ABSTRACT

NEUROENDOCRINE REGULATION OF FOOD INTAKE
FOLLOWING ACUTE STRESS IN THE TILAPIA,
OREOCHROMIS MOSSAMBICUS

The general stress response principally results in the release of cortisol, with the overall effect of mobilizing metabolic energy and redirecting it towards homeostatic maintenance. Consequently, an important factor altered by stress is food intake. In several teleost species, a decrease in food intake following stress has been observed, though the neuroendocrine mechanism controlling the reduction in food intake during stress has yet to be determined. These studies were designed to investigate the effect of an acute stress on food intake and brain expression of corticotropin-releasing hormone (CRH), neuropeptide Y (NPY), ghrelin and ghrelin's receptor and whether these effects are mediated by cortisol. Therefore, metyrapone, a cortisol synthesis inhibitor, was administered via feed in three doses of 10, 25, and 50 mg/kg body weight for 1 wk prior to a 30 min crowding and handling stress. Following the stressor, fish were allowed to feed for 1 h. Stress reduced food intake, while elevating mRNA levels of CRH, an appetite suppressor. Additionally, metyrapone treatment dose-dependently blocked the stress-induced reduction in food intake. While NPY and ghrelin levels were unchanged, an increase in ghrelin receptor activity suggests a function for ghrelin in regulating overall metabolism in response to stress. The elevation of CRH mRNA levels was also reversed with metyrapone treatment, suggesting cortisol and CRH play a role in mediating reduction of food intake during stress in tilapia.

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NEUROENDOCRINE REGULATION OF FOOD INTAKE FOLLOWING ACUTE STRESS IN THE TILAPIA, *OREOCHROMIS MOSSAMBICUS*

by

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For the Department of Biology:

We, the undersigned, certify that the thesis of the following student meets the required standards of scholarship, format, and style of the university and the student's graduate degree program for the awarding of the master's degree.

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TABLE OF CONTENTS

Page

LIST OF TABLES vi

LIST OF FIGURES vii

CHAPTER 1: INTRODUCTION 1

Hypothalamic Pituitary Interrenal Axis 2

Regulation of Food Intake During Stress 3

Objectives 7

Hypotheses 7

CHAPTER 2: ACUTE STRESS INHIBITS FOOD INTAKE AND ALTERS GHRELIN REGULATION IN AN ADVANCED TELEOST, *OREOCHROMIS MOSSAMBICUS* 8

Materials and Methods 10

Results 14

Discussion 19

CHAPTER 3: ROLE FOR CORTISOL IN MEDIATING STRESS-INDUCED REDUCTION OF FOOD INTAKE IN AN ADVANCED TELEOST, *OREOCHROMIS MOSSAMBICUS* 25

Materials and Methods 26

Results 30

Discussion 36

CHAPTER 4: CONCLUSION 41

REFERENCES 43

LIST OF TABLES

Page

Table 1: Primer sequences..... 13

LIST OF FIGURES

Page

Figure 1. Representation of how stimulation of the autonomic nervous system and hypothalamic-pituitary-interrenal axis mobilizes energy during stress. 4

Figure 2. Diagram of tilapia brain with regions of interest labeled. 12

Figure 3. Effect of acute stress on food intake. Vertical bars represent means ±SEM (n = 12). **Significantly different from control at P<0.01 (one-way ANOVA). 15

Figure 4. Effect of acute stress on plasma cortisol levels (A) and plasma glucose levels (B). Vertical bars represent means ±SEM (n = 12). ***Significantly different from control at P<0.001 (one-way ANOVA). 15

Figure 5. Effect of acute stress on plasma ghrelin levels (A) and stomach ghrelin mRNA levels (B). Vertical bars represent means ±SEM (*n* = 12). 16

Figure 6. Effect of acute stress on telencephalon/pre-optic area levels of CRH mRNA (A) and optic tectum/hypothalamus CRH mRNA (B). Vertical bars represent means ±SEM (*n* = 12). 17

Figure 7. Effect of acute stress on telencephalon/pre-optic area levels of NPY mRNA (A) and optic tectum/hypothalamus NPY mRNA (B). Vertical bars represent means ±SEM (*n* = 12). 17

Figure 8. Effect of acute stress on telencephalon/pre-optic area levels of ghrelin mRNA (A) and optic tectum/hypothalamus ghrelin mRNA (B). Vertical bars represent means ±SEM (*n* = 12). *Significantly different from control at *P*<0.05 (one-way ANOVA). 18

Figure 9. Effect of acute stress on telencephalon/pre-optic area levels of GHSR hnRNA (A), GHSR1a-LR mRNA (B) and GHSR1b-LR mRNA (C). Vertical bars represent means ±SEM (*n* = 12). **Significantly different from control at *P*<0.01 (one-way ANOVA). 18

Figure 10. Effect of acute stress on optic tectum/hypothalamus levels of GHSR hnRNA (A), GHSR1a-LR mRNA (B) and GHSR1b-LR mRNA (C). Vertical bars represent means ±SEM (*n* = 12). **,***Significantly different from control at *P*<0.01 and *P*<0.001, respectively (one-way ANOVA). 19

Figure 11. Diagram of tilapia brain with regions of interest labeled. 28

Page

Figure 12. Effect of metyrapone on food intake following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at P<0.05. 31

Figure 13. Effect of metyrapone on plasma cortisol levels (A) and plasma glucose levels (B) following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at P<0.05. 31

Figure 14. Effect of metyrapone on telencephalon/pre-optic area levels of CRH mRNA (A) and hypothalamic CRH mRNA (B) following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at P<0.05..... 32

Figure 15. Effect of metyrapone on telencephalon/pre-optic area levels of NPY mRNA (A) and hypothalamic NPY mRNA (B) following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at P<0.05. 33

Figure 16. Effect of metyrapone on telencephalon/pre-optic area levels of ghrelin mRNA (A) and hypothalamic ghrelin mRNA (B) following acute stress. Vertical bars represent means ±SEM (n = 10). 34

Figure 17. Effect of metyrapone on telencephalon/pre-optic area levels of GHSR hnRNA (A), GHSR1a-LR mRNA (B) and GHSR1b-LR mRNA (C) following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at P<0.05. 35

Figure 18. Effect of metyrapone on hypothalamic levels of GHSR hnRNA (A), GHSR1a-LR mRNA (B) and GHSR1b-LR mRNA (C) following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at P<0.05..... 36

CHAPTER 1: INTRODUCTION

Fish encounter a variety of stressors such as suboptimal water quality and temperature, competition, and lack of food availability. The stress response that ensues is characterized by a switch from an anabolic to a catabolic state, which appears to be fairly conserved from fish to mammals (Wendelaar Bonga 1997). The immediate response is mediated by the sympathetic nervous system (i.e. catecholamines), while a relatively prolonged response is orchestrated by the hypothalamic-pituitary-adrenal (HPA) axis, or hypothalamic-pituitary-interrenal (HPI) axis in fish, resulting in the release of corticosteroids. Catecholamines and corticosteroids (primarily cortisol in fish) have a general overall effect of mobilizing metabolic energy and redirecting it to processes needed to cope with an immediate stressor. While the actions of the stress response, as well as its components, are necessary to maintain homeostasis in an ever-changing environment, even a brief handling stress was shown to divert approximately 25% of energy available for metabolic activity to this maintenance (Schreck 2010). As a result, food intake is commonly altered as a mechanism of maintaining energy homeostasis during stress (Woods et al. 1998).

Food intake results from a set of complex neuroendocrine signals that integrate external stimuli with the internal milieu of the animal. Corticotropin-releasing hormone (CRH), the initiating factor of the stress axis, is also an important anorexigenic (appetite suppressing) factor in both mammals and fish (Volkoff et al. 2005). CRH stimulates adrenocorticotropic hormone (ACTH) release from the pituitary resulting in the release of corticosteroids, including cortisol from the interrenal gland in fish. In rats, CRH also stimulates the transcription of the polypeptide precursor to ACTH, pro-opiomelanocortin

(POMC) (Lightman 2008), which is also an anorexigenic factor. NPY is the primary orexigenic (appetite stimulating) factor in all vertebrates and seems to mediate the central actions of another potent orexigen, ghrelin (Chen et al. 2004; Miura et al. 2006). Ghrelin is primarily a gut hormone, but is also produced in the brain, where its receptor is expressed on NPY-containing neurons in mammals (Kohno et al. 2003). In mammals, a general pattern of increased food intake in response to acute or mild stressors and decreased food intake in response to chronic or severe stressors is well documented (Valles et al. 2000). Interestingly, fish exhibit decreases in food intake regardless of stressor intensity or duration (Bernier 2006). However, the neuroendocrine mechanism controlling the reduction in food intake during stress in fish has yet to be determined.

Hypothalamic Pituitary Interrenal Axis

In response to sensory perception of a stressor, the nonspecific portion of the stress response is activated and generally consists of both neuronal and hormonal responses (Schreck 2010). Usually within seconds, the sympathetic nervous system is triggered to release catecholamines, epinephrine and norepinephrine, ("fight or flight" hormones) from adrenal medullary cells in mammals or homologous chromaffin cells in fish (Reid et al. 1998). This response is generally supported within minutes by a relatively prolonged response orchestrated by the HPA axis (HPI axis in fish; Fig. 1). In all vertebrates, activation of this axis increases secretion of CRH from the hypothalamus, which acts as an ACTH secretagogue in the anterior pituitary, and ultimately releases corticosteroids from adrenal cortical cells in mammals (Chrousos 1998) or homologous interrenal cells in teleosts (Basu et al. 1965). Catecholamines as well as corticosteroids (e.g. cortisol) have a general overall effect of mobilizing

metabolic energy, primarily in the form of glucose, in order to cope with the stressor at hand. Cortisol also exhibits negative feedback on CRH by binding to centrally located glucocorticoid receptors (Chrousos 1998). This is a convenient route, as pulsatile activation of the same neuroendocrine pathways is the mechanism of energy allocation in normal situations as well (Wendelaar Bonga 1997). While the actions of the stress response, as well as its components, are necessary to maintain homeostasis in a plastic environment, including conditions of acute stress, they can be detrimental during times of severe or chronic stress (Belanger et al. 2001). Even if a fish appears to adapt to a stressor, long-term costs may accumulate in the form of decreased overall fitness, including developmental, growth, and reproductive capabilities (Schreck 2000). As a result, food intake is commonly altered as a mechanism of maintaining energy homeostasis during stress (Woods et al. 1998). Mammals exhibit both increases and decreases in food intake depending on the type of stressor (Varma et al. 1999). For example, while tail-pinch stress caused increased food intake in rats (Asakawa et al. 2001), restraint resulted in decreased food intake (Zylan and Brown 1996). In fish, however, food intake is reduced regardless of the stressor (Barton et al. 1987; Schreck et al. 1997; Pankhurst et al. 2008).

