stress, compared to sham implanted, heat stressed tilapia.
2.5 Discussion

The levels of plasma Cortisol (Table 2.1) in this experiment were consistent with other values reported for resting, stressed, and cortisol implanted trout and tilapia (Barton and Iwama, 1991; Vijayan et al., 1997b; Vijayan et al., 1997c; Ackerman et al., 2000). The major finding of this study was that high concentrations of cortisol suppressed or inhibited the heat stress-induced levels of hsp70 in hepatic and gill tissues of trout, and gill tissues of tilapia (Figures 2.1A, 2.2A, and 2.2B), thus providing evidence that a relationship between the cellular and organismal stress responses may exist in fish.

In trout, but not tilapia, fish injected with the vehicle carrier (coconut butter) had altered levels of hsp70 in hepatic and gill tissues compared to control tissues (Figures 2.1A and 2.2A). Since implants composed of coconut butter are routinely used to deliver drugs and hormones into fish (Specker et al., 1994), there is a need to further explore the molecular and immunological effects of this vehicle carrier, and the stress response associated with injecting fish.

Circulating levels of cortisol are elevated in fish during periods of stress to maintain homeostasis by activating the central nervous system, and mobilizing glucose and other energy reserves (Wendelaar Bonga, 1997; Mommsen et al., 1999). However, recent data in salmonids suggest that in addition to its effects on systemic pathways, cortisol also mediates the cellular responses to stress. Previous studies reported that high levels of cortisol suppressed heat stress-induced increases of hsp90 mRNA in rainbow trout hepatocytes.
(Sathiyaa et al., 2001) and gill hsp30 in cutthroat trout (Ackerman et al., 2000). The data contained within this chapter provide evidence, in vivo, that validates in vitro findings in trout hepatocytes (Sathiyaa et al., 2001), and is comparative to the in vivo findings by Ackerman et al. (2000). Collectively, these studies indicate that high levels of cortisol attenuate the cellular stress response (hsp30, hsp70, and hsp90) in trout (in vivo and in vitro) and tilapia (in vivo) during times of physiological stress.

Most of our knowledge about the organismal and cellular stress responses in fish has been obtained from studying salmonid species (Barton, 1997). Much less information is available on the stress physiology of tropical finfish, such as tilapia, and hardly any studies have explored the cellular stress responses in tropical species. For comparative purposes, these two species were exposed to similar test conditions. However, the results contained within this chapter indicated that in tilapia, a +12°C heat stress only mildly elevated plasma levels of cortisol (Table 2.1), and did not significantly affect levels of hsp70 in hepatic and gill tissues (Figures 2.1B and 2.2B). Tilapia are a stress tolerant species of fish that prefer an optimal temperature range between 22°C and 30°C (Bruton and Boltt, 1975). Given that Lindquist (1986) reported most organisms up-regulated hsp70 when exposed to conditions that are 5°C to 10°C above their optimal temperature, perhaps our heat-stress (34°C) was not high enough to invoke a significant stress response in tilapia. Unpublished work (A.E. Todgham, University of British Columbia) suggested that maximal hsp70 induction in trout occurs at 21°C, regardless of season, magnitude of heat stress, or acclimation temperatures, and I suspect that a similar threshold for induction exists for tilapia that is most likely greater than 34°C. Nonetheless, the findings of a relationship between the organismal and
cellular stress responses in tilapia gill tissues were similar to observations in trout hepatic and gill tissues (Figures 2.1A, 2.2A, and 2.2B), and these reinforce the notion that hsps have ubiquitous and highly conserved functions in nature. The findings of this experiment will set the foundation for future projects that explore the physiological significance of the cellular stress response in tilapia, and other non-salmonid species.

The relationship between the organismal and cellular stress responses has been better described in other vertebrate organisms (Brunt et al., 1990; Blake et al., 1991; Ciocca and Luque, 1991; Matic, 1995). These observations are not surprising because the cellular stress response, especially hsp70, is highly conserved and has similar functions across a wide range of organisms (Lindquist, 1986; Feder and Hofmann, 1999). Given the possibility that these two levels of stress response are linked, studies are underway to understand the cellular interactions and physiological significance of this relationship in many test organisms (reviewed by Matic, 1995; Iwama et al., 1999).

The possible molecular mechanisms governing these findings are still unclear. Elevated levels of cortisol in fish can down-regulate the number of cellular glucocorticoid receptor heterocomplexes (Pottinger, 1990; Maule and Schreck, 1991). Hsp70 is bound to the non-activated glucocorticoid receptor (Pratt, 1993; Hutchinson et al., 1994), and the glucocorticoid receptor in this state mediates the physiological effects of cortisol. After cortisol binds to the glucocorticoid receptor, hsp70 is displaced from the mature receptor complex (Hutchinson et al., 1994) and this free hsp70 can act through a negative feedback loop to inhibit the trimerization of heat shock factors and ensuing hsp induction (Morimoto
et al., 1992). With increased amounts of circulating cortisol (due to implants or physiological stress), the cellular levels of hsp70 may be further depleted since levels of non-activated or unbound glucocorticoid receptor are reduced. Another possible scenario to explain these observations may involve the ability of glucocorticoids to protect an organism against its normal defense reactions to stressors by preventing or inhibiting these defense systems from over-shooting their function and disturbing homeostasis (Munck et al., 1984; Matic, 1995). Along these lines, glucocorticoid receptor-mediated gene expression can inhibit various genes (Bamberger et al., 1996; Adcock, 2000), and these may include components of the cellular stress response (Li et al., 1999). However, these hypothetical scenarios of hsp control and regulation require further experimental support and validation.

Paroo et al. (1999) conducted a study that is analogous to this experiment, and noticed that the steroid hormone, estradiol, diminished post-exercise levels of hsp72 in male rat tissues. They postulated that exercise stress increased levels of reactive oxygen species, known inducers of the cellular stress response, but pretreatment of cells with estradiol prior to exercise stress reduced the cellular stress response due to this steroid hormone’s influence on antioxidant pathways. Recent data is emerging that various glucocorticoids are associated with antioxidant pathways (Yoshioka et al., 1994; Urayama et al., 1998; Valen et al., 2000) and this may explain why high levels of cortisol suppressed stress-induced alterations of hsp70.

The examination of stress in fish is important in environmental risk assessment, fish health management, and aquaculture operations. Given the highly conserved nature of hsp genes, their ubiquity in the biological environment, and overall protective function, it is
likely that hsp7 are involved in the generalized stress response of the whole organism. It is also probable that the expression of various hsp7 can be used as an indicator of stressed states in fish. The results of this chapter indicate that high concentrations of cortisol suppressed stress-induced tissue levels of hsp70, and this relationship between the organismal and cellular stress responses may be widespread among different species of fish.
Plasma cortisol values (ng/mL) in trout and tilapia injected with cortisol, and exposed to heat stress. ‘Heat Stress’ refers to fish exposed to a 2 h, +12°C heat stress, and ‘Cortisol’ and ‘Sham’ refer to treatment implants. Values represent treatment means ± SEM. Treatment differences (p<0.05) at a given time period are indicated by letters. n = 6 fish per treatment.
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<th>Trout</th>
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<th>Tilapia</th>
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<td></td>
<td>3 h post-stress</td>
<td>24 h post-stress</td>
<td>72 h post-stress</td>
<td>4 h post-stress</td>
<td>24 h post-stress</td>
<td>96 h post-stress</td>
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<td>Control</td>
<td>1.7 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.7 ± 10.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.9 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.1 ± 8.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Heat Stress</td>
<td>127.8 ± 8.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63.6 ± 24.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71.5 ± 17.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>108.6 ± 33.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>96.1 ± 25.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.8 ± 15.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Sham</td>
<td>63.9 ± 12.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86.1 ± 11.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77.4 ± 26.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.3 ± 14.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58.2 ± 13.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.2 ± 11.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Sham + Heat Stress</td>
<td>47.4 ± 7.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.1 ± 10.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.6 ± 28.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>104.5 ± 21.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>25.9 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Cortisol</td>
<td>807.0 ± 319.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>575.7 ± 168.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>222.3 ± 55.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>645.5 ± 116.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>208.5 ± 40.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>114.2 ± 11.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Cortisol + Heat Stress</td>
<td>2035 ± 260.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>960.4 ± 191.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>527.6 ± 76.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>908.4 ± 324.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>174.4 ± 30.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.0 ± 14.2&lt;sup&gt;a&lt;/sup&gt;</td>
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Figure 2.1A  Hepatic hsp70 levels in trout injected with cortisol, and exposed to heat stress. ‘Heat Stress’ refers to trout exposed to a 2 h, +12°C heat stress, and ‘Cortisol’ and ‘Sham’ refer to treatment implants. Bars represent treatment means, relative to control samples as quantified by SDS-PAGE and immunoblotting. Error bars represent SEM. Treatment differences (p<0.05) are indicated by letters. n = 6 fish per treatment.

Figure 2.1B  Hepatic hsp70 levels in tilapia injected with cortisol, and exposed to heat stress. ‘Heat Stress’ refers to tilapia exposed to a 2 h, +12°C heat stress, and ‘Cortisol’ and ‘Sham’ refer to treatment implants. Bars represent treatment means, relative to control samples as quantified by SDS-PAGE and immunoblotting. Error bars represent SEM. Treatment differences (p<0.05) were not observed. n = 6 fish per treatment.
Time post stress (h)

A

Trout hepatic hsp70

B

Tilapia hepatic hsp70

Time post stress (h)
Figure 2.2A  Gill hsp70 levels in trout injected with cortisol, and exposed to heat stress. ‘Heat Stress’ refers to trout exposed to a 2 h, +12°C heat stress, and ‘Cortisol’ and ‘Sham’ refer to treatment implants. Bars represent treatment means, relative to control samples as quantified by SDS-PAGE and immunoblotting. Error bars represent SEM. Treatment differences (p<0.05) are indicated by letters. n = 6 fish per treatment.

