THE ROLES OF SOCIAL STATUS, MATERNAL STRESS, AND PARENTAL INVESTMENT IN MODULATION OF HYPOTHALAMIC-PITUITARY-INTERRENAL AXIS FUNCTION IN TELEOST FISH

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DEDICATION

During the last year of my undergrad, I lost an important mentor to whom I wish to dedicate this thesis. Boyd Jeffrey was never a scientist but was enthusiastic and encouraged my interest in all things biology. Most of all he was a great dad, who supported and pushed me to pursue and accomplish my goals.

ABSTRACT

In teleost fish, the main glucocorticoid stress hormone, cortisol, is released via the hypothalamic-pituitary-interrenal (HPI) axis. This thesis examined the premise that social status, maternal experience, and reproductive status affect HPI axis function in fish. Social stress in juvenile rainbow trout (*Oncorhynchus mykiss*) chronically elevates circulating cortisol levels. In this thesis, social subordinance as well as exogenous cortisol treatment resulted in decreased plasma adrenocorticotropic hormone (ACTH) levels, consistent with a negative feedback role of cortisol in modulating HPI axis activity. At the target tissue level, liver glucocorticoid receptor 2 (GR2) mRNA and total GR protein levels were lower in subordinate fish. Although subordinate fish exhibited elevated resting cortisol levels, cortisol and glucose responses to an acute stressor were attenuated. Using an *in vitro* head kidney preparation, this attenuated cortisol response was attributed to lower ACTH-stimulated production of cortisol. By contrast, dominant status regulated genes associated with cortisol biosynthesis. The consequences of maternal social status on offspring HPI axis function were investigated in zebrafish (*Danio rerio*). At 48 hours post-fertilization (hpf), when *de novo* cortisol synthesis becomes possible, larvae of dominant females exhibited lower baseline cortisol levels accompanied by lower mRNA levels of corticotropin-releasing factor and cytochrome P450 side chain cleavage enzyme. Offspring of subordinate females exhibited attenuated stress-induced levels of cortisol at 144 hpf, perhaps as an adaptive response to maternal stress experience. Finally, modulation of HPI axis function was explored as a mechanism underlying attenuation

of the stress response during early paternal care in smallmouth bass (*Micropterus dolomieu*). In response to a stressor, males guarding free-swimming fry but not eggs elevated plasma ACTH and cortisol as well as mRNA levels of key HPI axis genes. These results point to a hypoactive HPI axis in males during early parental care as a mechanism for resistance to stress in these fish. Collectively, the results of this thesis emphasize the adaptive plasticity of the HPI axis. Activity of the HPI axis in teleost fish can be modulated by the individual's experience (e.g., social status) or circumstances (e.g., parental care), as well as by maternal stress.

RÉSUMÉ

Chez les téléostéens, le cortisol, principale hormone glucocorticoïde de stress, est secrété comme produit final de l'axe hypothalamo-pituitaire-interrénal (HPI). Cette thèse explore la prémisse que le statut social, l'expérience maternelle et le statut reproductif d'un poisson affectent le fonctionnement de l'axe HPI. Le stress social chez les truites arc-en-ciel juvéniles (*Oncorhynchus mykiss*) résulte en une élévation chronique des niveaux de cortisol en circulation. Cette thèse révèle que la subordination sociale et le traitement avec du cortisol entraina une réduction de l'hormone adrénocorticotrope (ACTH), en accord avec un rôle de rétrocontrôle négatif du cortisol sur l'activité de l'axe HPI. Au niveau des tissus cibles, l'ARNm du récepteur glucocorticoïde 2 (GR2) et le niveau total de protéine GR furent réduits dans le foie des poissons subordonnés. Bien que les poissons subordonnés démontrèrent une élévation du cortisol basale, leurs niveaux de cortisol et de glucose en réponse à un stress aigu furent réduits. De plus, l'utilisation d'une préparation *in vitro* du rein antérieur révéla que cette atténuation était attribuable à une réduction de la production de cortisol stimulée par ACTH. Par ailleurs, le statut dominant fut associé à une régulation des gènes associés à la biosynthèse du cortisol. Les conséquences du statut social maternel sur l'axe HPI de leur progéniture furent étudiées chez le poisson zèbre (*Danio rerio*). A 48 heures post-fécondation (hpf), le stade où la synthèse de cortisol *de novo* est possible, les embryons de femelles dominantes démontrèrent une baisse des niveaux de cortisol de base, ainsi qu'une réduction de l'ARNm de corticolibérine et de l'enzyme cytochrome p450 responsable du clivage de la chaine latérale. La progéniture des