Regulation of Food Intake During Stress

Satiety and appetite signals from a variety of peripheral origins are integrated within the hypothalamus, where peptidergic systems including populations of neurons secreting both CRH and NPY mediate anorexigenic and orexigenic effects, respectively (Berthoud 2002; Matsuda 2009). This points to the hypothalamus as the feeding regulation center in both mammals (Konturek et al. 2004) and fish (Bernier 2006). NPY is the most potent orexigenic factor in

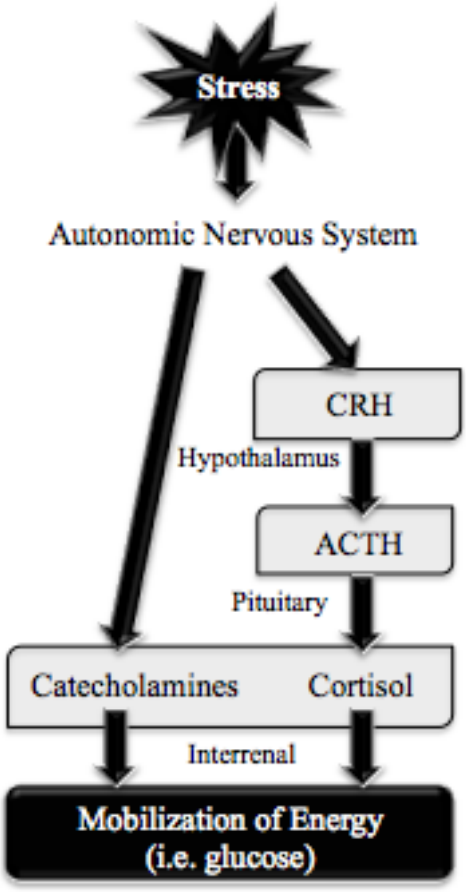

Figure 1. Representation of how stimulation of the autonomic nervous system and hypothalamic-pituitary-interrenal axis mobilizes energy during stress.

vertebrates, and its secretion is increased during fasting in pufferfish (Matsuda 2009) indicating involvement in energy homeostasis in fish (Schwartz et al. 2009).

After increases in expression following stress, NPY stimulates secretion of CRH and inhibits secretion of catecholamines in mammals (Chrousos 1998). Accordingly, CRH and NPY expression is increased in subordinate rainbow trout (Doyon et al. 2003). Although these actions of NPY seem counterintuitive, similar to the negative feedback actions of cortisol, they represent a self-restraint system

on the stress response and allow for multiple sites of integration as well as adaptation (Charmandari et al. 2004).

Along with initiation of the stress axis, centrally administered CRH decreases food intake in rodents and fish (Bernier 2006). In response to an actual stressor, however, fish seems to show increased CRH only after prolonged or severe physical stress (Huising et al. 2004; Doyon et al. 2005). Hypothalamic CRH stimulates production and secretion of POMC (Lightman 2008), the precursor to ACTH as well as alpha-melanocyte stimulating hormone (α-MSH), an anorexigenic factor in mammals (Stanley et al. 2005) that has been shown to have similar actions in trout (Volkoff et al. 2006) and goldfish (Matsuda 2009). Increases in POMC are also exhibited in response to low-water stress in channel catfish (Karsi et al. 2005). Further, hypothalamic neurons containing CRH, NPY and POMC all contain receptors for another appetite regulator, ghrelin (Stanley et al. 2005).

Ghrelin was recently discovered as an endogenous ligand for the growth hormone secretagogue receptor (GHSR) and, as the name implies, elicits a variety of actions contributing to growth and energy balance, including food intake, in rats (Kojima et al. 1999) and fish (Kaiya et al. 2008). Ghrelin is produced primarily in the gut, but also in the brain. Therefore ghrelin signaling in the hypothalamus could be the result of either peripherally or centrally derived peptides. Ghrelin's regulation of food intake, however, seems to be localized to NPY-containing neurons in mammals (Stanley et al. 2005). Similarly, treatment with the NPY receptor antagonist reverses ghrelin-induced feeding in goldfish (Matsuda 2009). Ghrelin also appears to be involved in mediating aspects of CRH's effects on the stress response in mammals. Centrally and peripherally administered ghrelin elevated hypothalamic CRH mRNA levels and plasma cortisol levels, resulting in

an increase in anxious behavior in mice. These anxiogenic effects were blocked by a CRH receptor antagonist treatment (Asakawa et al. 2001). Interestingly, rat ghrelin has also been shown to have NPY-stimulating effects in the brain only when the stress indicator cortisol is present (Goto et al. 2006), suggesting a link between ghrelin, stress, and food intake in mammals. Although Pankhurst et al. (2008) observed increases in cortisol and decreases in ghrelin following confinement stress in rainbow trout, the relationship between the neuroendocrine regulators of food intake and the HPI axis during acute stress remains uncertain in any teleost.

Although corticosteroid release is just one outcome of the stress response, many studies treat with corticosteroids to induce stress-like symptoms or use circulating levels as a measure of stress (Barton et al. 2002). These studies have shown that corticosteroids play a role in mediating the neuroendocrine changes affecting food intake during stress. Treatment with dexamethasone, a synthetic corticosteroid, decreased CRH and increased NPY in the hypothalamus of rats (Adams and Epels 2007). The same pattern was observed in Telen/POA mRNA levels in response to increased cortisol in goldfish (Bernier et al. 2004) and both were associated with an increase in food intake. Conversely, long-term cortisol treatment decreased food intake in rainbow trout (Gregory and Wood 1999). Therefore although a role for cortisol in mediating the effects of stress on food intake appears to be conserved, the effects mediated appear to be clade and even species specific. Nevertheless, few studies in fish have studied the involvement of and mechanism behind cortisol in stress-induced reduction in food intake in fish.

Objectives

1. Investigate the effect of acute stress on the neuroendocrine regulation of food intake.

2. Investigate the role of cortisol in mediating the effects of acute stress on food intake.

Hypotheses

Hypothesis I: Acute stress will decrease food intake

Hypothesis II: Acute stress will decrease the expression of orexigenic factors (NPY, ghrelin) and increase the expression of anorexigenic factor (CRH) in the telencephalon and/or hypothalamus region resulting in a decrease in food intake

Hypothesis III: Treatment with a cortisol synthesis blocker, metyrapone, will reverse the stress-induced reduction in food intake

CHAPTER 2: ACUTE STRESS INHIBITS FOOD INTAKE AND ALTERS GHRELIN REGULATION IN AN ADVANCED TELEOST, *OREOCHROMIS MOSSAMBICUS*

Fish encounter a variety of stressors in natural and artificial ecosystems. The stress response that ensues is characterized by a switch from an anabolic to a catabolic state in order to mobilize energy, which appears to be fairly conserved across vertebrates (Wendelaar Bonga 1997). While the actions of the stress response are necessary for allostasis, an adaptive stasis maintained during conditions that prevent an organism from achieving normal homeostasis, they come at the expense of other physiological functions. Many studies detail negative effects of the stress response on reproduction, growth, and immunity in fish (see review, Schreck 2010). These are all vegetative functions that are put on hold during stress. Likewise, food intake is also commonly altered to maintain energy homeostasis during stress (Woods et al. 1998), although the mechanisms behind this regulation are less well understood, especially in fish (Bernier and Peter, 2001)

Satiety and appetite signals from a variety of peripheral origins and different brain regions are integrated within the hypothalamus, the primary feeding regulation center in both mammals (Konturek et al. 2004) and fish (Bernier 2006). Here, peptidergic systems including populations of neurons secreting both CRH and NPY mediate anorexigenic and orexigenic effects, respectively (Berthoud 2002; Matsuda 2009). Sources of central input into this system are found in mammals and are suggested in fish, with implications of the telencephalon region contributing to energy homeostasis and appetite (Peter 1979; Lin 2000; Kulczykowska 2010). Other factors involved in the central regulation of food intake include ghrelin, a multifaceted hormone whose central actions are mediated

by its receptor (GHSR), located on NPY-containing neurons in mammals (Stanley et al. 2005). Accordingly, treatment with an NPY receptor antagonist reverses ghrelin-induced feeding in goldfish (Matsuda 2009). These factors appear to regulate food intake under normal as well as stressful conditions.

The effects of stress on food intake in mammals are highly variable, with opposite effects induced depending on the type of stressor (Marti 1994) and the current physiological state of the animal (Adam and Epel 2007). Conversely, fish consistently exhibit decreased food intake regardless of the stressor, although the mechanisms behind these observations are not clear (Bernier 2006).

The CRH and NPY neuroendocrine pathways have long been implicated in the mediation of food intake during the mammalian stress response (Chrousos 1998) and there is growing evidence that these roles are at least partially conserved in fish, with altered CRH and NPY mRNA levels in response to stress and cortisol treatment in rainbow trout (Doyon et al. 2003) and goldfish (Bernier et al. 2004).

Ghrelin appears to be involved in mediating aspects of the CRH effects on the stress response in mammals. Centrally and peripherally administered ghrelin elevated hypothalamic CRH mRNA levels and plasma cortisol levels, resulting in an increase in anxious behavior in mice. These anxiogenic effects were blocked by a CRH receptor antagonist treatment (Asakawa et al. 2001). Interestingly, rat ghrelin has also been shown to have NPY-stimulating effects in the brain only when the stress indicator cortisol is present, as demonstrated by an increase in NPY expression in response to ghrelin treatment in sham but not adrenalectomized rats (Goto et al. 2006), suggesting a link between ghrelin, stress, and food intake in mammals. Although Pankhurst et al. (2008) observed increases in cortisol and decreases in ghrelin following confinement stress in rainbow trout, the relationship

between the neuroendocrine regulators of food intake and the hypothalamic-pituitary-interrenal (HPI) axis during acute stress remains uncertain in any teleost. Therefore, this study was designed to investigate the effect of an acute stress on food intake and mRNA expression of neuroendocrine appetite regulators (CRH, NPY, ghrelin, and GHSRs) in the tilapia (*Oreochromis mossambicus*).

Materials and Methods

Animals

Male and female tilapia (*Oreochromis mossambicus*) weighing 75-85 gm, were reared in freshwater semi-recirculating tanks maintained at 25°C with simulated photoperiod (14h:10h; light:dark). Experimental tanks (22 L) on the same system were separated with opaque dividers so that treatments could be administered without affecting fish in adjacent tanks. Fish were fed a known amount of excess feed twice daily at 1000 and 1700 h for 2 weeks prior to experimental protocol (Silvercup, Murray, UT). To entrain feeding behavior, fish were allowed to feed for 1 h at each feeding period, after which time excess food was removed. The experiment commenced once all tanks were consuming similar amounts of feed.

Acute Stress Experiments

To investigate the effect of acute stress on food intake, food was withheld 24 h prior to the experiment so that an accurate measure of food intake could be determined. This procedure was based on observations by Fox et al. (2009), which demonstrated tilapia empty their stomach within 14 h of eating. Replicate tanks were randomly chosen to receive either stress or control treatment. On the day of the experiment at 0930 h, water levels were lowered in stress tanks such that

dorsal fins were exposed. In addition, these fish were netted and held out of the water for 10 s every 5 min for a 30-min period. This protocol subjected the fish to an air emersion and crowding stressor, as well as simulated handling stressors that take place in aquaculture settings (Belanger et al. 2001). At 1000 h, the water level was returned to 22 L and all fish (control and stress groups) were fed a known amount of excess feed and allowed to feed for 1 h. Following feeding, fish were rapidly decapitated, weighed, and stomach content was removed and dried at 72˚C for 28 h. Dried stomach contents were weighed and grams of dried individual stomach content was normalized to grams body weight of each fish (Fox et al. 2009).