Figure 2.2B  Gill hsp70 levels in tilapia injected with cortisol, and exposed to heat stress. ‘Heat Stress’ refers to tilapia exposed to a 2 h, +12°C heat stress, and ‘Cortisol’ and ‘Sham’ refer to treatment implants. Bars represent treatment means, relative to control samples as quantified by SDS-PAGE and immunoblotting. Error bars represent SEM. Treatment differences (p<0.05) are indicated by letters. n = 6 fish per treatment.
CHAPTER 3. Altered stress responses in rainbow trout following a dietary administration of cortisol and β-napthoflavone

3.1 Abstract

Previous research has demonstrated that fish inhabiting polluted waterways often have an impaired stress response at the organismal level. Given the possible link between the organismal (i.e. cortisol) and cellular (i.e. hsp) stress responses, this study was conducted to examine the ability of rainbow trout (O. mykiss) to respond to a 2 h, +14°C heat stress challenge following a 28 d, sub-chronic exposure to increased concentrations of cortisol (5 mg/kg body weight), β-napthoflavone (bnf; 50 mg/kg body weight), and a combination of both (mixture), through the diet (1.5% body weight every 48 h). While control fish responded to the heat stress by significantly increasing components of the organismal (cortisol, glucose, and lactate) and cellular (hepatic hsp70 protein) stress responses 6 h and 24 h post-stress, cortisol-, bnf-, and mixture-fed fish had impaired stress responses at both levels of organization. Additionally, hepatic hsp70 levels were significantly reduced 6 h post-stress in cortisol-fed fish that were exposed to the heat stress. While bnf-fed fish had significantly higher ethoxyresorufin-O-deethylase (EROD) activity, cortisol potentiated EROD activity in the mixture-fed fish. Similarly,

3 This chapter was adapted from a manuscript submitted for publication on September 15, 2001 to Fish Physiology and Biochemistry: Basu, N., Kennedy, C.J., Hodson, P.V., and Iwama, G.K. Altered stress responses in rainbow trout following a dietary administration of cortisol and β-napthoflavone.
plasma cortisol concentrations in the mixture-fed fish were significantly lower relative to cortisol-fed fish. These data are the first to indicate that sub-chronically stressed fish can have impaired stress responses at both the organismal and cellular levels. These enclosed findings raise questions regarding: a) the universal and simple applicability of biological indicators of stress in fish; b) the possible functional relationship between these two levels of stress responses; and c) the importance of hsps in the generalized stress response of the whole organism.
3.2 Introduction

Fish are often exposed to stressful conditions in their natural and anthropogenic environments. Consequently, they have developed a series of biochemical and physiological mechanisms to cope with various stressors. The most widely studied stress response in fish exists at the organismal, or neuro-endocrine level (reviewed by Sumpter, 1997; Wendelaar Bonga, 1997). The characteristic feature of this organismal stress response is the rapid release of stress hormones (e.g. cortisol) and their subsequent, well-characterized effects on organ systems and metabolic processes (Barton and Iwama, 1991; Mommsen et al., 1999). Since this organismal stress response has been observed in a wide range of animals exposed to a variety of physiological and psychological stressors, it is often referred to as a generalized stress response.

Numerous stressors, including toxicant exposure, can cause stress in fish (Adams, 1991; Wendelaar Bonga, 1997). In a series of studies on polluted lakes in Quebec, researchers demonstrated that yellow perch (Perca flavescens) and northern pike (Esox lucius) residing in these lakes had an impaired organismal stress response (e.g. altered levels of plasma cortisol and atrophied pituitary corticotropes) when challenged with a physical stressor (Hontela et al., 1992; Hontela et al., 1997). Another field study by Lappivaara and Oikari (1999) demonstrated that whitefish (Coregonus lavaretus) exposed to bleached kraft mill effluent (BKME) had an altered organismal stress response. Vijayan et al. (1997a) isolated rainbow trout (O. mykiss) hepatocytes from fish exposed to 3,3',4,4'-tetrachlorobiphenyl (TCBP) and found the catabolism and clearance
of cortisol was enhanced. Furthermore, Wilson et al. (1998) demonstrated that the polycyclic aromatic hydrocarbon (PAH), β-napthoflavone (bnf), alters cortisol metabolism by decreasing the responsiveness of interrenal cells to adrenocorticotropin hormone (ACTH) stimulation in rainbow trout. Taken together, these studies demonstrate that environmental toxicants may impair the ability of fish to mount physiologically important responses that are necessary to cope with stressors.

In addition to the organismal stress response, there is evidence suggesting that a generalized stress response exists at the cellular level, based on the actions of various hsp’s (Hightower, 1991; Feder and Hofmann, 1999). These proteins have general “housekeeping” functions within the cell, related to ensuring proper folding, transport, and degradation of cellular proteins. In addition to their constitutive functions, many hsp’s are induced when cells are exposed to stressors, other than heat. In fish, environmentally common pollutants, such as heavy metals (Williams et al., 1996; Duffy et al., 1999), industrial effluents (Janz et al., 1997; Vijayan et al., 1998), pesticides (Sanders, 1993; Hassanein et al., 1999), and PAHs (Vijayan et al., 1997b; Vijayan et al., 1998) are known inducers of hsp’s.

Recent findings have demonstrated that high levels of cortisol can suppress the heat stress-related increase of hsp’s in fish (Ackerman et al. 2000; Sathiyaa et al., 2001). In addition to these studies, I have shown that high levels of cortisol can suppress or prevent the heat stress-related increases of hsp70 in gill and hepatic tissues in rainbow trout (Figures 2.1A and 2.2A) and gill tissues in mossambique tilapia (Figure 2.2B).
Since these two levels of stress response may be linked, and toxicants may impair the organismal stress response, this present study was conducted to characterize both the organismal and cellular stress responses in fish that were sub-chronically (28 d) exposed to cortisol and bnf, singly and in combination.
3.3 Materials and methods

Fish

Juvenile rainbow trout (27.3 ± 0.6 g) were purchased from Spring Valley Trout Farm (Abbotsford, BC, Canada) and acclimated for two weeks in dechlorinated city water (5 – 7 °C; pH 6.9; 100% O₂ saturation) at the Alcan Aquatic Facility at Simon Fraser University (Burnaby, BC, Canada). Fish were held at a stocking density ranging from 15 to 20 g/L and fed 1.5% body weight every 48 h.

Feed Preparation

Fish pellets used during the acclimation period were used as the vehicle for drug delivery during the exposure period. Chemicals were incorporated into feed at a ratio of 333 μg cortisol per g feed, and 3.33 mg bnf per g feed. Assuming that individual fish consumed 1.5% body weight, we aimed to achieve internal concentrations of 5 mg cortisol/kg body weight fish (cortisol) and 50 mg bnf/g body weight fish (bnf). Cortisol and bnf were mixed at the same concentrations mentioned above to provide a mixture treatment (mixture). All chemicals were dissolved in acetone. In order to incorporate chemicals into the feed pellets, pellets were immersed in the acetone-chemical solutions and placed in a fume hood until the acetone had evaporated. Pellets were stored at −20°C until required.
Treatments and Exposure

Fish were randomly divided into five groups, each consisting of six replicate tanks, and held at a stocking density of approximately 25 g/L. Fish were fed treatment specific diets of 1.5% body weight every 48 h at 11:00 am on odd numbered days for a period of 28 d (i.e. 14 feeding days). Diets included: control (non-treated pellets), sham (pellets immersed in acetone), cortisol, bnf, and mixture.

Following a 28 d exposure period, 50% of the fish in each treatment group (i.e. three replicate tanks per treatment) were exposed to a 2 h, +14°C heat stress challenge (immediate change from 6 to 20°C). During this period, the remaining fish (non-heat stressed) were moved to a different tank for 2 h to simulate the handling stress experienced by the heat stressed fish. All fish were returned to their holding tanks at ambient temperatures following the heat stress period.

Fish Sampling

Six to nine fish were sampled from each treatment group on days 14 and 28 of the exposure period, and 6 h and 24 h post-heat stress. At each sampling period fish were quickly removed from their tanks, sacrificed by an overdose of buffered tricaine methanesulfonate (500 mg/L), and their weights and lengths were recorded. Approximately 0.2 mL of blood was obtained by caudal puncture using a heparinized syringe and spun at 10,000 rpm for 3 min to obtain blood plasma. Four gill arches and
whole hepatic tissues were excised quickly from all fish. All plasma and tissues were immediately frozen on dry ice and stored at –80°C until analysis.

**Analytical Measurements**

**Plasma cortisol, glucose, and lactate**

Plasma cortisol was measured using an enzyme-linked immunosorbent assay kit from Neogen Corporation (Lansing, MI, USA). Cortisol was measured directly from plasma samples on a Molecular Devices SpectraMax 340PC microplate reader ($\lambda = 450$ nm). An YSI 2300 Stat Plus probe was used to simultaneously measure plasma lactate and glucose concentrations. All plasma samples were assayed in duplicate.

**Ethoxyresorufin-O-deethylase (EROD) assay**

The activity of cytochrome P4501A1 (CYP1A) proteins was quantified via the EROD assay according to the methods described by Hodson *et al.* (1996). Hepatic tissues were homogenized in 500 µL HEPES-KCl buffer (pH 7.4, 0.15 M KCl, 0.02 M HEPES), and the homogenates were centrifuged at 9,000 g for 20 min at 4°C to obtain supernatant fractions. EROD activity was measured fluorimetrically by the de-ethylation of 7-ethoxyresorufin (7-ER) to resorufin in the hepatic supernatant fractions. The samples were assayed in triplicate on 96-well microplates. Reaction mixtures contained 50 µL of hepatic supernatant fraction and 50 µL of HEPES reaction buffer (10 µM 7-ER in 0.01 M HEPES...
buffer). The plates were incubated for 10 min and the reaction was initiated by addition of 10 μL of 24 mM aqueous NADPH. Fluorescence was measured once per minute for 12 min with a SoftMax Pro II microplate fluorometer ($\lambda_{\text{excitation}} = 530$ nm; $\lambda_{\text{emission}} = 590$ nm). Each plate contained resorufin standards in duplicate. Positive controls included hepatic supernatant fractions from individual rainbow trout (5 - 10 g) injected with 10 μg bnf (a model inducer) per gram body weight prepared in sunflower oil, and negative controls were supernatant fractions from rainbow trout injected with sunflower oil.