femelles subordonnées montra une atténuation de l'induction de cortisol à 144 hpf, ce qui pourrait révéler une réponse adaptive relative à l'expérience maternelle du stress. Finalement, la modulation de l'axe HPI fut explorée en tant que mécanisme causant l'atténuation de la réponse au stress lors du début des soins paternels chez l'achigan à petite bouche (*Micropterus dolomieu).* En réponse à un stress, les males durant la phase de garde des larves et non des oeufs élevèrent leurs niveaux plasmatique d'ACTH et cortisol, de même que les niveaux d'ARNm de gènes importants de l'axe HPI. Ces résultats suggèrent qu'une hypoactivité de l'axe HPI durant le début des soins paternels est un mécanisme de résistance au stress chez ces poissons. Collectivement, les résultats de la présente thèse mettent en évidence la plasticité adaptive de l'axe HPI. Chez les poissons téléostéens, l'axe HPI apparait donc modulable par l'expérience de l'individu (par ex. le statut social), les circonstances (par ex. les soins parentaux), ainsi que par le stress maternel.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
α-MSH	α-melanocyte-stimulating hormone
β-END	β-endorphin
β-LPH	β-lipotrophic hormone
µg	micrograms
µl	microlitres
5-HT	5-hydroxytryptamine
8-OH-DPAT	8-hydroxy-2-(di-*n*-propylamino)-tetralin
11β-HSD	11β-hydroxysteroid dehydrogenase
11β-hyd	11β-hydroxylase
AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
AVP	arginine vasopressin
AVT	arginine vasotocin
bp	base pairs
BSA	bovine serum albumin
bw	body weight
°C	degrees celsius
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CLIP	corticotropin-like intermediate lobe peptide
cm	centimetre
CNSS	caudal neurosecretory system
con	control
cort	cortisol
CpG	cytosine-phosphodiester-guanosine
CRF	corticotropin-releasing factor

CRF-BP	corticotropin-releasing factor binding protein
CRF-R	corticotropin-releasing factor receptor
CRH	corticotropin-releasing hormone
Ct	threshold cycle
d	days
DOC	11-deoxycorticosterone
dom	dominant
dpf	days post-fertilization
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphoshate
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme-linked immunoassay
elps	elipsoid
fast	fasted
g	grams
g	gravity
G	gauge
GR	glucocorticoid receptor
h	hours
HDL	high density lipoprotein
hpf	hours post-fertilization
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HPI	hypothalamic-pituitary-interrenal
HR	high stress responsiveness
IgG	immunoglobulin G
IR	interrenal
IST	isotocin
IU	international unit
kg	kilograms

L	litres
LDL	low density lipoprotein
LR	low stress responsiveness
m	metres
M	molar
MC2R	melanocortin 2 receptor
MEM	minimum essential media
min	minute
mg	milligrams
MG-FE	males guarding fresh eggs
MG-FSF	males guarding free-swimming fry
ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimole
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
N	sample size
ng	nanograms
P450scc	cytochrome P450 side chain cleavage enzyme
PC	prohormone convertase
PCR	polymerase chain reaction
PKA	protein kinase A
POA	preoptic area
POMC	proopiomelanocortin
PVC	polyvinyl chloride
PVN	paraventricular nucleus
RIA	radioimmunoassay
RM	repeated measures
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction

RU486	mifepristone
s	seconds
SDS–PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
StAR	steroidogenic acute regulatory protein
S-QPCR	semi-quantitative real-time reverse transcriptase polymerase chain reaction
sub	subordinate
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TRH	thyrotropin-releasing hormone
U	urotensin
Ucn	urocortin
V	volts

CHAPTER 1

General Introduction

The reciprocal relationship between an animal's behaviour and its physiology has been the subject of extensive research effort. Teleost fish provide an interesting and diverse taxon in which to study such questions, because teleost fish differ greatly in their social structures, reproductive behaviours, and physiology (reviewed by Johnsson et al., 2006; Oliveira, 2006). Importantly for the purpose of this thesis, the stress axis [hypothalamic-pituitary-interrenal (HPI) axis in teleost fish and hypothalamic-pituitary-adrenal (HPA) axis in tetrapods], which is responsible for the synthesis and release of glucocorticoid stress hormones, is well conserved across taxa and across species within a taxon. Although the stress axis itself is well conserved, increasingly, research suggests that activity of the stress axis can be modulated by a variety of factors. This thesis examined potential regulators of HPI axis function using two domesticated and one wild teleost species. Chapters 2 and 3 studied the effects of social status and social stress on HPI axis function at rest and following exposure to an acute stressor using juvenile rainbow trout (*Oncorhynchus mykiss*). Chapter 4 continued this work to the next generation by examining the role of maternal social status in modulating development of the HPI axis and the stress response in embryonic and larval zebrafish (*Danio rerio*). Finally, Chapter 5 investigated the possible mechanisms underlying attenuation of the stress response during early parental care in male smallmouth bass (*Micropterus dolomieu*). In each case, the impact of naturally-occurring stressors or aspects of a fish's life history on HPI axis function was studied with the aim of testing the general hypothesis that the HPI axis exhibits plasticity. This general introduction will briefly review the key concepts providing a framework for this research, including stress, the stress response, the HPI axis, and

cortisol synthesis, in addition to social hierarchies, maternal stress, and parental care, with a primary focus on teleost species.