To investigate the effect of acute stress on the neuroendocrine regulators of appetite, the above experiment was replicated with the following modifications. Following the stressor at 1000 h, water levels were raised to 22 L and all fish (control and stress groups) were immediately sampled in order to measure pre-prandial changes in response to stress that may regulate food intake. Sodium bicarbonate-buffered (200 mg/l) tricaine methanesulfonate (100 mg/l), was added directly to the tanks to inhibit a stress response during subsequent handling (Vijayan et al. 1997). Blood samples were taken from caudal vasculature using Na^+-heparin (200 U/ml; Sigma, St. Louis, MO) coated syringes. Plasma was isolated from whole blood by centrifugation at 10K × g for 10 min at 4°C, then stored at -20°C until analysis of cortisol, ghrelin and glucose levels. Immediately after blood sampling, fish were rapidly decapitated. Brains from each fish were extracted and two regions of interest were isolated by microscopic dissection: the telencephalon/pre-optic area (Telen/POA) and optic tectum/hypothalamic (OT/HYP) regions (Fig. 2). These sections were placed in TRI-Reagent (Ambion, Austin, TX), snap frozen in liquid nitrogen, and stored at -80°C until analysis of

mRNA levels of ghrelin, GHSR-LR isoforms (GHSR1a-LR and GHSR1b-LR), heteronuclear GHSR (hnGHSR), NPY, and CRH by quantitative real-time PCR (qPCR). Stomach was collected for measurement of ghrelin mRNA levels.

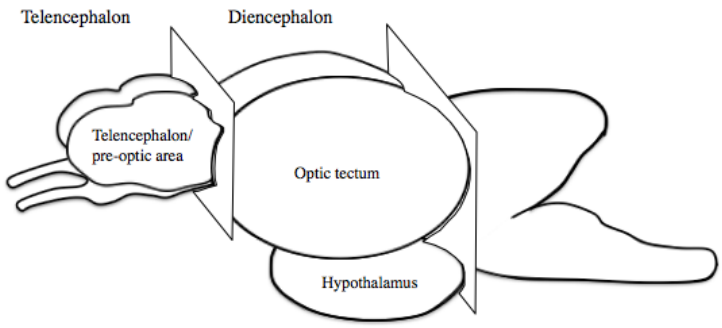

Figure 2. Diagram of tilapia brain with regions of interest labeled.

RNA Extraction, cDNA Synthesis and qPCR

Total RNA was extracted from tissue samples using TRI Reagent (Ambion) and diluted to 200 ng/µl. Subsequent cDNA synthesis was carried out using High Capacity cDNA Synthesis Kit (Applied Biosystems, Carlsbad, CA). A cDNA sample was run with no reverse transcriptase as a control for genomic contamination. cDNA was amplified by quantitative PCR (qPCR), for which each reactions reaction contained 7.5 µl of Power SYBR Green (ghrelin, NPY, CRH) or TaqMan Master Mix and probe (GHSR1a-LR, GHSR1b-LR, hn-GHS-R), along with 0.3 µl of appropriate gene specific primers (see Table 1), and 3 µl of cDNA sample or standard (15 µl total volume). Standards were produced from serial dilutions of purified plasmid DNA for each mRNA of interest. A no-template control was included to check for contamination. All qPCRs were run on the 7300 Real Time PCR System (Applied Biosystems). Data was normalized to total RNA added to each qPCR reaction and expressed as relative to control (Picha et al. 2008).

Table 1: Primer sequences

Target mRNA	Sequence	Annealing Temp (ºC)
CRH	F: 5'-GGAGAGGCTTGGGGAGGA-3' R: 5'-TGCCCTGTAAAAGACGCCG-3'	60
Ghrelin	F: 5'-GGGTTGGTTCAGCTGTCATT-3' R: 5'-ACGTTTGGGTGTTTGGTAGAC-3'	60
NPY	F: 5'-ATGCATCCTAACTTGGTGAG-3' R: 5'-GTCTTGTGATGAGGTTGATG-3'	55
Ghrelin	F: 5'-GGGTTGGTTCAGCTGTCATT-3' R: 5'-ACGTTTGGGTGTTTGGTAGAC-3'	60
GHSR1a-LR	F: 5'-AAGATGCTGGTGGTTGTTGTG-3' R: 5'-TGGCGGCAGGCTGTCGGTCAG-3' Probe: 5'-CGCTCTCTGGATGCTCCTTCACC-3'	58
GHSR1b-LR	F: 5'-ATGGCTCTACAGCCTGATAGG-3' R: 5'-TTGCGCGTTCTGGAAACTTAC-3' Probe: 5'-CTGTGGCAAAGGCACCGAGAGACG-3'	54
GHSR-intron	F: 5'-GACAGATGAAAGACACCTTAGGGC-3' R: 5'-GGTTAGTCAAAGGAGGTGCTGATT-3'	58

Plasma Analysis

Plasma ghrelin levels were measured using a ghrelin radioimmunoassay established by Riley et al. (2008). One hundred microliters of rat ghrelin standards and plasma samples were incubated with 200 µl anti-rat ghrelin (from H. Hosada) at a dilution of 1:750,000. After incubation at 4°C overnight, 100 µl of ^{125}I-human ghrelin (Millipore, St. Charles, MO) was added before an additional overnight incubation at 4°C. Finally, 100 µl anti-rabbit IgG goat serum at 1:35 (with 10% polyethylene glycol) was added, incubated for 2 h at room temperature, and then centrifuged at 3000 × g for 30 min to separate free and bound tracers. Radioactivity of aspirated pellet was then quantified using a gamma counter (Packard, Palo Alto, CA).

Plasma cortisol radioimmunoassay was carried out using commercially available ImmuChem cortisol ^{125}I kit (MP Biomedicals, Orangeburg, NY).

Plasma glucose levels were measured using the Infinity Glucose reagent (ThermoElectron, Pittsburgh, PA). Two microliters of glucose standards (10, 5, 2.5 1.5 and 0 mg/ml) and plasma samples were added to a 96-well plate along with 300 µl of assay reagent. The plate was then incubated at room temperature for 5 min before subsequent reading at 340 nm (ELx800 plate reader, Biotek, Winooski, VT).

Statistical Analysis

Statistical analyses were carried out using one-way ANOVA (analysis of variance) tests (STATISTICA, Statsoft, Tulsa, OK). Significant treatment means were found using a Fischer least common difference test. Data are expressed as means ± S.E.M. Differences were considered significant at $P<0.05$.

Results

Fish exposed to an acute handling and crowding stress exhibited a reduction ($P<0.01$) in food intake when compared with unstressed controls (Fig. 3). Based upon this finding, we then investigated the effect of this stressor on the endocrine control of food intake. Plasma cortisol and glucose levels were measured as indicators of stress in these animals. There was no difference in plasma cortisol between the control and stressed groups (Fig. 4A). However, plasma glucose levels were elevated in the stressed group ($P<0.001$; Fig. 4B), suggesting the protocol was a sufficient stressor.

Stomach ghrelin mRNA was unaltered by the stress treatment (Fig. 5A). Accordingly, circulating ghrelin levels were also unaffected (Fig. 5B), indicating gastric ghrelin secretion is not regulating food intake immediately following a stressor. Both CRH and NPY mRNA levels in both the Telen/POA and OT/HYP regions were unaffected by the stressor (Figs. 6 and 7), indicating changes in

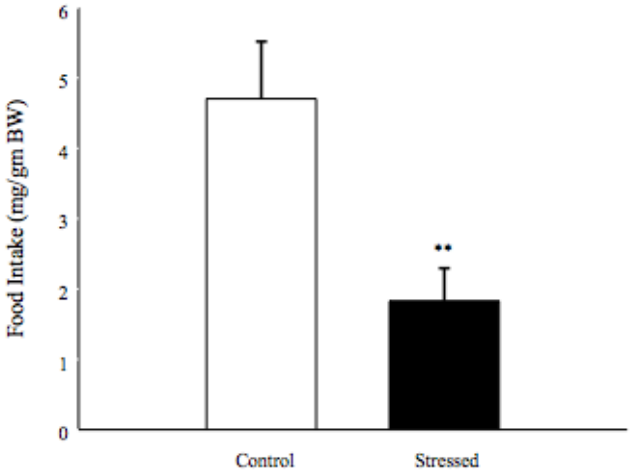

Figure 3. Effect of acute stress on food intake. Vertical bars represent means ±SEM (n = 12). **Significantly different from control at P<0.01 (one-way ANOVA).

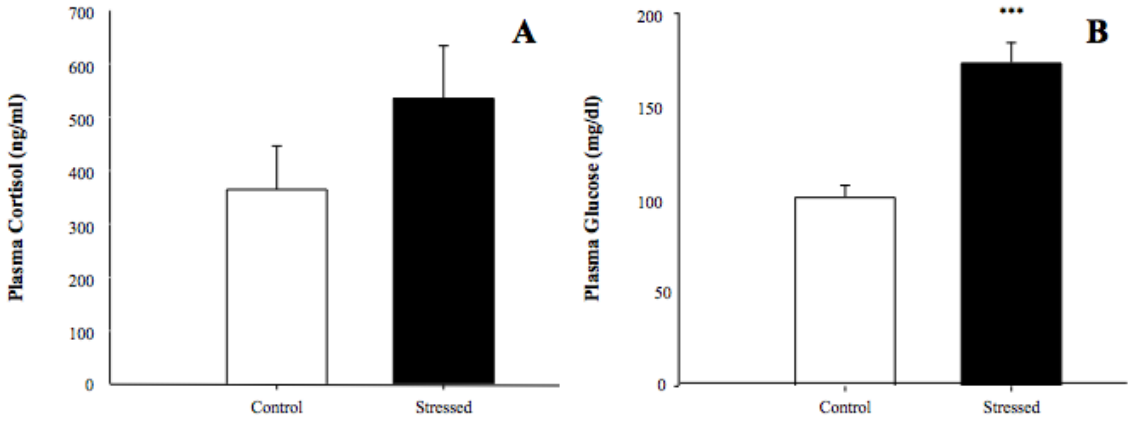

Figure 4. Effect of acute stress on plasma cortisol levels (A) and plasma glucose levels (B). Vertical bars represent means ±SEM (n = 12). ***Significantly different from control at P<0.001 (one-way ANOVA).

expression are not regulating the decrease in food intake. Ghrelin mRNA levels were elevated (P<0.05) in the Telen/POA following the stressor (Fig. 8A), but no effect was observed in the OT/HYP (Fig. 8B), suggesting altered ghrelin regulation following stress. Further, analysis of the Telen/POA revealed elevated levels (P<0.01) of GHS-R hnRNA (Fig. 9A), while levels of both GHSR isoforms (GHSR1a-LR and GHSR1b-LR2) were unaltered following the stressor (Fig. 9B and C, respectively), indicating rapid activation of receptor expression. The stressor induced a different pattern in the OT/HYP than in the Telen/POA, with the GHSR hnRNA levels being suppressed (P<0.001), GHSR1a-LR mRNA levels being elevated (P<0.01), and no effect on GHSR1b-LR mRNA levels (Fig. 10 A, B, and C, respectively), although it is unclear whether hnRNA levels reflect regulation of expression or turnover to mRNA.