**SDS-PAGE and Immunoblotting**

Hepatic and gill tissues were homogenized in ice-cold lysis buffer (containing 100 mM Tris-HCl (pH 7.5), 0.1% SDS, 1 mM ethylenediamine tetra acetic acid (EDTA), 1 μM pepstatin A, 1 mM α-toluenesulfonyl fluoride (PMSF), 1 μM leupeptin, and 0.01 μM aprotinin). The tissue lysates were cleared in a microcentrifuge at 16,500 g for 3 min at room temperature. Ten μL of supernatant was taken for protein determination with the bicinchoninic acid (BCA) assay using bovine serum albumin (BSA) as a reference. A 1:1 solution of supernatant and SDS-sample dilution buffer (Laemmli, 1970) was boiled for 3 min and then frozen at −80°C until hsp70 quantification.

Levels of hsp70 protein were measured using the discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemmli (1970). Proteins (20 μg) were resolved with a 4% stacking and 12.5% resolving gel on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). Prestained molecular
weight markers (Gibco-BRL, Burlington, ON, Canada) were loaded to every gel. Tissue samples from six control (non-heat stressed) fish were pooled and loaded to every gel to normalize the data. Following electrophoretic separation, the proteins were transferred onto nitrocellulose membranes for immunoblotting, as detailed by Forsyth et al. (1997). Briefly, membranes were incubated for 1 h in a polyclonal rabbit antibody raised against rainbow trout (RTG-2) hsp70. Following the incubation in primary antibody, membranes were incubated in an alkaline phosphatase-conjugated goat anti-rabbit IgG (Gibco-BRL, Burlington, ON, Canada) secondary antibody for 1 h. Both antibodies were diluted with 2% skim milk powder in TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5). Reactivity of the primary antibody with rainbow trout hsp70 has been previously demonstrated (Forsyth et al., 1997; Figures 2.1 and 2.2).

Statistical Analysis

Results are reported as mean ± SEM. A p-value of equal to, or less than 0.05 was considered significant for all statistical tests. Treatment comparisons were tested using one-way analysis of variance (ANOVA). Student-Newman-Keuls tests were applied to discern differences among means for those treatments that had statistically significant differences. All EROD data were log transformed for statistical analysis since the variance of CYP1A activity among treatments is non-homogeneous (Hodson et al., 1996), as shown by the Bartlett test. Following statistical analysis, EROD results were back-transformed to the original units (i.e. as geometric means). For hsp70 quantification, band intensities were obtained using Sigma Gel software (Jandel
Scientific, San Rafael, CA, USA) and values were normalized using band intensities from control (non-heat stressed) fish samples that were run concurrently on each gel.
3.4 Results

Plasma

Levels of plasma cortisol in control fish not exposed to heat stress were relatively unchanged (< 20 ng/mL) throughout the experiment (Table 3.1). There were no significant differences in plasma cortisol levels between sham and control fish that were not exposed to heat stress. Heat stress significantly increased levels of plasma cortisol 6 h post-stress in the control and sham fish.

Cortisol-fed fish had significantly higher concentrations of plasma cortisol, relative to control fish (Table 3.1). Following the final feeding event (i.e. d 27 of the exposure period), levels of plasma cortisol remained high in cortisol-fed fish, relative to control fish, up to 3 d post-feeding (i.e. d 30 of the study are fish sampled 24 h post-stress). During that period, levels of cortisol decreased significantly in a time-dependent manner. Heat stress did not significantly increase levels of plasma cortisol in the cortisol-fed fish, relative to cortisol-fed (non-stressed) fish, at both 6 h and 24 h post-stress.

Bnf-fed fish had higher levels of plasma cortisol relative to control fish, but this increase was only statistically significant on d 28 (Table 3.1). Mixture-fed fish had significantly higher levels of plasma cortisol relative to control fish throughout the experiment. While plasma cortisol levels in mixture-fed fish were significantly higher
than fish fed bnf alone, plasma cortisol levels in the mixture-fed fish were significantly lower than those measured in the cortisol-fed fish. Heat stress did not significantly increase levels of plasma cortisol in the bnf- and mixture-fed fish, relative to bnf- and mixture-fed, non-stressed fish, 6 h and 24 h post-stress.

Mean plasma levels of glucose in non-stressed, control fish ranged between 4.5 and 6.8 mmol/L (Table 3.1). There were no significant differences in plasma glucose levels among control (non-stressed), sham (non-stressed), and bnf-fed (stressed and non-stressed) fish, 6 h and 24 h post-stress. Cortisol- and mixture-fed fish had higher levels of plasma glucose, relative to control, non-stressed fish, but this increase was not statistically significant. Heat stress significantly increased levels of plasma glucose 6 h and 24 h post-stress only in the control and sham fish. Heat stress did not significantly affect plasma glucose in cortisol-, bnf-, and mixture-fed fish.

Mean plasma levels of lactate in non-stressed, control fish ranged between 1.2 and 2.1 mmol/L (Table 3.1). While heat stress significantly increased levels of plasma lactate 6 h and 24 h post-stress in the control and sham fish, levels of plasma lactate in cortisol-, bnf-, and mixture-fed fish were not significantly changed following the heat stress.

**Tissues**

Mean hepatic EROD activity was less than 5 pmol/mg/min in all treatments, except for the bnf-fed fish (Table 3.2). EROD activity was significantly elevated (i.e. >
13 pmol/mg/min) in fish exposed to bnf, singly and in the mixture, relative to control fish. Exposure of fish to heat stress did not significantly change EROD activity within a given treatment.

Hsp70 was detected in hepatic tissues of all fish sampled, including control fish (Figure 3.1). Levels of hsp70 were not significantly different among control, sham, and cortisol-fed fish during the exposure period (Figure 3.1A). Bnf- and mixture-fed fish had significantly higher levels of hsp70 throughout the experiment, relative to control fish. Heat stress significantly increased levels of hsp70 only in the control and sham groups 6 h post-stress (Figure 3.1B). Levels of hepatic hsp70 were significantly decreased in cortisol-fed fish that were exposed to the heat stress, relative to cortisol (non-stressed) fish 6 h post-stress (Figure 3.1B).

Hsp70 was detected in gill tissues of all trout sampled, including controls (Figure 3.2). Levels of hsp70 were slightly elevated in control and sham groups following heat stress stress (Figures 3.2B and 3.2C), but this increase was not statistically significant.
3.5 Discussion

Indices of stress, including levels of plasma cortisol and hepatic hsp70, in non-stressed, control fish were consistent with values reported for resting and stressed fish in previous studies (Barton and Iwama, 1991; Ackerman et al., 2000; Chapter 2: Table 2.1, Figures 2.1 and 2.2). The major finding of this chapter was that sub-chronically stressed fish (i.e. exposed to cortisol and/or bnf) had impaired stress responses at both the cellular and organismal levels following exposure to a heat stress challenge.

In my first study (Chapter 2), elevated levels of cortisol suppressed or prevented the stress-induced increases of hsp70 in fish (Figures 2.1A, 2.2A, and 2.2B). In that study, cortisol was introduced into the fish via intra-peritoneal (i.p.) injection and this procedure resulted in unexpected levels of plasma cortisol (Table 2.1) and hsp70 (Figure 2.1 and 2.2); most likely due to the inherent stress associated with anesthetizing, handling and injecting fish. To address this problem, in the current study I incorporated cortisol into the fish feed and found this to be an effective method to elevate levels of plasma cortisol (Table 3.1). Levels of plasma cortisol were elevated to expected concentrations, and the inter-fish variation associated with this uptake was low relative to plasma cortisol values observed following i.p. injections (Table 3.1; see review by Gamperl et al., 1994).

Comparable to the findings in Chapter 2 (Figures 2.1A, 2.2A, and 2.2B), hepatic hsp70 levels in cortisol-fed fish were attenuated following heat stress (Figures 3.1B and 3.1C). While the study in Chapter 2 elevated cortisol for an acute duration (24 h), in this
current experiment, cortisol was elevated for a sub-chronic period of 28 d in fish prior to the heat stress challenge. Given the consistency of these findings, a functional relationship most likely exists between the organismal and cellular responses to stress. These observations are not surprising given the highly conserved and predictable nature of these two levels of generalized stress responses (Wendelaar Bonga, 1997; Feder and Hofmann, 1999).

In order to gain an understanding of why cortisol suppresses the cellular stress response in fish, an examination into the association of hsp70 with the glucocorticoid receptor is required. Stress and cortisol-treatments can decrease the number of cellular glucocorticoid receptors in fish (Pottinger, 1990; Maule and Schreck, 1991), and stress has been demonstrated to alter the association between hsp70 and hsp90 with the rat glucocorticoid receptor (Cvoro et al., 1998a; Cvoro et al., 1998b; Dundjerski et al., 2000). After cortisol binds to the glucocorticoid receptor, hsp70 is displaced from the activated receptor heterocomplex (Pratt and Welsh, 1994) and this free hsp70 can act through negative feedback to inhibit the further activation of hsp70 genes (Morimoto et al., 1992). Collectively, these independent studies point to the existence of a complex relationship among stress, cortisol, the glucocorticoid receptor, and hsp70.

The synthesis of cortisol, and the metabolism of aromatic organic pollutants, such as bnf, are mediated by members of the cytochrome P450 family of proteins (reviewed by Buhler and Wang-Buhler, 1998). As expected, bnf-fed fish had significantly high EROD activity (Table 3.2). However, mixture-fed fish had significantly lower EROD activity.
relative to bnf-fed fish, even though both treatments were exposed to the same
congcentration of bnf. A similar potentiation effect of cortisol on bnf-induced activity of
EROD was observed in primary cultures of trout hepatocytes (Devaux et al., 1992).
Thus, cortisol may have an adaptive role in the biotransformation and elimination of
toxicants in fish. Furthermore, bnf exposure resulted in a decreased level of circulating
plasma cortisol in the mixture-fed fish, relative to cortisol-fed fish. These findings are
similar to those reported by Vijayan et al. (1997a) who observed that rainbow trout
exposed to TCBP, a polychlorinated biphenyl (PCB) congener, had enhanced metabolism
and clearance of cortisol. Therefore, certain toxicants may alter the metabolism of
cortisol. As a whole, these findings warrant further research into the complex interplay
among cortisol, toxicants, and the cytochrome P450 system.