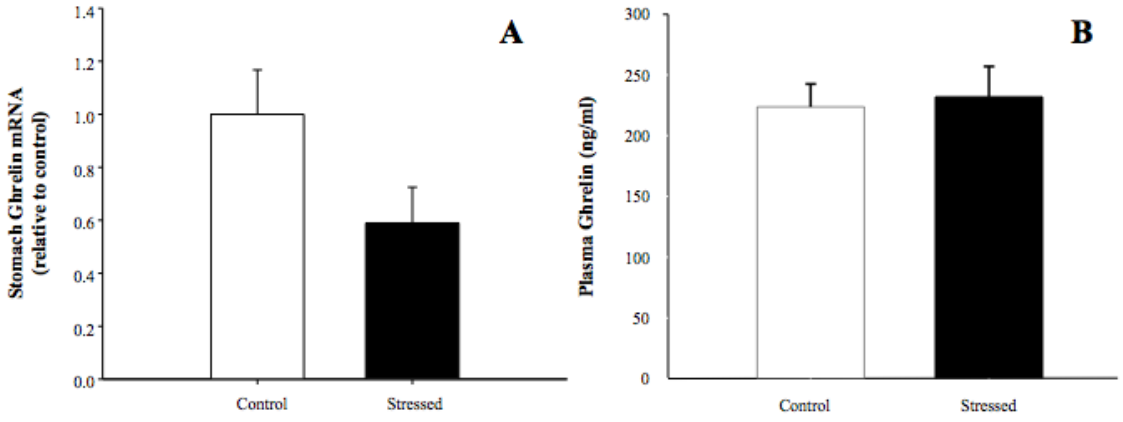

Figure 5. Effect of acute stress on plasma ghrelin levels (A) and stomach ghrelin mRNA levels (B). Vertical bars represent means ±SEM (n = 12).

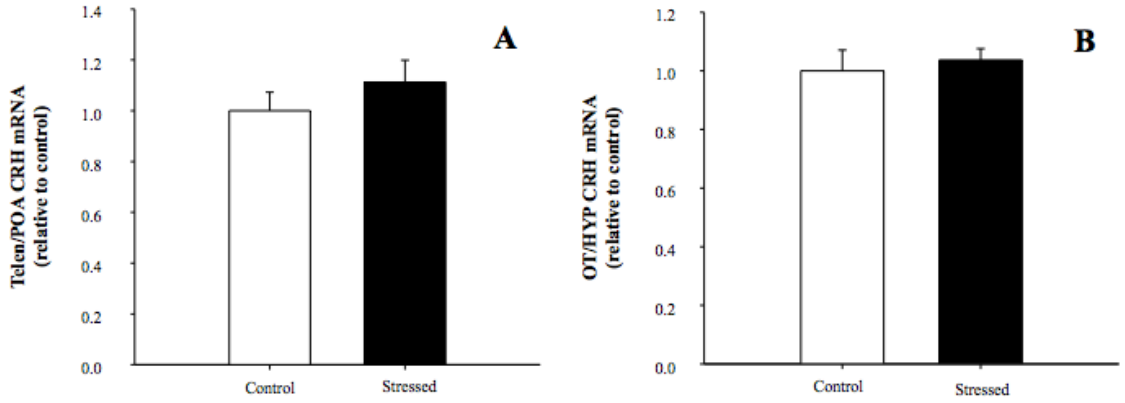

Figure 6. Effect of acute stress on telencephalon/pre-optic area levels of CRH mRNA (A) and optic tectum/hypothalamus CRH mRNA (B). Vertical bars represent means ±SEM (*n* = 12).

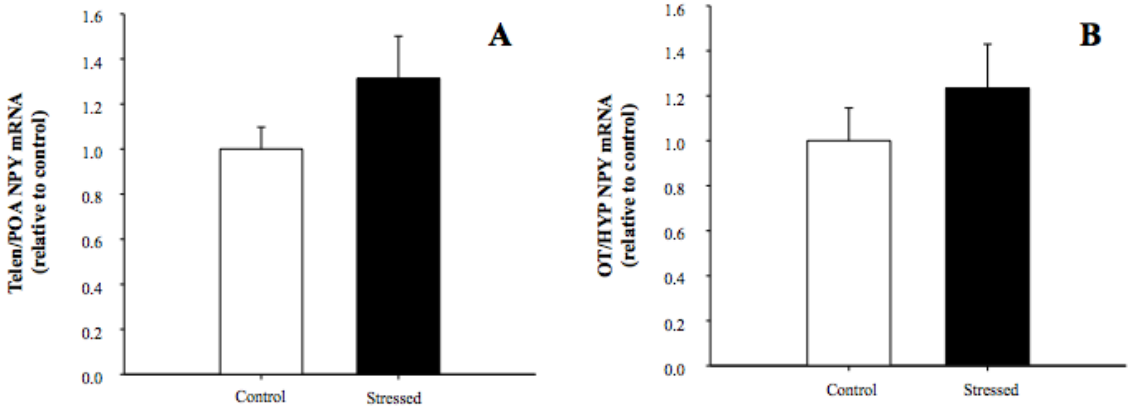

Figure 7. Effect of acute stress on telencephalon/pre-optic area levels of NPY mRNA (A) and optic tectum/hypothalamus NPY mRNA (B). Vertical bars represent means ±SEM (*n* = 12).

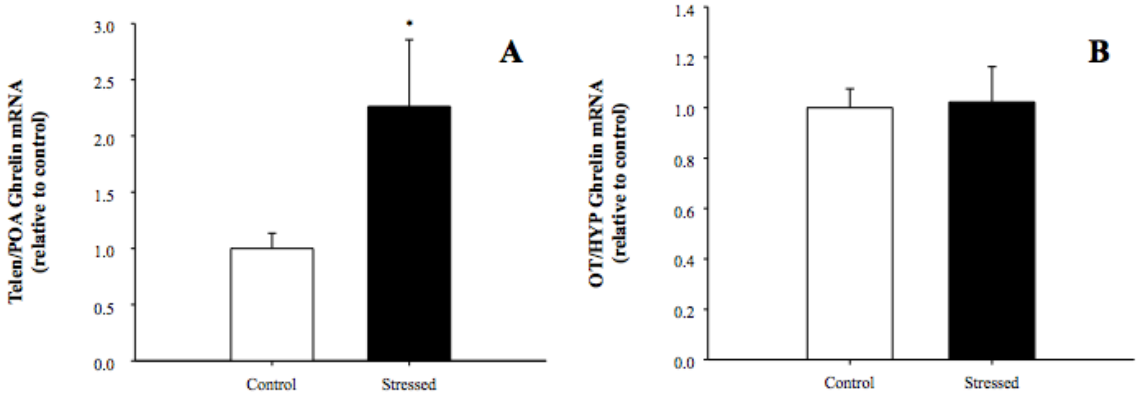

Figure 8. Effect of acute stress on telencephalon/pre-optic area levels of ghrelin mRNA (A) and optic tectum/hypothalamus ghrelin mRNA (B). Vertical bars represent means ±SEM (n = 12). *Significantly different from control at P<0.05 (one-way ANOVA).

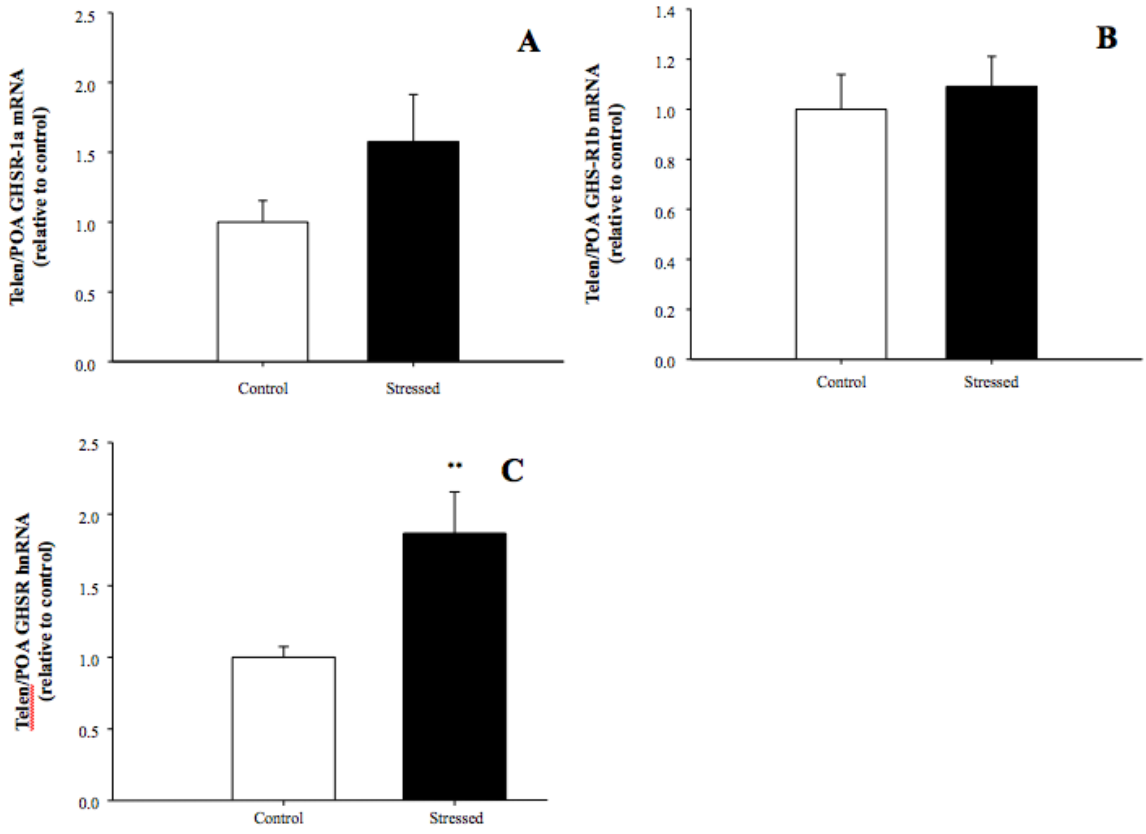

Figure 9. Effect of acute stress on telencephalon/pre-optic area levels of GHSR hnRNA (A), GHSR1a-LR mRNA (B) and GHSR1b-LR mRNA (C). Vertical bars represent means ±SEM (n = 12). **Significantly different from control at P<0.01 (one-way ANOVA).