Field studies have documented that fish chronically exposed to PCBs, PAHs,
mercury (Hontela et al., 1992; Hontela et al., 1997) and BKME (Lappivaara and Oikari,
1999) have an altered stress response at the organismal level. The results in this chapter
demonstrate that in a laboratory setting, fish sub-chronically exposed to bnf also have an
impaired organismal stress response, based on measurements of plasma cortisol, glucose,
and lactate (Table 3.1). While control fish that were exposed to heat stress had
significantly higher levels of components of the organismal stress response, stressed fish
(cortisol-fed) or those exposed to a toxicant (bnf-fed) had unchanged levels of plasma
cortisol, glucose, and lactate. One explanation for this possible impairment in stress
response may be prolonged (i.e. 28 d) hyperactivity of the organismal stress response in
these fish, ultimately leading to an exhaustion of the system.
While there is mounting evidence that chronic exposure of fish to toxicants can impair their organismal stress response, no information exists on how this exposure affects their cellular stress response. Environmentally persistent toxicants, such as bnf, are known inducers of various hsp's. This is most likely due to the inherent toxicity of these chemicals and their ability to cause oxidative stress within the cell. Byproducts of oxidative stress, such as radicals and scavengers, denature cellular proteins; a potent trigger for hsp gene activation (Freeman et al., 1999). Levels of hepatic hsp70 were raised approximately 2-3 times greater than control fish in bnf- and mixture-fed fish (Figure 3.1). However, when these fish were exposed to the heat stress challenge, they could not elevate levels of hepatic hsp70 any further. There is a possibility that levels of hepatic hsp70 were at a maximal cellular level, but I have previously demonstrated that hepatic hsp70 in rainbow trout can be raised in excess of five times greater than control fish (Figure 2.1A). Stress responses are energetically costly to an organism (Koehn and Bayne, 1989), and perhaps prolonged exposure to bnf exhausted the ability of trout to maintain a functional cellular stress response. Whether this potential energetic demand impairs physiological processes within the animal needs to be resolved, given the importance of hsp's in cellular homeostasis. In addition to hepatic tissues, I also observed that following heat stress, levels of gill hsp70 were increased in control and sham fish, while unchanged in cortisol-, bnf-, and mixture-fed fish (Figure 3.2). These tissue-specific responses (i.e. muted response in gill tissue) were similar to my earlier observations (Figure 2.2), and raise the possibility that hsp70 is differentially regulated in fish tissues.
The assessment of stress in fish is very important in the fields of environmental risk assessment, health management, and aquaculture operations. In the current literature, there is a bias towards the use of hsps and cortisol as bio-indicators of stress in fish, but little is known about their physiological function and relationship within the animal. The findings in this chapter demonstrate that fish that have sub-chronically elevated levels of cortisol, or are exposed to polluted environments, have an impaired stress response at both the organismal and cellular levels. The mechanisms underlying these findings need to be resolved, given the importance of the cellular and organismal stress responses in fish physiological and homeostatic processes.
Table 3.1 Measurements of organismal stress (cortisol, glucose, and lactate) in trout exposed to dietary cortisol, bnf, and a mixture of both, following heat stress. 'Day 14' and 'Day 28' refer to fish sampled during the exposure period, and '6 h' and '24 h' represent fish sampled 6 h and 24 h post-heat stress, respectively. 'Heat Stress' refers to fish that were exposed to a 2 h, +14°C heat stress. Numbers represent treatment means followed by SEM in brackets. Letters represent significant differences (p<0.05) among treatments at a given sampling time. n = 6 to 9 fish per treatment.
<table>
<thead>
<tr>
<th>Time:</th>
<th>Cortisol (ng/mL)</th>
<th>Glucose (mmol/L)</th>
<th>Lactate (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exposure Period</td>
<td>Post-Stress Period</td>
<td>Exposure Period</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td>Day 28</td>
<td>6 h</td>
</tr>
<tr>
<td>Control</td>
<td>6.4 (2.1) a</td>
<td>6.7 (0.7) a</td>
<td>15.5 (3.3) a</td>
</tr>
<tr>
<td>Control (+ heat stress)</td>
<td>52.8 (13.5) b</td>
<td>17.5 (7.1) a</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>6.0 (2.1) a</td>
<td>11.2 (2.0) a</td>
<td>16.5 (4.8) a</td>
</tr>
<tr>
<td>Sham (+ heat stress)</td>
<td>45.2 (9.0) b</td>
<td>17.3 (6.3) a</td>
<td></td>
</tr>
<tr>
<td>Cortisol</td>
<td>372.7 (54.1) b</td>
<td>487.3 (30.2) b</td>
<td>165.4 (46.1) c</td>
</tr>
<tr>
<td>Cortisol (+ heat stress)</td>
<td>123.4 (21.7) c</td>
<td>75.4 (3.5) b</td>
<td></td>
</tr>
<tr>
<td>Bnf</td>
<td>20.9 (7.6) a</td>
<td>24.4 (2.9) c</td>
<td>18.0 (5.3) a</td>
</tr>
<tr>
<td>Bnf (+ heat stress)</td>
<td>23.8 (6.5) a</td>
<td>17.9 (3.7) a</td>
<td></td>
</tr>
<tr>
<td>Mixture</td>
<td>62.4 (13.2) c</td>
<td>100.6 (7.8) c</td>
<td>99.7 (31.8) c</td>
</tr>
<tr>
<td>Mixture (+ heat stress)</td>
<td>77.1 (21.6) c</td>
<td>106.5 (47.6) b</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2  Hepatic ethoxyresorufin-O-deethylase (EROD) activity (pmol/min/mg) in trout exposed to dietary cortisol, bnf, and a mixture of both, following heat stress. ‘Day 14’ and ‘Day 28’ refer to fish sampled during the exposure period, and ‘6 h post-stress’ and ‘24 h post-stress’ represent fish sampled 6 h and 24 h post-heat stress, respectively. ‘Heat Stress’ refers to fish that were exposed to a 2 h, +14°C heat stress. Numbers represent treatment means followed by SEM in brackets. Letters represent significant differences (p<0.05) among treatments at a given sampling time. n = 6 to 9 fish per treatment.
<table>
<thead>
<tr>
<th></th>
<th>Day 14</th>
<th>Day 28</th>
<th>6 h post-stress</th>
<th>24 h post-stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No Stress</td>
<td>+ Heat Stress</td>
</tr>
<tr>
<td>Control</td>
<td>1.9 (1.4)\textsuperscript{a}</td>
<td>1.5 (1.4)\textsuperscript{a}</td>
<td>2.5 (1.4)\textsuperscript{a}</td>
<td>1.6 (1.5)\textsuperscript{a}</td>
</tr>
<tr>
<td>Sham</td>
<td>3.3 (1.4)\textsuperscript{a}</td>
<td>0.9 (2.7)\textsuperscript{a}</td>
<td>3.7 (1.2)\textsuperscript{a}</td>
<td>2.0 (1.3)\textsuperscript{a}</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.7 (1.3)\textsuperscript{a}</td>
<td>0.9 (1.5)\textsuperscript{a}</td>
<td>0.8 (1.6)\textsuperscript{a}</td>
<td>1.7 (1.3)\textsuperscript{a}</td>
</tr>
<tr>
<td>Bnf</td>
<td>13.4 (1.7)\textsuperscript{b}</td>
<td>14.6 (1.8)\textsuperscript{b}</td>
<td>18.9 (1.7)\textsuperscript{b}</td>
<td>31.7 (1.2)\textsuperscript{b}</td>
</tr>
<tr>
<td>Mixture</td>
<td>4.7 (1.2)\textsuperscript{ab}</td>
<td>3.2 (1.3)\textsuperscript{ab}</td>
<td>1.9 (1.3)\textsuperscript{a}</td>
<td>3.1 (1.5)\textsuperscript{a}</td>
</tr>
</tbody>
</table>
Figure 3.1A Hepatic hsp70 levels in trout exposed to cortisol, bnf, and a mixture of both, during a 28 d exposure period. Bars represent treatment means, relative to control samples as quantified by SDS-PAGE and immunoblotting. Error bars represent SEM. Treatment differences (p<0.05) are indicated by letters. n = 6 to 9 fish per treatment.

Figure 3.1B Hepatic hsp70 levels in trout exposed to cortisol, bnf, and a mixture of both, 6 h post-heat stress. ‘Heat Stress’ refers to trout exposed to a 2 h, +14°C heat stress. Bars represent treatment means, relative to control samples as quantified by SDS-PAGE and immunoblotting. Error bars represent SEM. Treatment differences (p<0.05) are indicated by letters. n = 6 to 9 fish per treatment.

Figure 3.1C Hepatic hsp70 levels in trout exposed to dietary concentrations of cortisol, bnf, and a mixture of both, 24 h post-heat stress. ‘Heat Stress’ refers to trout exposed to a 2 h, +14°C heat stress. Bars represent treatment means, relative to control samples as quantified by SDS-PAGE and immunoblotting. Error bars represent SEM. Treatment differences (p<0.05) are indicated by letters. n = 6 to 9 fish per treatment.
### A

**Exposure Period**

- **Day 14**
- **Day 28**

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative hepatic hsp70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>a</td>
</tr>
<tr>
<td>Sham</td>
<td>a</td>
</tr>
<tr>
<td>Cortisol</td>
<td>a</td>
</tr>
<tr>
<td>Bnf</td>
<td>b</td>
</tr>
<tr>
<td>Mixture</td>
<td>b</td>
</tr>
</tbody>
</table>

### B

**6 h Post-Heat Stress**

- Treatment
- Treatment + Heat Stress

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative hepatic hsp70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>a</td>
</tr>
<tr>
<td>Sham</td>
<td>a</td>
</tr>
<tr>
<td>Cortisol</td>
<td>a</td>
</tr>
<tr>
<td>Bnf</td>
<td>b</td>
</tr>
<tr>
<td>Mixture</td>
<td>b</td>
</tr>
</tbody>
</table>

### C

**24 h Post-Heat Stress**

- Treatment
- Treatment + Heat Stress

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative hepatic hsp70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>a</td>
</tr>
<tr>
<td>Sham</td>
<td>a</td>
</tr>
<tr>
<td>Cortisol</td>
<td>ab</td>
</tr>
<tr>
<td>Bnf</td>
<td>b</td>
</tr>
<tr>
<td>Mixture</td>
<td>b</td>
</tr>
</tbody>
</table>
Figure 3.2A Gill hsp70 levels in trout exposed to dietary cortisol, bnf, and a mixture of both, during a 28 d exposure period. Bars represent treatment means, relative to control samples as quantified by SDS-PAGE and immunoblotting. Error bars represent SEM. No treatment differences (p<0.05) were observed. n = 6 to 9 fish per treatment.

Figure 3.2B Gill hsp70 levels in trout exposed to dietary cortisol, bnf, and a mixture of both, 6 h post-heat stress. 'Heat Stress' refers to trout exposed to a 2 h, +14°C heat stress. Bars represent treatment means, relative to control samples as quantified by SDS-PAGE and immunoblotting. Error bars represent SEM. No treatment differences (p<0.05) were observed. n = 6 to 9 fish per treatment.

Figure 3.2C Gill hsp70 levels in trout exposed to dietary cortisol, bnf, and a mixture of both, 24 h post-heat stress. 'Heat Stress' refers to trout exposed to a 2 h, +14°C heat stress. Bars represent treatment means, relative to control samples as quantified by SDS-PAGE and immunoblotting. Error bars represent SEM. No treatment differences (p<0.05) were observed. n = 6 to 9 fish per treatment.
A

Exposure Period

Day 14
Day 28

Control Sham Cortisol Bnf Mixture

Relative gill hsp70

B

6 h Post-Heat Stress

Treatment Treatment + Heat Stress

Relative gill hsp70

Control Sham Cortisol Bnf Mixture

C

24 h Post-Heat Stress

Treatment Treatment + Heat Stress

Relative gill hsp70

Control Sham Cortisol Bnf Mixture
Chapter 4. The effects of stress on the association between heat shock protein 70 and the glucocorticoid receptor in rainbow trout

4.1 Abstract

Cortisol suppresses the stress-related induction of various hsp70 in fish, suggesting a functional relationship between the organismal and cellular stress responses. However, the molecular mechanisms governing this observation are not known. The purpose of this study was to characterize the association between hepatic hsp70 and the glucocorticoid receptor in rainbow trout that were exposed to heat stress, cortisol, and β-naphthoflavone (bnf). The glucocorticoid receptor represents a unique receptor complex in stress physiology since it contains elements of both the organismal (i.e. it mediates physiological effects of cortisol) and cellular (i.e. its assembly, function, and transport depends on various hsp70) stress responses. This study is the first to document that the glucocorticoid receptor heterocomplex in rainbow trout hepatic tissues contains hsp70. Heat stress significantly increased levels of total cellular hsp70, and by discerning the association of hsp70 with the glucocorticoid receptor, I demonstrated that heat stress significantly increased the amount of hsp70 not bound to the glucocorticoid receptor, while significantly decreasing the amount of hsp70 bound to the glucocorticoid receptor. By calculating the ratio of hsp70 bound to the glucocorticoid receptor, to the total number of glucocorticoid receptors, stress (heat stress and cortisol-treatment)

4 This chapter was adapted from a manuscript submitted for publication on October 20, 2001 to the American Journal of Physiology (Cell Physiology): Basu, N., Kennedy, C.J., and Iwama, G.K. The effects of stress on the association between hsp70 and the glucocorticoid receptor in rainbow trout.
promoted the association of hsp70 with the glucocorticoid receptor. The data contained within this chapter demonstrates a functional and structural link between hsp70 and the glucocorticoid receptor in rainbow trout, and raises questions regarding the existence of a complex, interrelated stress response that spans all levels of biological organization within the whole animal.
4.2 Introduction

Fish are constantly exposed to potentially stressful conditions, and as a consequence have evolved stress response systems at many levels of biological organization. At the organismal level, stress activates the nervous system and triggers a cascade of humoral reactions along the hypothalamo-pituitary-interrenal axis that ultimately results in the rapid release of corticosteroid stress hormones (primary stress response; Figure 1.1; Wendelaar Bonga, 1997; Mommsen et al., 1999). Corticosteroids are biosynthesized by various microsomal enzymes located in the interrenal cells of the head-kidney tissue, and their production is under the strict control of the pituitary gland (Donaldson, 1981). Once released into the circulatory system, corticosteroids can enter a cell by passive diffusion, but recent data suggests that carrier-mediated processes may facilitate this uptake (Vijayan et al., 1997a). The physiological effects of corticosteroids are regulated by the cellular glucocorticoid receptor; a ligand-inducible transcription factor that can activate or repress target genes (Munck et al., 1984; Adcock, 2000). Corticosteroid-mediated gene induction can influence a variety of physiological functions related to metabolism, immunity, behavior, osmoregulation, and cardiovascular transport (Wendelaar Bonga, 1997; Mommsen et al., 1999).

The assembly, functionality, and transport of the glucocorticoid receptor depends upon the actions of various hsp9s, including hsp90, hsp70, and hsp56, which combine with the glucocorticoid receptor to form a multi-protein heterocomplex (Pratt, 1993; Hutchinson et al., 1994; Pratt et al., 1994). Hsp70 and hsp90 appear to act together to facilitate and chaperone the folding of the hormone-binding domain of the glucocorticoid receptor, while hsp56 appears to have a role in the nuclear trafficking of the activated receptor complex. In addition to their role
in maintaining steroid receptors, hsps are required in many aspects of protein metabolism in the unstressed cell (reviewed by Lindquist, 1986; Morimoto et al., 1990; Nover, 1991; Hendrick et al., 1993; Fink et al., 1998). Hsps can be up-regulated when cells are exposed to a wide variety of stressors, particularly those that denature proteins; this is the hallmark feature of the cellular stress response (Hightower, 1991; Sanders, 1993; Welch, 1993). In fish, the induction of hsps have been reported in cell lines, primary cultures of cells, as well as in various tissues from whole animals (reviewed by Iwama et al., 1998; Iwama et al., 1999).

Steroid hormones have induced hsp genes in a wide range of laboratory models, including HeLa cells (Kasambalides and Lanks, 1983), chicken oviduct cells (Baez et al., 1987), breast cancer cells (Ciocca and Luque, 1991), fungi (Brunt et al., 1990), and rat myocytes (Sun et al., 2000), suggesting the existence of a functional relationship between steroid hormones and hsps. In cultured rainbow trout (O. mykiss) hepatocytes, Sathiyaa et al. (2001) demonstrated that elevated levels of cortisol, the primary corticosteroid stress hormone, suppressed the heat stress-related expression of hsp90 mRNA. Further studies, in vivo, demonstrated that elevated levels of cortisol inhibited the heat stress-related induction of gill hsp30 in cutthroat trout (O. clarki clarki; Ackerman et al. 2000), hepatic and gill hsp70 in trout (Figures 2.1A, 2.2A, and 3.1B), and gill hsp70 in tilapia (Figure 2.2B).

While there is some evidence to demonstrate that cortisol attenuates the cellular stress response in fish, a comprehensive understanding of the molecular mechanisms that underlie this phenomenon are not clear. Since the glucocorticoid receptor represents a unique receptor complex that contains elements of both the organismal (i.e. cortisol-inducible transcription
factor) and cellular (i.e. structural and functional association with various hspss) stress responses (Figure 1.2), an examination of the association between the glucocorticoid receptor and hspss may prove valuable. The purpose of this study was to quantify total cellular levels of hsp70, glucocorticoid receptor, hsp70 bound to the glucocorticoid receptor (bound hsp70), and hsp70 not bound to the glucocorticoid receptor (unbound hsp70) in the hepatic tissues of rainbow trout, following exposure of fish to heat stress, a stress hormone (cortisol), and a reference toxicant (bna). By characterizing the association of hsp70 and the glucocorticoid receptor, a better understanding of the functional relationship between the organismal and cellular stress response in fish will be gained.
4.3 Materials and methods

Fish

Juvenile rainbow trout (27.3 ± 0.6 g) were obtained from Spring Valley Trout Farm (Abbotsford, BC, Canada) and acclimated for two weeks in dechlorinated city water and constant aeration (5 – 7°C; pH 6.9) at the Alcan Aquatic Facility at Simon Fraser University (Burnaby, BC, Canada). Fish were held at a stocking density ranging from 15 to 20 g/L, exposed to a 16:8, light:dark photoperiod, and fed 1.5% body weight every 48 h.

Feed Preparation

Chemicals were incorporated into feed at a ratio of 333 μg cortisol per g feed, and 3.33 mg bnf per g feed. Assuming that individual fish consumed 1.5% body weight, I aimed to achieve internal body concentrations of 5 mg cortisol per kg body weight fish (cortisol) and 50 mg bnf per kg body weight fish (bnf). All chemicals were dissolved in acetone. In order to incorporate chemicals into the feed pellets, pellets were immersed in the acetone-chemical solutions and placed in a fume hood for approximately 2 h until the acetone had evaporated. Pellets were stored at -20°C until required.
Treatments and Exposure

Fish were randomly divided into four groups, each consisting of six replicate tanks. Fish were held at a stocking density of approximately 25 g/L, and fed treatment specific diets of 1.5% body weight every 48 h at 11:00 am on odd numbered days, for a duration of 28 d (i.e. 14 days of feeding). Treatments included: controls (non-treated feed), sham (acetone-immersed feed), cortisol, and bnf.

After a 28 d feeding period, 50% of the fish in each treatment group (i.e. three replicate tanks per treatment) were exposed to a 2 h, +14°C heat stress (immediate change from 6 to 20°C). During this period, the remaining fish (non-stressed) were moved to different tanks for 2 h to simulate the handling stress experienced by the fish exposed to the heat stress. All fish were returned to their holding tanks at ambient temperatures following the 2 h heat stress period.

Fish Sampling

Three fish from each replicate tank (three replicate tanks per treatment) were sampled 6 h post-stress. Fish were quickly removed from their tanks, sacrificed by an overdose of buffered tricaine methanesulfonate (500 mg/L), and their weights and fork lengths were recorded. Approximately 0.2 mL of blood was obtained by caudal puncture using a heparinized syringe and spun at 10,000 rpm for 3 min to obtain blood plasma. Whole hepatic
tissues were excised quickly from all fish. All plasma and tissues were immediately frozen on dry ice and stored at −80°C until analysis.

**Plasma cortisol**

Plasma cortisol was measured using an enzyme-linked immunosorbent assay kit from Neogen Corporation (Lansing, MI, USA) on a 96-well microplate reader (λ = 450 nm).