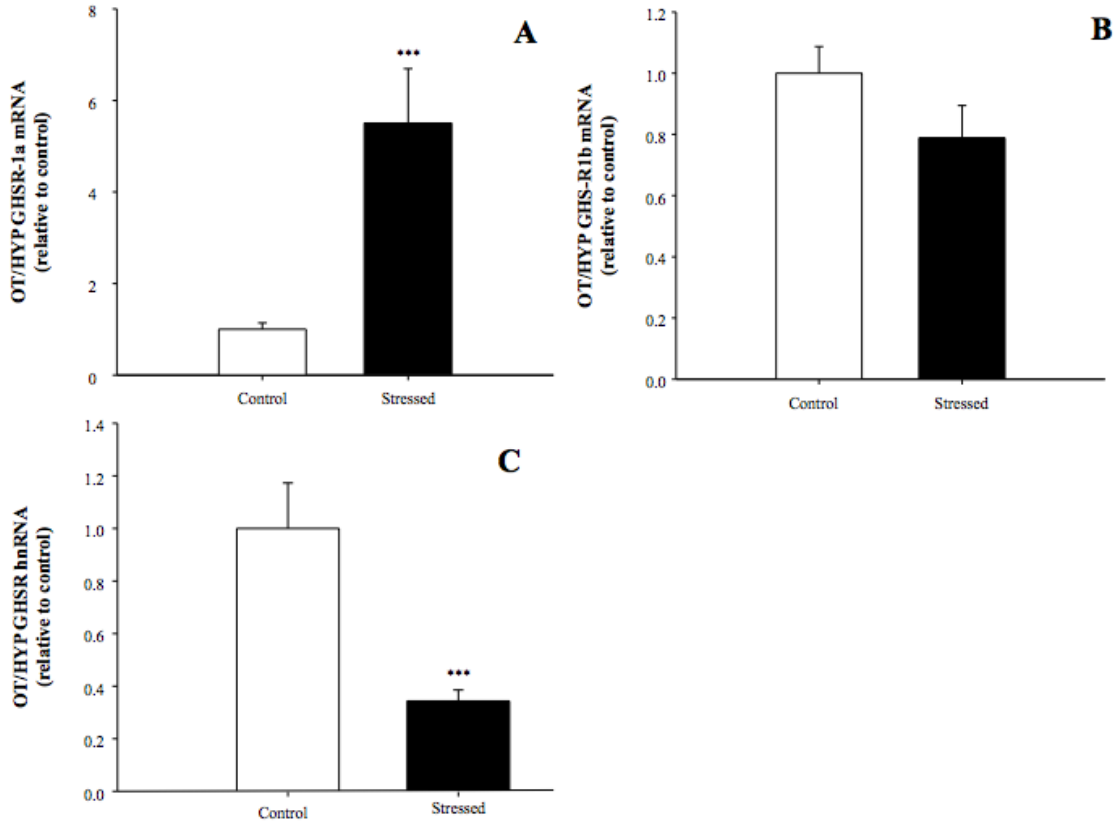

Figure 10. Effect of acute stress on optic tectum/hypothalamus levels of GHSR hnRNA (A), GHSR1a-LR mRNA (B) and GHSR1b-LR mRNA (C). Vertical bars represent means ±SEM (n = 12). **,***Significantly different from control at P<0.01 and P<0.001, respectively (one-way ANOVA).

Discussion

In the present study, immediate suppression of food intake followed an acute handling and crowding stress in tilapia. Similar findings have been shown in response to repeated once-daily acute stressors (1-5 min) after 17 d in Atlantic salmon (McCormick et al. 1998) and following 24 h exposure to hypoxic conditions in rainbow trout (Bernier and Craig 2005). However, only one other study measured food intake immediately following a stressor, showing a decrease after a 1 min restraint stress in brown trout (Pickering and Duston 1983). It is

unclear the mechanisms regulating these reductions in food intake following stress are unclear in fish.

Regulation of food intake during stress in mammals is fairly well characterized (Adams and Epel 2007). Increased food intake following tail pinch stress in rats, a model for stress-induced hyperphagia, is reversed with pre-treatment of CRH. Further, increases in food intake following tail pinch stress and NPY administration were potentiated with addition of CRH antagonist, suggesting CRH interacts with NPY and plays a role in regulating food intake and energy metabolism during stress (Heinrichs et al. 1992). There is some evidence that the roles of these appetite regulators are conserved in fish. Cortisol injections elevated NPY mRNA and suppressed CRH mRNA levels in the Telen/POA of goldfish (Bernier et al. 2004), resulting in an increase in food intake. On the other hand, both CRH and NPY expression was elevated in the POA of socially subordinate rainbow trout, indicating the involvement of a mechanism beyond cortisol in regulating signaling by these factors in response to social stress (Doyon et al. 2003). Although CRH and NPY mRNA levels were unchanged in the current study, it is likely that changes occurred at the protein level. Fish generally exhibit elevated CRH mRNA levels only after prolonged (e.g. subordinate stress) or severe physical stress (Huising et al. 2004; Doyon et al. 2005). Indeed, studies in the common carp have shown that CRH induces ACTH release from the pituitary almost immediately following a stressor, suggesting that stored protein is responsible for the initial stress response rather than altered gene expression (Huising et al. 2004). Significant CRH and NPY immunoreactivity has been localized to the telencephalon and optic tectum in tilapia (Pepels et al. 2004; Sakharkar et al. 2005) and optic tectum CRH has been implicated in behavioral modifications associated with stress in frogs, including feeding behavior (Carr et

al. 2006). Further, it has been long established that electric stimulation of the optic tectum induces feeding behavior in several fish species (Kulczykowska et al. 2010). However, our current findings do not indicate the reduction of food intake following stress is mediated by changes in either CRH or NPY expression, possibly a result of changes in the optic tectum masking changes in the hypothalamus.

Although ghrelin has been implicated as a regulator of behavioral responses to stress in mice (Asakawa et al. 2001), studies have not investigated this relationship in fish. Our lab has shown that following a 24 h cortisol injection, plasma and stomach ghrelin mRNA levels were significantly reduced (Janzen and Riley, 2011), suggesting that peripheral ghrelin regulates the appetite reducing effects of cortisol. Similar correlation of plasma levels of ghrelin and cortisol was also recognized in chronically stressed Atlantic salmon (Pankhurst et al. 2008). In the current study, acute stress did not alter plasma or stomach mRNA levels of ghrelin, indicating ghrelin is not playing a role in acute appetite regulation following a stress. However, an acute stress caused elevated ghrelin mRNA levels in the Telen/POA, which may have a role contributing to overall energy balance as suggested in mammals (Asakawa et al. 2005).

Ghrelin's actions are mediated in these areas by the active form of its receptor, GHS-R1a. However, alternate processing yields a second, assumedly non-functional transcript, GHS-R1b (Kaiya et al. 2008; Petersenn et al. 2001). We have previously identified both GHS-R-like receptor (GHSR-LR) transcripts in tilapia (Fox et al. 2007; Kaiya et al. 2009). Recently, Kaiya et al. (2009) determined tissue distribution of GHSR1a-LR and GHSR1b-LR in tilapia and found that GHSR1a-LR is expressed in significantly higher amounts than GHSR1b-LR in all tissues assayed, but expression levels of both transcripts are at

highest levels in the brain. We have shown previously that brain levels of GHSR1b-LR mRNA were altered during fasting in tilapia, though GHSR1a-LR levels remained unchanged (Riley et al. 2008), suggesting that GHSR1b-LR may exhibit some biological function in tilapia. It is proposed that GHS-R1b acts as a dominant negative by forming a heterodimer with GHS-R1a, rendering it biologically inactive (Leung et al. 2007). In support of this idea, black seabream GHS-R1b has been noted to impair GHS-R1a function when co-expressed in HEK293 cells (Chan et al. 2004). The GHS-R gene is first translated to heteronuclear RNA, which has a very short half-life and is spliced immediately to either GHS-R1a or GHS-R1b and can therefore act as a more sensitive indicator of activation (Goto et al. 2006). Significant decreases in GHSR hnRNA and increases in GHS-R1a transcript were detected in the OT/HYP in response to stress in the current study, suggesting an increase in conversion of GHS-R hnRNA to GHS-R1a mRNA. The significant increase in GHSR hnRNA in the Telen/POA exhibits distinctly alternative regulation from the OT/HYP and therefore may indicate different functions of ghrelin signaling in this region. Further, no change in either form of receptor mRNA was observed in this region, although it is unclear whether the mRNA levels reflect GHSR hnRNA processing or mRNA turnover. The absence of a difference in plasma cortisol levels between the stressed and unstressed fish is likely attributable to the almost immediate cortisol response documented in tilapia. Balm et al. (1994) showed the cortisol response to handling in tilapia peaks after only 5 min and therefore may be difficult to use as the ‘stress indicator’ for this species. However, the cortisol levels in both groups indicate stressed levels, considering basal levels in this species have been reported to range from 20-60ng/μl (Balm et al. 1994; Vijayan et al. 1997). Although a lethal dose of MS-222 has been deemed a viable option for obtaining unbiased cortisol levels in

trout (Pirhonen and Schreck 2002), it has been shown to elevate cortisol to stressed levels when used as an anesthetic in the hybrid striped bass (Davis et al. 2004). Our results indicate that our dose (100mg/L) may not have been sufficient as a lethal dose, resulting in the elevated plasma cortisol levels in our control fish. The same dose was previously used as a lethal dose in the same species of tilapia, although the fish were considerably larger (223 ± 8 g; Vijayan et al. 1997) than the fish (80 ± 5 g) used in the current study, indicating differences in dose-effectiveness relative to fish size. Plasma glucose levels were elevated following stress in the present study, similar to the pattern following a confinement stress in the same species (Vijayan et al. 1997). Glucose may be a better indicator of stress in this species because of its slow clearance rate (Riley et al. 2009). Although, any changes in glucose evoked by the stressor could be mediated not only by cortisol, but also by catecholamines. Studies in fish have shown that the adrenergic stress response is only evoked in certain severe or chronic situations; especially those that induce reduced blood oxygen content (Reid et al. 1998). Due to the hypoxic nature of the stress protocol implemented in the current study, it likely induced what Perry and Bernier (1999) consider acute severe stress, therefore warranting further study of these pathways in tilapia.

In summary, an acute severe stress decreased food intake in tilapia. This reduction in food intake is not reflected by changes in CRH or NPY mRNA levels in Telen/POA and OT/HYP, although regulation may take place at the protein level or via the sympathetic nervous system. The increase in ghrelin regulatory activity suggests a function for ghrelin in regulating overall metabolism in response to stress in different regions of the brain, as proposed in rats by Patterson et al. (2010). Though central injection has not been attempted in tilapia, long-term ghrelin treatment has been shown to increase food intake (Riley et al. 2005),

suggesting an orexigenic role for this species, therefore the increase in ghrelin activity may indicate a role in stress recovery. Tilapia are noted for their uniquely rapid and adaptive stress response (Basu 1965). This quick restoration of homeostasis may be the result of an early onset of restraining forces to counteract the costs of the general stress response.

CHAPTER 3: ROLE FOR CORTISOL IN MEDIATING STRESS-INDUCED REDUCTION OF FOOD INTAKE IN AN ADVANCED TELEOST, *OREOCHROMIS MOSSAMBICUS*

Food intake is an important component of an animal's overall energy metabolism, which is highly regulated by the integration of signals from central and peripheral origins (Laferrere et al. 2006). However, the major site of neuroendocrine control of appetite is the hypothalamus, which integrates opposing signals of anorexigenic (e.g. CRH) and orexigenic (e.g. NPY, ghrelin) factors (Crespi et al. 2004). Though the actions of these factors are fairly conserved across vertebrates, how these factors are regulated in response to stress varies among vertebrate lineages.

Previous studies in mammals point to cortisol as a mediator of the effects on food intake following a stressor. Glucocorticoids decrease CRH activity and release from the hypothalamus resulting in an increase NPY activity and subsequently in an increase in food intake (Krysiak et al. 1999). Indeed, adrenalectomy in rats inhibits the reduction in food intake by CRH (de Pedro et al. 1997) and inhibits stimulation of food intake by NPY (Krysiak et al. 1999), indicating a critical role for corticosteroids in regulating the neurohormonal control of food intake. While a relationship has been established between circulating levels of cortisol and ghrelin in humans (Otto et al. 2004; Azzam et al. 2010) and ghrelin's anxiety inducing effects are reduced by CRH antagonist in mice (Asakawa et al. 2001), very little is understood about interactions between ghrelin and the stress axis in any species.

The mechanisms regulating food intake following stress in non-mammalian vertebrates are less well understood. However, cortisol has also been implicated as the mediator of appetite-suppressing effects of stress in fish (Bernier et al. 2001).