**Tissue preparation**

Hepatic tissues were homogenized in ice-cold lysis buffer (150 mM NaCl, 1 % Triton X-100, 50 mM Tris, 1 mM ethylenediamine tetra acetic acid (EDTA), 1 μM pepstatin A, 1 mM α-toluenesulfonyl fluoride (PMSF), 1 μM leupeptin, and 0.01 μM aprotinin) under non-denaturing conditions with a hand-held homogenizer. The tissue lysates were cleared in a microcentrifuge at 12,000 g for 10 min at 4°C. Ten μL of supernatant was taken for protein determination with the bicinchoninic acid (BCA) assay using bovine serum albumin (BSA) as a reference. 100 μL of the supernatant was boiled for 3 min with 100 μL SDS-sample buffer (Laemmli, 1970). This solution was frozen at −80°C and used to determine total cellular levels of hsp70.
Immunoprecipitation of hsp70 to the glucocorticoid receptor

To eliminate non-specific binding of antigens to Protein-A Sepharose 6MB (Protein-A; Amersham Pharmacia Biotech AB, Uppsala, Sweden), supernatant from each sample was pre-cleared with 50 μL of Protein-A with over-end shaking for 1 h at 4°C. The pre-cleared supernatant was retrieved by centrifuging the sample for 20 sec (12,000 g at 4°C). During this time, 5 μL of a rabbit anti-rainbow trout glucocorticoid receptor antibody (1:1000 dilution; Tujague et al., 1998) was immunoadsorbed to 100 μL Protein-A for 1 h with over-end shaking at 4°C. Following both shaking events, 500 μL of supernatant (2.5 mg protein) was added to the glucocorticoid receptor antibody and Protein-A complex, and this solution was incubated overnight at 4°C with over-end shaking. The following day, the samples were centrifuged for 20 sec (12,000 g at 4°C). The supernatant (containing hsp70 not bound to glucocorticoid receptor; unbound) was removed and stored at −80°C. The pellet (containing hsp70 bound to glucocorticoid receptor; bound) was washed three times with lysis buffer, and the hsp70 proteins immunoadsorbed to the glucocorticoid receptor were extracted by boiling the sample in SDS-sample buffer (Laemmli, 1970).

SDS PAGE and Immunoblotting

Levels of hsp70 (total, unbound, and bound) and glucocorticoid receptor were measured using the discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemmli (1970). Proteins were separated with a 4% stacking and 12.5% resolving gel on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). Prestained
molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA) were loaded to every gel. Tissue samples from six control fish were pooled and loaded to every gel to normalize the data. Following electrophoretic separation, the proteins were transferred onto nitrocellulose membranes for immunoblotting, as detailed by Forsyth et al. (1997). Briefly, membranes were incubated overnight at 4°C in a polyclonal rabbit anti-rainbow trout (RTG-2) hsp70 antibody (Forsyth et al., 1997) or a rabbit anti-rainbow trout glucocorticoid receptor antibody (Tujague et al., 1998). Following the incubation in primary antibody, membranes were incubated in an alkaline phosphatase conjugated goat anti-rabbit IgG secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. All antibodies were diluted with 2% w/v skim milk powder in TTBS (20 mM Tris, 500 mM NaCl, 0.05% v/v Tween-20, pH 7.5). Reactivity of the hsp70 (Forsyth et al., 1997; Figures 2.1 and 3.1) and glucocorticoid receptor (Tujague et al., 1998) antibodies have been previously demonstrated in rainbow trout.

**Statistical Analysis**

Results are reported as mean ± SEM. A p-value of equal to, or less than 0.05 was considered significant for all statistical tests, unless otherwise stated. The glucocorticoid receptor data was tested at a p-value of equal to, or less than 0.10 to show trends in the data. Treatment comparisons were tested using one-way analysis of variance (ANOVA). Student-Newman-Keuls tests were applied to discern differences among means for those treatments that had significant differences. Band intensities for the hsp70 and glucocorticoid receptor proteins were obtained using densitometry software (Jandel Scientific, San Rafael, CA, USA) and
values were normalized using band intensity values from control fish that were run concurrently on each gel.
4.4 Results

To characterize the stress-status of individual fish in the experimental treatments, levels of cortisol in diluted blood plasma samples were measured (Figure 4.1). The measured levels of plasma cortisol in control and sham fish were not different from each other, and heat stress significantly increased levels of cortisol in control and sham fish by 241% and 174%, respectively. Cortisol-fed fish had significantly higher levels of plasma cortisol, relative to controls, and heat stress did not alter these values. Bnf-fed fish had levels of plasma cortisol that were not significantly different from control fish, and heat stress did not change these levels.

There were no significant differences in glucocorticoid receptor levels between control and sham fish, and heat stress caused a non-significant decrease (p<0.1) of glucocorticoid receptor in control and sham fish by 34% and 55%, respectively (Figure 4.2). Cortisol-fed fish had 35% lower levels (non-significant; p<0.15) of glucocorticoid receptor, relative to control fish, and heat stress further reduced levels of glucocorticoid receptor in this treatment. Bnf-fed fish had levels of glucocorticoid receptor that were similar to values measured in control fish, and heat stress did not change these levels.

There were no differences in levels of total hsp70 between control and sham fish, and heat stress significantly increased total hsp70 in control and sham fish by 72% and 65%, respectively (Figure 4.3). Heat stress significantly reduced total hsp70 levels by 42% in the cortisol-fed fish, relative to non-stressed, cortisol-fed fish. Bnf-fed fish had significantly higher
levels of total hsp70, relative to control fish, and heat stress did not change hsp70 levels in this treatment.

By immunoadsorbing the glucocorticoid receptor antibody with Protein-A, the glucocorticoid receptor and associated molecules (i.e. hsp70 bound to this receptor) could be selectively captured from a sample. No glucocorticoid receptor was detected in the samples classified as “unbound” (i.e. samples that contain hsp70 not bound to the glucocorticoid receptor); demonstrating that all glucocorticoid receptor protein had immunoadsorbed to the Protein-A and glucocorticoid receptor antibody complex. There were no significant differences in the levels of unbound hsp70 among control, sham, cortisol-fed, and bnf-fed treatments (non-stressed treatments) (Figure 4.4A). However, heat stress significantly increased levels of unbound hsp70 in control, sham, and bnf-fed fish by 64%, 62%, and 142%, respectively, and significantly decreased levels of unbound hsp70 in cortisol-fed fish by 44%.

We could not detect hsp70 in control (non-immune) samples that were immunoprecipitated in the absence of glucocorticoid receptor antibody, thus demonstrating that non-specific binding of hsp70 to Protein-A did not occur. Levels of bound hsp70 were not significantly different between control and sham fish (Figure 4.4B), and heat stress significantly reduced levels of bound hsp70 in control and sham fish by 29% and 27%, respectively. Cortisol-fed fish had higher (non-significant; p<0.1) levels of bound hsp70, relative to control fish, and heat stress did not affect these values. Heat stress significantly decreased levels of bound hsp70 by 53% in bnf-fed fish.
By exploring the ratio of hsp70 and glucocorticoid receptor, I found that cortisol-fed fish had the highest ratio of bound hsp70 to glucocorticoid receptor, and heat stress further increased this ratio (Figure 4.5). Heat stress significantly increased the ratio of bound hsp70 to glucocorticoid receptor in all treatments, except for bnf-fed fish.
4.5 Discussion

It has been previously established that cortisol inhibits the stress-related induction of hsp30 (Ackerman et al., 2000), hsp70 (Figures 2.1A, 2.2A, 2.2B, and 3.1B), and hsp90 (Sathiyaa et al., 2001) in fish, and here the cellular interactions that may govern these observations was explored. This study is the first to document that the glucocorticoid receptor heterocomplex in rainbow trout hepatic tissues contains hsp70 (Figure 4.4B); a fact already established in mammalian models (Hutchinson et al. 1994; Cvoro et al. 1998b). A major finding of this chapter was that the association between hsp70 and the glucocorticoid receptor can be altered in a stress-specific manner, following exposure of fish to heat stress, stress hormones (i.e. cortisol), or toxicants (i.e. bnf).

Stress in fish, such as that caused by exposure to heat stress, has been demonstrated to induce hsp70 (Figure 4.3; reviewed by Iwama et al., 1998), while reducing the levels of glucocorticoid receptor (Figure 4.2; Pottinger, 1990; Maule et al., 1991) within the cell. These data confirmed the trend that stress, or cortisol treatment can reduce (p<0.1) the number of cellular glucocorticoid receptors, but this reduction was not statistically significant to a p-value of equal to, or less than 0.05. By characterizing the association between hsp70 and glucocorticoid receptor, these findings demonstrated that heat stress in control fish increased the levels of unbound hsp70 (Figure 4.4A), while causing levels of bound hsp70 to decrease (Figure 4.4B). The observed increase in unbound hsp70 needs to be further explored to determine what percentage of these hsps exist in a “free pool” that are available as molecular chaperones versus those occupied in other cellular systems. A complete characterization of the
sub-cellular localizations and quantitative amounts of hsp70 will greatly aid in the understanding of the cellular stress response.

Cortisol-fed fish exhibited the highest relative ratio of bound hsp70 to the glucocorticoid receptor, and this ratio was increased in all the treatments, except for bnf-fed fish, following heat stress (Figure 4.5). Similar to these findings, a study on hepatic cytosols from male rats revealed that heat stress increased the ratio of bound hsp70 to glucocorticoid receptor (Cvoro et al., 1998b). These observations imply that cortisol, or stress, may promote the association between hsp70 and glucocorticoid receptor. Whether these findings were a result of stoichiometric and conformational alterations in the glucocorticoid receptor heterocomplex, or an increased affinity of the glucocorticoid receptor to bind hsp70 needs to be clarified. The specific functional roles of hsp70 in the glucocorticoid receptor heterocomplex are still unclear, but it is very likely that hsp70 is essential in the assembly and maintenance of the glucocorticoid receptor heterocomplex (Hutchinson et al. 1994; Pratt and Welsh, 1994). In the case of hsp90, Whitesell and Cook (1996) suggested that the binding of hsp90 to the glucocorticoid receptor stabilizes the receptor heterocomplex against proteolytic degradation, and these findings were supported by the study of Dundjerski et al. (2000) who studied the effects of cadmium on the association of hsp90 and glucocorticoid receptor. Steroid receptors can bind hormones in the absence of hsps, but there is considerable evidence that hsps increase the binding capacity of the steroid receptor, facilitate nuclear translocation of the receptor complex, and increase the proteolytic half-life of the receptor complex (Pratt and Welsh, 1994; Czar et al., 1997; Smith et al., 1998). Perhaps the ability of cortisol, or stress to promote the
association of hsp70 with the glucocorticoid receptor is an adaptive mechanism evolved by the organism to enhance the functionality of the glucocorticoid receptor.