Treatment with cortisol reduced food intake in rainbow trout (Gregory and Wood 1999) and channel catfish (Peterson et al. 2005). Conversely, goldfish exhibit increased food intake following low-dose cortisol (50 g/kg) treatment (Bernier et al. 2004). Metyrapone, a cortisol synthesis inhibitor, has been used to successfully inhibit the actions of corticosteroids in a number of vertebrates (Janssens 1967; de Pedro et al. 1997; Milligan 1997), including fish (Bernier and Peter, 2001).

In the current study, we assessed the effect of inhibiting cortisol synthesis using metyrapone on the regulation of food intake during an acute handling and crowding stress in tilapia. Tilapia are an economically important species of teleost that, as we previously demonstrated, exhibit the characteristic decrease in food intake following stress observed in other teleosts.

Materials and Methods

Animals

Male and female tilapia (*Oreochromis mossambicus*) weighing 85-115 gm, were reared in freshwater semi-recirculating tanks maintained at 25°C with simulated photoperiod (14h:10h; light:dark). Experimental tanks (46 L) on the same system were separated with opaque dividers so that treatments could be administered without affecting fish in adjacent tanks. Fish were fed a known amount of excess feed (Silvercup, Murray, UT) twice daily at 1000 and 1700 h for 2 weeks prior to experimental protocol. To entrain feeding behavior, fish were allowed to feed for 1 h at each feeding period, after which time excess food was removed. The experiment commenced once all tanks were consuming similar amounts of feed.

Metyrapone Experiments

To investigate the effect of metyrapone on food intake during acute stress, metyrapone (Enzo, Plymouth Meeting, PA) was administered via feed to avoid handling stress associated with injections (Bennett and Rhodes 1985). Three doses (10, 25, and 50 mg/kg BW) of metyrapone were each dissolved in 10 ml 95% ethanol before being sprayed onto the feed and allowed to dry overnight before use in the experiment. 10 ml 95% ethanol was sprayed on the control feed. Fish were fed the treated feed (as described above) for 1 wk prior to a 30 min crowding and handling stress. Fish were not fed 24 h prior to the experiment so that an accurate measure of food intake could be determined. This procedure is based on observations by Fox et al. (2009), which demonstrated that tilapia empty their stomach within 14 h of eating. Replicate tanks were randomly assigned to receive control or 10, 25, or 50mg/kg metyrapone-treated feed. On the day of the experiment at 0930 h, water levels were lowered in stress and metyrapone treated tanks such that dorsal fins were exposed. In addition, these fish were netted and held out of the water for 10 s every 5 min for a 30-min period. This protocol subjected the fish to an air emersion and crowding stressor, as well as simulated handling stressors that take place in aquaculture settings (Belanger et al. 2001). At 1000 h, the water level was returned to 46 L and all fish (control and stress groups) were fed a known amount of excess feed and allowed to feed for 1 h. Following feeding, fish were sacrificed by rapid decapitation, weighed, and stomach content was removed and dried at 72˚C for 28 h. Dried stomach contents were weighed and grams of dried individual stomach content was normalized to grams body weight of each fish (Fox et al. 2009).

To investigate the effect of metyrapone on the neuroendocrine regulators of appetite during acute stress, the above experiment was replicated with

modifications. Following the stressor at 1000 h, water levels were raised to 46 L and all fish (control and stress groups) were immediately sampled in order to measure pre-prandial changes in response to stress that may regulate food intake. Sodium bicarbonate-buffered (200mg/l) tricaine methanesulfonate (100mg/l) was added directly to the tanks to inhibit a stress response during subsequent handling (Vijayan et al. 1997). Blood samples were taken from caudal vasculature using Na^{+}-heparin (200U/ml; Sigma) coated syringes. Plasma was isolated from whole blood by centrifugation at 10K × g for 10 min at 4°C, then stored at -20°C until analysis of cortisol, ghrelin and glucose levels. Immediately after blood sampling, fish were rapidly decapitated. Brains from each fish were extracted and two regions of interest were isolated by microscopic dissection: the telencephalon/pre-optic area (Telen/POA) and hypothalamic (HYP) regions (Fig. 11). These sections were placed in TRI-Reagent (Ambion), snap frozen in liquid nitrogen, and stored at -80°C until analysis of mRNA levels of ghrelin, GHSR-LR isoforms (GHSR1a-LR and GHSR1b-LR), heteronuclear GHSR (hn-GHSR), NPY, and CRH by quantitative real-time PCR (qPCR). Stomach was collected for measurement of ghrelin mRNA levels.

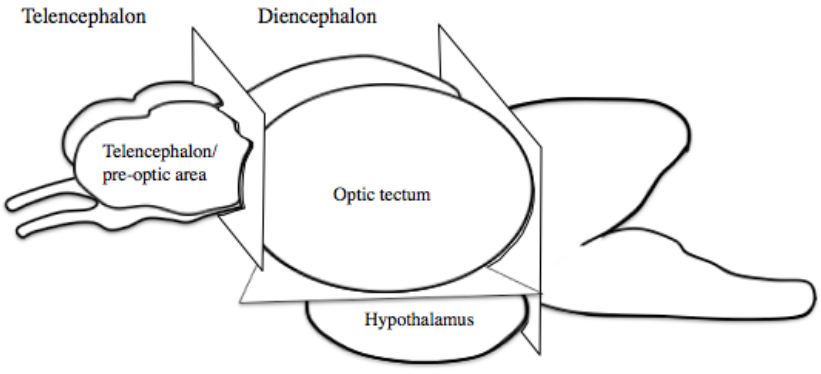

Figure 11. Diagram of tilapia brain with regions of interest labeled.

RNA Extraction, cDNA Synthesis and qPCR

Total RNA was extracted from tissue samples using TRI Reagent (Ambion) and diluted to 200 ng/µl. Subsequent cDNA synthesis was carried out using High Capacity cDNA Synthesis Kit (Applied Biosystems). A cDNA sample was run with no reverse transcriptase as a control for genomic contamination. cDNA was amplified by quantitative PCR (qPCR), for which each reactions reaction contained 7.5 µl of Power SYBR Green (ghrelin, NPY, CRH) or TaqMan Master Mix and probe (GHSR1a-LR, GHSR1b-LR, hn-GHS-R), along with 0.3 µl of appropriate gene specific primers (see Table 1, p. 13), and 3 µl of cDNA sample or standard (15 µl total volume). Standards were produced from serial dilutions of purified plasmid DNA for each mRNA of interest. A no-template control was included to check for contamination. All qPCRs were run on the 7300 Real Time PCR System (Applied Biosystems). Data was normalized to total RNA added to each qPCR reaction and expressed as relative to control (Picha et al. 2008).

Plasma Analysis

Plasma ghrelin levels were measured using a ghrelin radioimmunoassay established by Riley et al. (2008). One hundred microliters of rat ghrelin standards and plasma samples were incubated with 200 µl anti-rat ghrelin (from H. Hosada) at a dilution of 1:750,000. After incubation at 4°C overnight, 100 µl of ^{125}I-human ghrelin (Millipore) was added before an additional overnight incubation at 4°C. Finally, 100 µl anti-rabbit IgG goat serum at 1:35 (with 10% polyethylene glycol) was added, incubated for 2 h at room temperature, and then centrifuged at 3000 × g for 30 min to separate free and bound tracers. Radioactivity of aspirated pellet was then quantified using a gamma counter (Packard, Palo Alto, CA).

Plasma cortisol radioimmunoassay was carried out using commercially available ImmuChem cortisol ^{125}I kit (MP Biomedicals).

Plasma glucose levels were measured using the Infinity Glucose reagent (ThermoElectron). Two microliters of glucose standards (10, 5, 2.5 1.5 and 0 mg/ml) and plasma samples were added to a 96-well plate along with 300 µl of assay reagent. The plate was then incubated at room temperature for 5 min before subsequent reading at 340 nm (ELx800 plate reader, Biotek).

Statistical Analysis

Statistical analyses were carried out using one-way ANOVA (analysis of variance) tests (STATISTICA, Statsoft). Significant treatment means were found using a Fischer least common difference test. Data are expressed as means ± S.E.M. Differences were considered significant at $P<0.05$.

Results

Mirroring our previous experimental results, stressed fish exhibited reduced ($P<0.01$) food intake when compared with unstressed controls. Metyrapone treatment dose-dependently blocked the stress-induced reduction in food intake, with the 25 and 50 mg/kg doses reversing the negative effect of stress on food intake ($P < 0.05$ and 0.001, respectively; Fig. 12). There was no difference in plasma cortisol levels between control and stressed groups, although the 10 and 25 mg/kg metyrapone treatments resulted in higher cortisol levels than unstressed controls ($P<0.01$; Fig. 13A). There was, however, an increase ($P<0.001$) in plasma glucose levels between control and stressed fish, suggesting fish were sufficiently stressed. Although, elevated levels of glucose persisted in all metyrapone treatments ($P<0.001$; Fig. 13B), suggesting activation of the sympathetic nervous system.

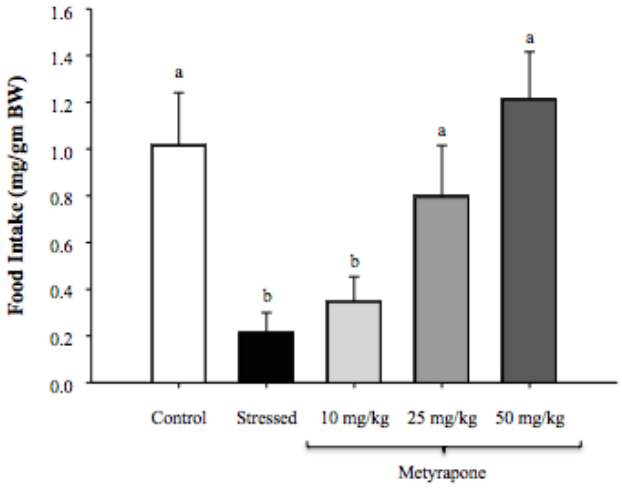

Figure 12. Effect of metyrapone on food intake following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at P<0.05.

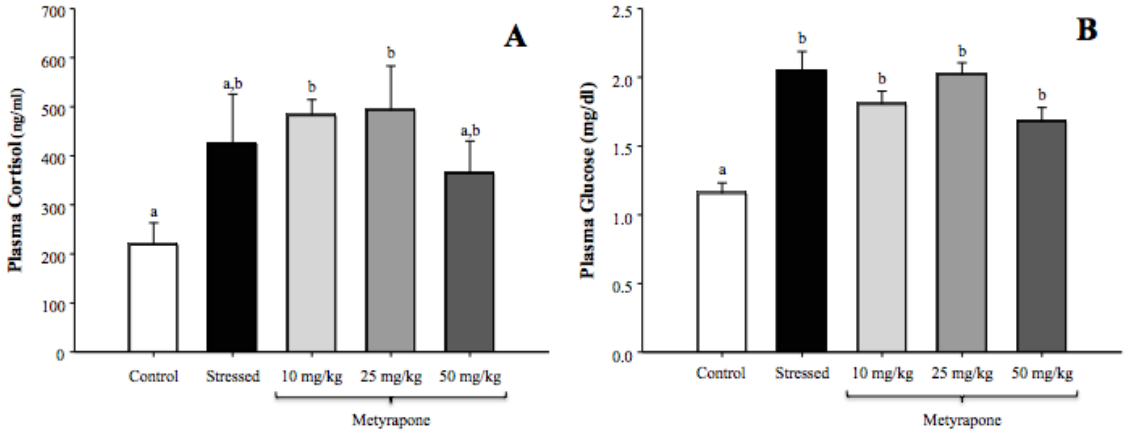

Figure 13. Effect of metyrapone on plasma cortisol levels (A) and plasma glucose levels (B) following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at P<0.05.

Telen/POA CRH mRNA levels were unchanged between stress and control groups. Interestingly, CRH mRNA levels were reduced in the 25 mg/kg metyrapone treatment compared with control (P<0.01) and stressed (P<0.05) groups (Fig. 14A). CRH mRNA levels in the HYP were elevated (P<0.001) in response to the stressor and reversed to unstressed levels with all metyrapone doses (Fig. 14B), suggesting a role for CRH following stress in the hypothalamus. Conversely, Telen/POA NPY mRNA levels were elevated (P<0.01) in the stressed and 10 and 25 mg/kg metyrapone treatments compared with controls. NPY mRNA levels returned to control levels following treatment with the 50 mg/kg dose (Fig. 15A). There was no change in NPY mRNA levels in the HYP between control and stressed fish, suggesting a role for NPY in the telencephalon following stress. In addition, there was no clear effect of metyrapone treatment on NPY mRNA levels, although the 10 mg/kg metyrapone treatment lowered (P<0.01) NPY mRNA levels compared with stressed fish (Fig. 15B).

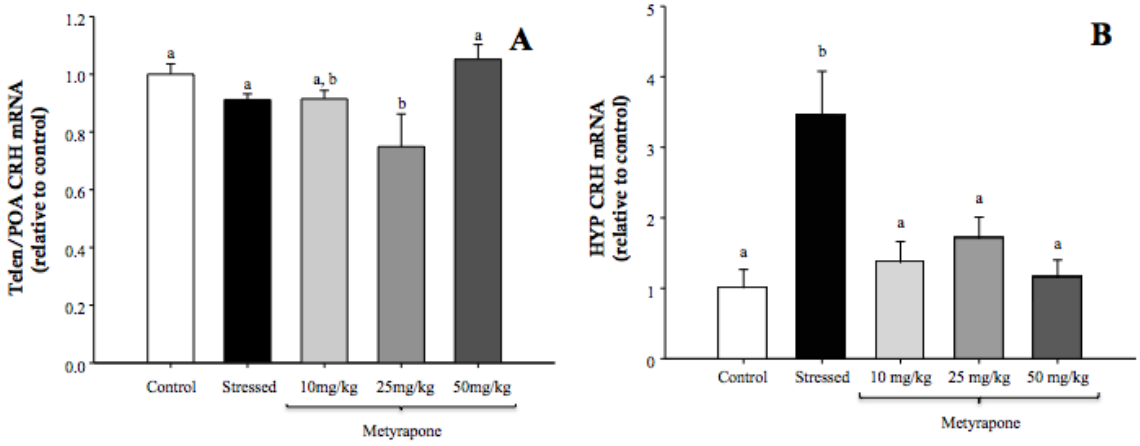

Figure 14. Effect of metyrapone on telencephalon/pre-optic area levels of CRH mRNA (A) and hypothalamic CRH mRNA (B) following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at P<0.05.

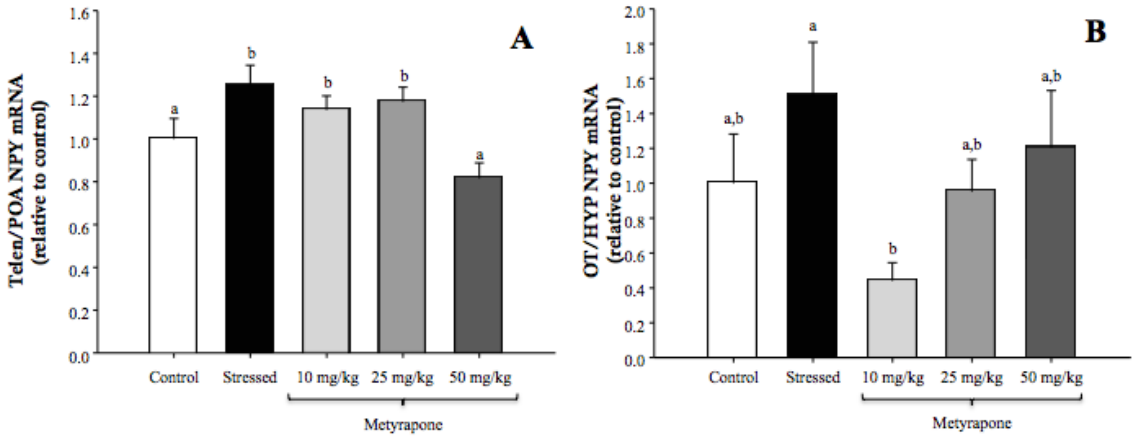

Figure 15. Effect of metyrapone on telencephalon/pre-optic area levels of NPY mRNA (A) and hypothalamic NPY mRNA (B) following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at P<0.05.

Ghrelin mRNA levels were unaltered in response to both stress and metyrapone treatment in both the Telen/POA and HYP (Figs. 16A and 16B, respectively), which does not support a role for ghrelin in regulating food intake following stress. However, in the Telen/POA, GHSR hnRNA levels were elevated (P<0.001) in stressed animals as well as in all metyrapone treated fish (P<0.05) compared with controls (Fig. 17A), suggesting that regulation of ghrelin activity is rapidly altered following stress and appears not to be mediated by cortisol in this region. GHSR1a-LR mRNA levels were not different between control and stressed fish, but the 10 mg/kg metyrapone treatment increased (P<0.05) mRNA levels compared with control fish (Fig. 17B). While these data do not support a role for GHSR1a-LR in the Telen/POA, there was a clear pattern of GHSR1b-LR regulation in response to stress, with GHSR1b-LR mRNA levels elevated (P<0.001) in stressed fish. Further, metyrapone treatment dose-dependently reduced mRNA to control levels (P<0.01; Fig. 17C), suggesting cortisol plays a role in mediating this response. Similar to the Telen/POA, GHSR hnRNA levels in

the HYP were elevated (P<0.01) in stressed fish. The 10 and 50 mg/kg metyrapone treatments returned GHSR hnRNA to control levels, while the 25 mg/kg treatment was ineffective in lowering hnRNA levels (Fig. 18A). These results indicate that regulation of ghrelin activity is rapidly altered following stress in the hypothalamus, although it appears cortisol may be involved in mediating this response in this region. GHSR1a-LR and GHSR1b-LR mRNA levels in the HYP were unaltered by stress or metyrapone treatment when compared with controls, although the 50 mg/kg metyrapone treatment lowered (P<0.05) both mRNA levels relative to the stressed group (18B and 18C, respectively), showing no clear role for these receptor isoforms in this region.

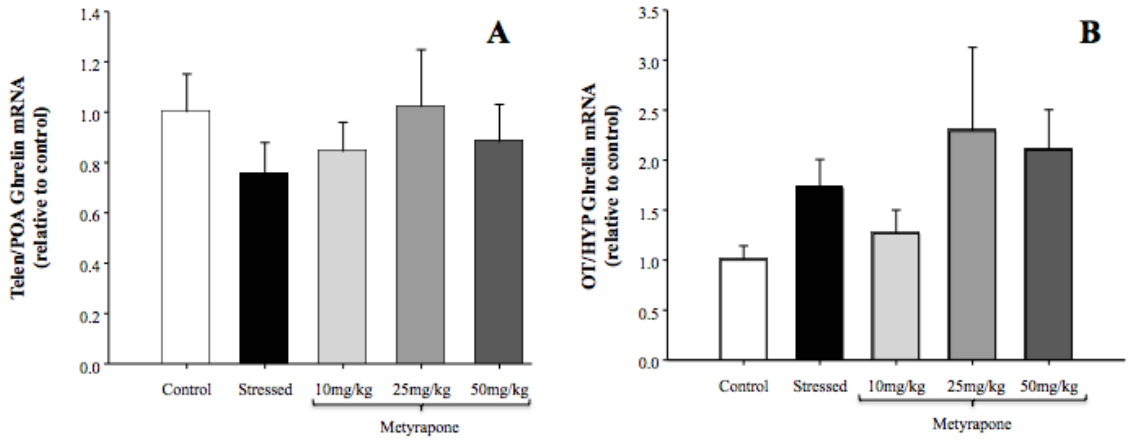

Figure 16. Effect of metyrapone on telencephalon/pre-optic area levels of ghrelin mRNA (A) and hypothalamic ghrelin mRNA (B) following acute stress. Vertical bars represent means ±SEM (n = 10).

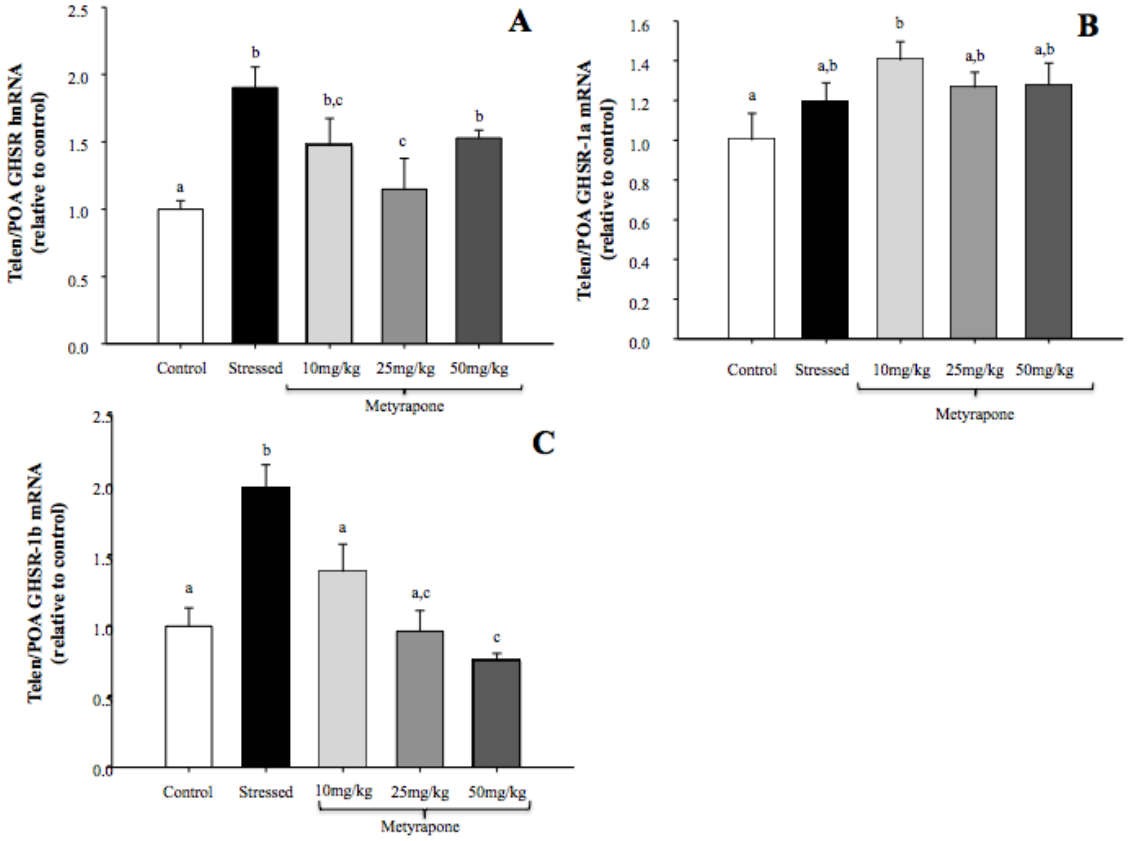

Figure 17. Effect of metyrapone on telencephalon/pre-optic area levels of GHSR hnRNA (A), GHSR1a-LR mRNA (B) and GHSR1b-LR mRNA (C) following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at $P<0.05$.