One possible function of glucocorticoids is to protect an organism against its normal defense reactions to stressors by preventing or inhibiting these reaction systems from over-shooting their function and disturbing homeostasis (Munck et al., 1984; Matic, 1995). Following this line of reasoning, high levels of cortisol may inhibit the induction of hsp70 (a cellular defense mechanism) following periods of stress. It has been postulated that glucocorticoid receptor-mediated gene expression can inhibit various genes (Bamberger et al., 1996), and this may include components of the cellular stress response (Li et al., 1999). In this study, cortisol-fed fish exposed to the heat stress had significantly lower levels of unbound hsp70 (Figure 4.4A); observations (i.e. suppressed levels of hsp70) similar to those made earlier in my thesis (Figures 2.1A, 2.2A, 2.2B, and 3.1B).

Environmental toxicants, such as bnf, can damage cellular components and cause oxidative stress (Freeman et al., 1999); a potent trigger for hsp gene activation. Given their sensitivity to a wide variety of pollutants (e.g. metals, industrial effluents, organic hydrocarbons, and pesticides), the application of hsps as biomarkers of environmental contamination have been explored in comprehensive review articles (Sanders, 1993; Hightower, 1998; Iwama et al., 1998; Bierkens, 2000). Bnf-fed fish in this study had levels of total hsp70 that were two times higher than values observed in control fish, and heat stress did not alter these values (Figure 4.3). However, by discerning the association of hsp70 with the glucocorticoid receptor, heat stress significantly increased levels of unbound hsp70 (Figure
4.4A), while reducing the amount of bound hsp70 (Figure 4.4B). These findings signified that the cellular damage caused by heat stress, in addition to the toxicant exposure, encouraged hsp70 to disassociate from the glucocorticoid receptor complex and join the "free pool". In doing so, more hsp70 may be available to maintain cellular homeostasis, but further experimental evidence is required to support this statement.

While prior studies have established that steroid hormones, including cortisol, alter levels of hsp70, few have explored the molecular interactions underlying their findings. The major findings of this chapter were that hsp70 is a component of the glucocorticoid receptor heterocomplex in hepatic tissues of rainbow trout, and stress (heat stress or cortisol exposure) promoted the interaction of hsp70 with the glucocorticoid receptor. These results demonstrate that a functional and structural link exists between the organismal and cellular stress responses in fish, and raises questions regarding the existence of a complex, interrelated stress response that spans all levels of biological organization within the whole animal.
Figure 4.1  Plasma cortisol (ng/mL) values in trout exposed to cortisol and bnf, following a heat stress challenge. ‘Heat Stress’ represents trout exposed to a 2 h, +14°C heat stress. Bars represent treatment means, and error bars represent SEM. Letters denote significant differences among treatments (p<0.05). n = 3 replicate tanks per treatment (3 fish per each tank).
Plasma Cortisol (ng/mL)

- **Treatment**
- **Treatment + Heat Stress**

**Control**
- a

**Sham**
- a
- b

**Cortisol**
- c

**Bnf**
- a
- a
Figure 4.2 Levels of hepatic glucocorticoid receptor in trout exposed to cortisol and bnf, following a heat stress challenge. Values were quantified by SDS-PAGE and immunoblotting, and normalized to hsp70 levels from control samples. 'Heat Stress' refers to trout exposed to a 2 h, +14°C heat stress. Bars represent treatment means and error bars represent SEM. Treatment differences (NOTE: p<0.1) are indicated by letters. n = 3 replicate tanks per treatment (3 fish per each tank).
Hepatic Glucocorticoid Receptor

Control  Sham  Cortisol  Bnf

- Treatment
- Treatment + Heat Stress
Figure 4.3  Levels of total hepatic hsp70 in trout exposed to cortisol and bnf, following a heat stress challenge. Values were quantified by SDS-PAGE and immunoblotting, and normalized to hsp70 levels from control samples (non-stressed trout). ‘Heat Stress’ refers to trout exposed to a 2 h, +14°C heat stress. Bars represent treatment means and error bars represent SEM. Treatment differences (p<0.05) are indicated by letters. n = 3 replicate tanks per treatment (3 fish per each tank).
Figure 4.4A  Levels of hepatic hsp70 not bound to the glucocorticoid receptor (unbound hsp70) in trout exposed to cortisol and bnf, following a heat stress challenge. Values were quantified by SDS-PAGE and immunoblotting, and normalized to hsp70 levels from control samples. ‘Heat Stress’ refers to trout exposed to a 2 h, +14°C heat stress. Bars represent treatment means and error bars represent SEM. Treatment differences (p<0.05) are indicated by letters. n = 3 replicate tanks per treatment (3 fish per each tank).

Figure 4.4B  Levels of hepatic hsp70 bound to the glucocorticoid receptor (bound hsp70) in trout exposed to cortisol and bnf, following a heat stress challenge. Values were quantified by SDS-PAGE and immunoblotting, and normalized to hsp70 levels from control samples. ‘Heat Stress’ refers to trout exposed to a 2 h, +14°C heat stress. Bars represent treatment means and error bars represent SEM. Treatment differences (p<0.05) are indicated by letters. n = 3 replicate tanks per treatment (3 fish per each tank).
**Figure A**

Unbound Hepatic hsp70 (Hsp70 Not Bound to Glucocorticoid Receptor)

- Control
- Sham
- Cortisol
- Bnf

**Figure B**

Bound Hepatic hsp70 (Hsp70 Bound to Glucocorticoid Receptor)

- Control
- Sham
- Cortisol
- Bnf

Legend:
- Treatment
- Treatment + Heat Stress
Figure 4.5  Ratio of bound hsp70 to total levels of glucocorticoid receptor in hepatic tissues of trout exposed to cortisol and bnf, following a heat stress challenge. Values were quantified by SDS-PAGE and immunoblotting, and normalized to hsp70 levels from control samples. ‘Heat Stress’ refers to trout exposed to a 2 h, +14°C heat stress. Bars represent treatment means and error bars represent SEM. Treatment differences (p<0.05) are indicated by letters. n = 3 replicate tanks per treatment (3 fish per each tank).
Treatment

Treatment + Heat Stress

Bound hsp70:GR (relative ratio)

- Control
- Sham
- Cortisol
- Bnf

Legend:
- Treatment
- Treatment + Heat Stress

Legend:
- a
- b
- c
5.0 DISCUSSION

5.1 General discussion

The major finding of this thesis is that the organismal (i.e. cortisol) and cellular (i.e. hsp70) stress responses in fish are related at functional and structural levels. This was clearly demonstrated by: (1) studying a temperate salmonid (rainbow trout) and a tropical finfish (mossambique tilapia); (2) measuring the stress responses in hepatic and gill tissues; (3) exogenously introducing cortisol into fish via intra-peritoneal injection or dietary incorporation; (4) artificially raising levels of cortisol for acute (24 h) and sub-chronic (28 d) periods; (5) utilizing different types of stressors, including heat stress, stress hormones (i.e. cortisol), and toxicants (i.e. β-napthoflavone); (6) characterizing the stress response at the organismal (i.e. cortisol, glucose, and lactate) and cellular (i.e. hsp70 and glucocorticoid receptor) levels; and (7) applying novel laboratory methodologies to characterize the association between hsp70 and the glucocorticoid receptor.

As each chapter contains in-depth discussions that are relevant to its respective contents, I will summarize the findings of my thesis and explore the relevance of my data in this chapter.

The organismal and cellular stress responses are ubiquitous in nature and their functions have been well characterized in a wide range of organisms. The high degree of similarity in
responses that are exhibited by distant organisms when confronted by a threatening situation is truly remarkable, and enforces the adaptive significance of stress responses in evolution. For example: (1) the “fight or flight” response has been observed in organisms across a wide range of habitats (Canon, 1929; Goligorsky, 2001); (2) hsp70 have been detected in every species in which they have been sought (Feder and Hofmann, 1999); and (3) cDNA sequences for hsp70 are more than 50% similar among bacteria, yeast, and Drosophila (Craig et al., 1979). My thesis demonstrated that high levels of cortisol can suppress or prevent the heat stress-related increases of hsp70 in fish under a wide range of experimental conditions (Figures 2.1A, 2.2A, 2.2B, 3.1B, 3.1C, and 4.4). These findings are comparable to earlier observations in rainbow trout hepatocytes (Sathiyaa et al., 2001) and gill tissues of cutthroat trout (Ackerman et al., 2000). Collectively, these data reinforce the conserved and ubiquitous nature of the organismal and cellular stress responses in different species of fish.

The ability to exogenously administer drugs and hormones into fish has greatly improved our understanding of many physiological processes. In order to characterize the effects of cortisol on the cellular stress response, cortisol was injected intra-peritoneally into fish as slow releasing pellets (see Chapter 2). While the injection of cortisol into trout resulted in significantly high levels of plasma cortisol, unexpectedly high levels of plasma cortisol and hepatic hsp70 were measured in sham-injected, control trout (Table 2.1 and Figure 2.1A). These unexpected results were most likely due to the inherent stress associated with netting, handling, air exposing, and injecting fish. To address this problem, cortisol and bnf were incorporated into feed pellets and introduced to fish via the diet (see Chapter 3). The major benefit of introducing drugs via the diet is that no anaesthetic, handling, or surgical procedures
are required, all of which can cause stress in fish (Barton and Iwama, 1991; Gamperl et al., 1994). Chapter 3 of this thesis demonstrated that dietary incorporation was a highly effective method to artificially raise levels of cortisol and bnf in fish, without causing any measurable stress to the animal (Table 3.1 and Figure 3.1).