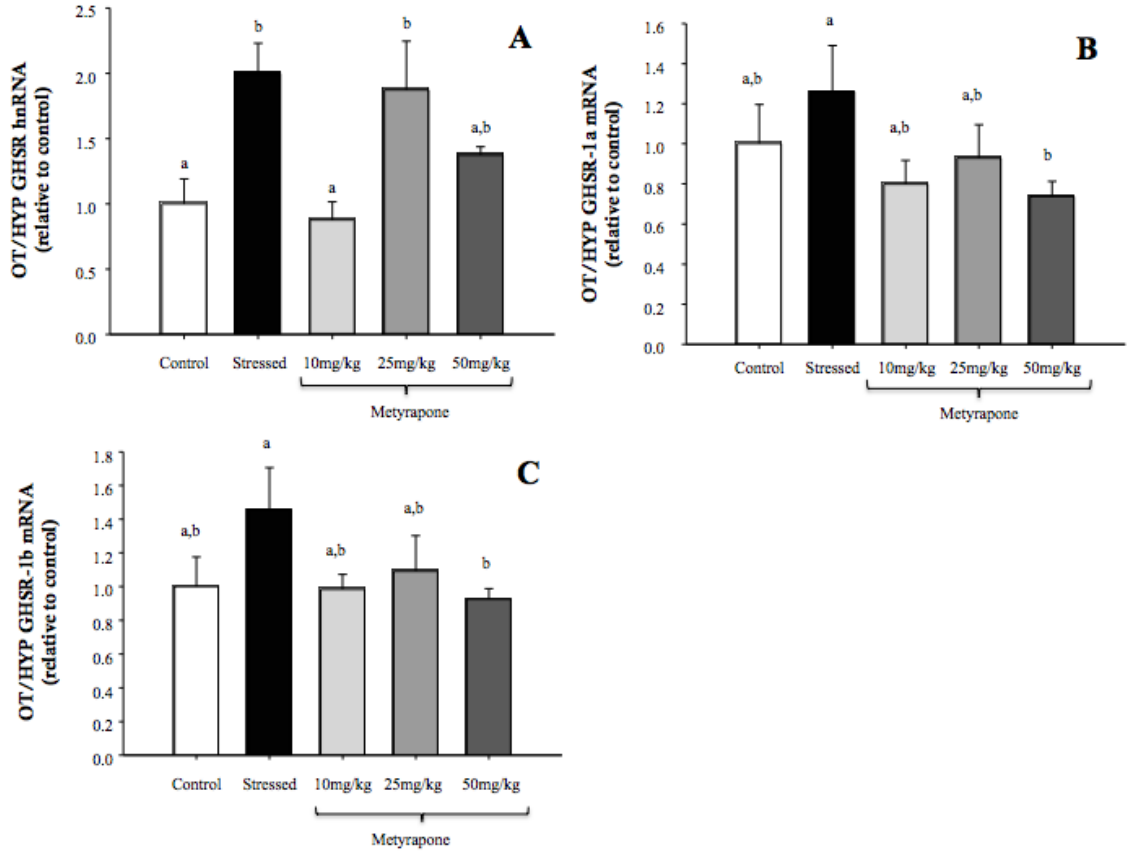

Figure 18. Effect of metyrapone on hypothalamic levels of GHSR hnRNA (A), GHSR1a-LR mRNA (B) and GHSR1b-LR mRNA (C) following acute stress. Vertical bars represent means ±SEM (n = 10). Vertical bars not sharing a letter differ significantly at $P<0.05$.

Discussion

The current study showed a decrease in food intake following an acute handling and crowding stressor, an effect that we previously demonstrated in this species and that conforms to an established paradigm in fish (Bernier et al. 2006). Additionally, metyrapone treatment dose-dependently blocked the stress-induced reduction in food intake, with the 25 and 50 mg/kg doses significantly reversing the negative effect of stress on food intake. Metyrapone has also been used to reverse a corticosterone-induced increase in food intake in frogs (Yao et al. 2008). These data suggest that cortisol synthesis was successfully reduced by metyrapone

treatment and that cortisol is, at least in part, mediating the decrease in food intake following an acute stress in this species.

An increase in CRH mRNA levels in the HYP was accompanied by a decrease in food intake in stressed fish, suggesting this highly conserved anorexigenic peptide is mediating the observed decrease in food intake. This is in contrast to our previous study, in which no change was observed in CRH mRNA levels in the OT/HYP between control and stressed fish. However, the section sampled in the first study included the optic tectum and changes in this region may have been masking the effects observed in the second study. Indeed, significant CRH and NPY immunoreactivity has been localized to the optic tectum in tilapia (Pepels et al. 2004; Sakharkar et al. 2005). Interestingly, there was an increase in NPY and no change in CRH mRNA levels in Telen/POA following stress. This conflicts with previous studies in goldfish, where cortisol injection caused no change HYP mRNA levels, but did increase Telen/POA NPY and decrease CRH mRNA levels, resulting in an increase in food intake (Bernier 1999) similar to mammals (Krysiak et al. 1999). In addition, CRH and NPY mRNA levels in the Telen/POA were significantly elevated in socially subordinate rainbow trout. However, these animals were subjected to a chronic stressor and elevations in CRH mRNA in this region of the brain may be playing a role in maintaining the HPI axis over a long period of time (Doyon et al. 2003). The same study showed elevated Telen/POA NPY mRNA levels in subordinate fish, which was proposed to play a role in regulating food intake (Doyon et al. 2003). The stressed fish in the current study exhibited this same elevation and food intake was also affected, further supporting a role for NPY in food intake in this region.

Cortisol has been proposed as an important mediator of food intake during stress in fish (de Pedro et al. 1997). In the current study, all metyrapone treatments

reversed the stress induced increase in HYP CRH mRNA to unstressed levels, suggesting that cortisol mediates the decrease in food intake following stress through an increase in CRH mRNA levels in the HYP. Interestingly, in goldfish stressed levels of plasma cortisol are suggested to decrease CRH expression in the telencephalon, resulting in an increase in food intake (de Pedro et al. 1997). Similarly, corticosterone treatment reduced CRH, while metyrapone increased CRH in the pre-optic area of frogs (Yao et al. 2008). Together with our results, these data suggest that cortisol's effect on mediating CRH activity is regionally specific in the brain (Bernier et al. 1999) as well as lineage-specific.

Although our previous study showed an increase in Telen/POA ghrelin mRNA levels in response to stress, there were no changes in Telen/POA or HYP regions in this study in response to stress or metyrapone treatment. These differing responses between studies suggest ghrelin may regulate overall energy metabolism rather than food intake during acute stress and may reflect the differences in fish size and/or tank density used in the studies. The same conclusions can be drawn from the GHSR-LR isoform data. There were different responses in the Telen/POA region between studies and no clear effect of metyrapone treatment, suggesting they are not being regulated by cortisol following acute stress. However, the difference in regulation in the HYP region in the current study may also be due to the elimination of the optic tectum region. Although cortisol does not seem to mediate the effects on ghrelin regulation following acute stress, the rapid changes in both GHSR hnRNA and GHSR-LR isoforms in both the Telen/POA and HYP in response to acute stress suggest that acute stress alters ghrelin signaling in the brain. Currently, it is not clear what role ghrelin is playing following an acute stressor.

Plasma cortisol levels were not elevated in stressed fish compared to controls, which may result from handling stress during sampling following treatments. It has been shown in tilapia that the cortisol response takes occurs within 5 min (Balm et al. 1994; Pepels et al. 2004). However, elevated plasma glucose levels in response to the stressor demonstrate the efficiency of the stressor. Interestingly, cortisol levels were also elevated in metyrapone-treated fish. Similar results have been shown in rat and it is suggested to be a result of metyrapone not specifically being a cortisol synthesis inhibitor, but rather a general blocker of P450s, which are involved in variety of metabolic reactions, and may be inducing a pharmacological stress (Rotllant et al. 2002). Therefore, further study with an alternative treatment, such as RU486, which acts as a glucocorticoid receptor antagonist, should be carried out to further elucidate the role of cortisol in the stress-induced reduction of food intake observed in the current study. Additionally, plasma glucose levels were elevated to stress levels in all metyrapone treatments, which may suggest activation of the sympathetic nervous system in response to acute stress.

In summary, stress elevated CRH mRNA levels in the HYP while reducing food intake. Additionally, metyrapone treatment dose-dependently blocked the negative effects of stress on food intake. The elevated CRH mRNA levels were also reversed by all metyrapone treatments, suggesting that cortisol's negative effects on food intake are mediated by elevated CRH activity in the HYP during stress in tilapia. Changes in ghrelin following a stress do not seem to be mediated by cortisol, though rapid elevation of GHSR hnRNA and and GHSR-LR isoforms in both the Telen/POA and HYPO in response to acute stress nonetheless suggest an important role for ghrelin following an acute stress response. Further studies

manipulating the stress response are suggested to elucidate the role of cortisol in mediating the reduction of food intake following stress in tilapia.

CHAPTER 4: CONCLUSION

These studies have shown that tilapia exhibit reduced food intake following a stress, adhering to a consistent pattern among fish (Bernier et al. 2006). These data have also demonstrated an important role for cortisol in mediating the effects of acute stress on food intake in a vertebrate of basal lineage, a role that seems to be a highly conserved from fish to mammals (Bernier et al. 2001; Adams and Epels 2007). Metyrapone treatment dose-dependently blocked the stress-induced reduction in food intake, reversing the effect of stress on food intake and an elevation of CRH mRNA levels in stressed animals was also reversed in all metyrapone treatments, suggesting that cortisol and CRH play a role in mediating the observed reduction in food intake during stress. The observed reduction in food intake was not reflected by changes in NPY and ghrelin mRNA levels in the OT/HYP, although regulation may take place at the protein level or via the sympathetic nervous system. However, consistent suppression of food intake and alternative elevation of Telen/POA mRNA levels of ghrelin in one study and NPY in a repeat of the study, suggest that these factors may regulate overall energy metabolism rather than food intake during acute stress and may reflect the differences in fish size and/or tank density used in the studies. The increase in ghrelin regulatory activity suggests a function for ghrelin in regulating overall metabolism in response to stress in different regions of the brain, as proposed in rats by Patterson et al. (2010). This is the first observation of a role for ghrelin regulation following stress in fish.

Because the protocol used in these studies appears to induce both the HPI axis as well as an adrenergic stress response, making it unclear whether the results are the outcome of the actions of cortisol, catecholamines, or both, further studies

will be necessary to elucidate the pathways leading to these results. Further attempts to block the HPI axis and sympathetic stress response should be made, the former possibly with adrenergic receptor antagonists, the latter with the glucocorticoid receptor antagonist, RU-486, which has been successfully used in goldfish (Bernier et al. 2001).

Although the generalized stress response is consistent throughout the vertebrate lineage, with most of the aforementioned neuroendocrine factors exhibiting highly conserved genetic sequences, the differential utilization in response to various environments is key to predicting how microevolution may take place in the future (Denver et al. 2009) and enhance our understanding of the effect of stress on energy metabolism in vertebrates and how these responses have evolved and adapted over time.

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