Most studies concerning stress in fish have focused on salmonid species and have been restricted to regions of North America and Europe (Barton, 1997). The 1999 world market of aquaculture was valued at approximately $53.5 billion (US), and this included the production of approximately 21.5 tonnes of fish (FishStat Plus, FAO, 2001). However, a majority of these species were non-salmonid (e.g. carp, tilapia, and milkfish) and localized to developing nations (e.g. China and India). Given these facts, more research on the stress physiology of commercially important species is required. Such projects will undoubtedly minimize the potential health and environmental impacts associated with aquaculture, and improve the profitability of these industries. Chapter 2 of this thesis was an adaptation of the first published report to measure hsp70 levels in tilapia. Given the potential application of hsps as a bio-indicator of stress in fish, more studies are required to resolve their function in non-salmonid models.

A review of the current literature on hsps in fish reveals that most of the research is heavily biased towards the application of hsps as bio-indicators of stress and pollution exposure. However, I must caution that before hsps are routinely used as a tool in risk assessment, a better understanding of their function and significance within the whole animal needs to be gained. For example, little is known about the kinetics of hsp induction following
exposure to toxicants, and hsp levels are highly influenced by numerous factors, such as the thermal history of an organism (Nakano, 2000) and developmental stage (Lele et al., 1997). Prior studies have demonstrated that fish inhabiting polluted waterways have an altered stress response at the organismal level (i.e. impaired synthesis of cortisol; Hontela et al., 1992; Hontela et al., 1997; Lappivaara and Oikari, 1999). The data in this thesis demonstrated that, in addition to an impaired organismal stress response, fish sub-chronically exposed to bnf have an impaired stress response at the cellular level (i.e. impaired ability to induce hepatic hsp70; Table 3.1 and Figure 3.1B). These data raise questions regarding the applicability of cortisol and hsps as bio-indicators of stressed states of fish that inhabit polluted waters.

The relationship among physiological stress, steroid hormones, and hsps has been briefly explored in non-piscine models. Dr. Robert Udelsman’s laboratory (The Johns Hopkins Hospital, Baltimore, USA) showed that hsp expression in rats is under the direct control of the hypothalamo-pituitary-adrenal axis (Blake et al., 1991; Udelsman et al., 1993; Udelsman et al., 1994). These researchers demonstrated that restraint stress induced hsp70 in adrenal cortical and thoracic aorta tissues of rats, but this induction was not observed in hypophysectomized rats. However, the induction of hsp70 in these tissues was re-established following the addition of adrenocorticotropic hormone (ACTH; see Figure 1.1). Dr. Earl Noble’s laboratory (University of Western Ontario, London, Canada) demonstrated that estradiol, a steroid hormone, can inhibit the exercise stress-related increase of hsp70 in various tissues of male rats (Paroo et al., 1999; Paroo et al., 2001), a finding that is analogous to my observations (Figures 2.1A, 2.2A, 2.2B, 3.1B, and 4.4). They postulated their results were most likely due to the indirect ability of estradiol, and possibly other steroid hormones, to activate antioxidant
mechanisms and stabilize cellular membranes. The activation of these cellular defense systems by estradiol may reduce the need for hsps following stress. The research group of Dr. Gordana Matic (Institute for Biological Research, Belgrade, Yugoslavia) has spent the past decade exploring the relationship among the glucocorticoid receptor, physiological stress, and hsps. In a series of experiments on the hepatic tissues of male Wistar rats, this group has demonstrated that heat stress and cadmium exposure can lead to a functional impairment of the glucocorticoid receptor heterocomplex, and alter the association of hsp70 and hsp90 with the glucocorticoid receptor (Matic et al., 1990; Matic et al., 1995; Cvoro et al., 1998a; Cvoro et al., 1998b; Dundjerski et al., 2000).

In order to explore the molecular mechanisms that link the organismal and cellular stress responses, the association of hsp70 with the glucocorticoid receptor was explored in Chapter 4. In stress physiology, the glucocorticoid receptor heterocomplex is unique since it mediates the physiological effects of cortisol (Matic et al., 1990; Mommsen et al., 1999), and its assembly, functionality, and transport is dependent upon hsp70, hsp90, and hsp56 (see Figure 1.2; Pratt, 1993). To study the effects of stress on the association between hsp70 and the glucocorticoid receptor, a rainbow trout glucocorticoid receptor antibody (Tujague et al., 1998) was immuno-adsorbed with Protein-A Sepharose. By doing this, the glucocorticoid receptor and associated molecules (i.e. hsp70) could be selectively isolated (co-precipitated). After validating this technique, I demonstrated for the first time that the glucocorticoid receptor heterocomplex contains hsp70 in rainbow trout hepatic tissues. Additionally, heat stress increased the ratio of hsp70 that are bound to the glucocorticoid receptor, and cortisol-fed fish had the highest ratio of hsp70 that were bound to the glucocorticoid receptor (Figure 4.5).
These findings suggest that cortisol and heat stress can promote the binding of hsp70 with the glucocorticoid receptor. It is known that hsps can prevent the proteolytic degradation of steroid receptors (Whitesell and Cook, 1996), and facilitate the binding of hormones to receptors (Pratt, 1993; Pratt and Welsh, 1994; Pratt, 1997). Perhaps, the ability of stress and cortisol to protect the glucocorticoid receptor by recruiting hsp70 to the receptor complex is an adaptive mechanism to ensure that cortisol-mediated gene transcription remains functional following periods of environmental stress.

The recurring observations that cortisol suppresses or inhibits the heat stress-related induction of hsp70 may be an adaptive mechanism employed by the organism to maintain homeostasis. It has been postulated that one possible function of glucocorticoids is to protect an organism against its normal defense reactions to stressors (i.e. cellular stress response) by preventing or inhibiting these reaction systems from over-shooting their function and disturbing homeostasis (Munck et al., 1984; Matic, 1995). While there is no comprehensive experimental evidence to explain these observations, three likely mechanisms need to be considered. First, very high concentrations of cortisol may increase glucocorticoid receptor-mediated gene activation to pharmacological levels, such that too much energy is mobilized during the secondary stress response. To control this output, it has been proposed that low (Picard et al., 1990) or high (Kang et al., 1999) levels of cellular hsp90 can alter the hsp:glucocorticoid receptor ratio, thus regulating the ligand-binding capacity of the receptor. Second, evidence is emerging that glucocorticoid hormones can influence antioxidant pathways (Yoshioka et al., 1994; Urayama et al., 1998; Valen et al., 2000). In doing so, antioxidants may confer additional protection to the cell during stress, and thus, reduce the necessity for a cellular stress
response. Third, glucocorticoid-mediated gene induction can repress, rather than activate, the transcription of certain genes through a type 2 mechanism (Bamberger et al., 1997; Adcock, 2000). Affected genes may encode products that have a direct, or indirect, effect on the cellular stress response.

5.2 Future directions

A review of the current literature on hsps in fish reveals that a majority of these studies have been in vitro and/or utilized these proteins as non-specific biomarkers of environmental stress. Additionally, many of these studies describe the induction of hsps following exposure of test organisms to certain experimental conditions, without exploring or resolving the mechanisms underlying their observations. The main limitation of our present knowledge still lies in the scarcity of data available on the significance of hsps in the generalized stress response and overall physiology of the whole animal. My thesis demonstrated that hsps are functionally associated with the neuro-endocrine stress response in fish, but more data are required to clarify the importance of hsps in fish physiology.

It is apparent that there are some major gaps in our knowledge about bio-indicators of stress in fish. Hsps (Sanders, 1993; Feder and Hofmann, 1999) and cortisol (Wendelaar Bonga, 1997; Mommsen et al., 1999) are both influenced by a broad range of environmental (e.g. acclimation temperature and water quality) and genetic (e.g. strain differences and selective breeding practices) factors that need to be considered. For example, the induction of hsps can vary, depending on: tissue differences, cellular localization, multiple isoforms of common hsps,
and degree of inducibility (i.e. constitutive, partially induced following stress, or stress-induced). The findings contained within this thesis demonstrated that hsp70 levels could be influenced by circulating concentrations of cortisol (Figure 2.1A), toxicant exposure (Figure 3.1B), and the number of glucocorticoid receptors within a cell (Figure 4.4). Therefore, the universal applicability of bio-indicators of stress in fish requires a thorough understanding of how their levels are influenced by factors that are both internal and external to the organism.

Fundamental questions still exist on how an animal senses a stress. There is general consensus that hsps are regulated at the transcriptional level (Morimoto et al., 1992; Fink and Goto, 1998), but the mechanisms underlying this activation are not well understood. Similarly, there is little information regarding the changes that occur in the vertebrate brain following the perception of a stress, and how this can activate the HPI axis (Sumpter, 1997) or the cellular stress response. By discovering the mechanisms by which an animal senses a stressor and elicits a stress response, we will gain a better understanding of the extent to which the organismal and cellular stress responses are related in fish.

There are many potential applications for hsps in the aquatic sciences. They hold great promise as biomarkers of generalized stress in populations exposed to environmental pollutants, pathogens, or poor water quality (Sanders, 1993; Bierkens, 2000). Present measures of stress in fish, such as increased plasma concentrations of cortisol and glucose can be greatly influenced by handling procedures, while tissue levels of hsp70 are not affected (Vijayan et al., 1997b). Hsps may also be effective in aquaculture management strategies to enhance the stress tolerance and health of farmed fish, and there are efforts underway examining the potential use
of hsps in the development of vaccines against infectious diseases (Iwama et al., 1998). While beneficial applications for hsps exist in industrial and environmental practices, the first step is for researchers to resolve their physiological significance.

5.3 Concluding remarks

Given the adaptive similarities between the organismal and cellular stress responses, the purpose of my thesis was to determine if a functional relationship exists between these two levels of stress response. By studying the cellular and organismal stress responses in fish under a wide variety of experimental conditions, I have demonstrated that: (1) cortisol has a suppressive effect on the stress-induced levels of hsp70; (2) fish that are sub-chronically stressed have an impaired organismal and cellular stress response when challenged to a stressor; and (3) stress can promote the association between hsp70 and the glucocorticoid receptor. While these statements are simplistic interpretations of my findings, collectively they point to the existence of a functional and structural relationship between the organismal and cellular stress responses in fish. In order to gain a holistic understanding of how the activities of hsps at the cellular level relate to physiological systems at the organismal level, we need to continue studies that bridge the disciplines of molecular biology and animal physiology. Once we have gained a clear understanding of the physiological significance of hsps and what implications their functions may have on the performance and health of fish populations and ecosystems, we may confidently apply them in practical situations.
LITERATURE CITED


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