1.1 Stress and the stress response in teleosts

The concept of stress has evolved over the past two and a half millennia, beginning with ideas conceptualized by Heracleitus and Hippocrates, and leading to more recent concepts defined by Walter Cannon and Hans Selye (reviewed by Chrousos and Gold, 1992). Historically, stress was defined as a condition in which intrinsic or extrinsic factors (i.e., stressors) threaten the maintenance of a dynamic equilibrium, or homeostasis, of an organism (reviewed by Chrousos and Gold, 1992; Wendelaar Bonga, 1997). Exposure to a stressor results in a stress response, that is, the suite of behavioural and physiological responses used by an organism to compensate for, or adapt to, a perceived threat. The acute stress response is thought to be adaptive; however, when stressors are severe or prolonged, the stress response becomes dysfunctional, and may result in an inhibition of growth, reproduction, and/or immune function (reviewed by Wendelaar Bonga, 1997). Although the concept of stress is based largely on mammalian studies, the teleost stress response shares a high degree of commonality with the mammalian stress response (reviewed by Wendelaar Bonga, 1997).

The integrated stress response is comprised of primary, secondary, and tertiary events (reviewed by Barton, 2002; Wendelaar Bonga, 1997). The primary response to a stressor involves release of catecholamines from chromaffin cells and activation of the HPI axis resulting in the increased synthesis and release of glucocorticoids from

steroidogenic interrenal cells. Catecholamine levels rise and peak within the first few minutes of exposure to the stressor, whereas glucocorticoid levels rise more slowly allowing for easier measurement of both resting and stress-induced levels compared to catecholamines. Release of catecholamine and glucocorticoid stress hormones leads to the secondary response, in which these hormones act at the tissue level to increase cardiac output and oxygen uptake, and mobilize energy resources. Tertiary responses, which seem largely to be restricted to situations where the stressor is prolonged or repeated, occur at the whole animal or population levels with potential consequences for survival, and include inhibition of growth, reproduction, and immune function as well as the impaired ability of an organism to respond to subsequent or additional stressors. Although the catecholamine response plays an important role in the integrated stress response, this thesis focuses on prolonged stressors (e.g., social stress), where glucocorticoids are likely to play a more important role than catecholamines (e.g., Sloman et al., 2001; Thomas and Gilmour, 2006). Thus, this general introduction will focus on mechanisms that control HPI axis synthesis and release of glucocorticoids during stress, and review the stress response from a teleost perspective, making comparisons to mammalian and avian systems.

1.2 Hypothalamic-pituitary-interrenal axis in teleost fish

Cortisol is the primary glucocorticoid in teleost fish and is synthesized as the end product of the HPI axis (Fig. 1.1A) (reviewed by Barton, 2002; Mommsen et al., 1999; Wendelaar Bonga, 1997). Activation of the HPI axis begins with the release of corticotropin-releasing factor (CRF) from the hypothalamus, which leads to

adrenocorticotropic hormone (ACTH) release from the pituitary. Unlike in mammals, teleost fish do not have a hypothalamic-pituitary portal vessel system; instead, CRF-releasing neurons, with their cell bodies in the preoptic area (POA) of the brain, extend to directly innervate corticotropes in the teleost pituitary (e.g., Anglade et al., 1993; Olivereau and Olivereau, 1988; Pepels et al., 2002; Peter et al., 1990; Zupanc et al., 1999). In most teleost fish, the CRF system consists of two G protein-coupled receptors for CRF, CRF-R1 and CRF-R2 [a third CRF receptor, CRF-R3, has also been identified in bullhead, *Ameiurus nebulosus* (Arai et al., 2001)], and a binding protein, CRF-BP (Alderman and Bernier, 2007; Alderman et al., 2008; Flik et al., 2006; Huising et al., 2004). Recently, two isoforms of CRF-R1 (CRF-R1.1 and CRF-R1.2) and CRF-R2 (CRF-R2.1 and CRF-R2.2) were found in common carp (Manuel et al., 2014). Manuel et al. (2014) suggested distinct functions for each receptor due to differences in their responsiveness to CRF and CRF-like peptides (see below). In mammals, CRF-BP plays an inhibitory role in HPA axis activation by limiting the bioavailability of CRF for its receptors (Potter et al., 1991) and CRF-BP appears to act similarly in fish (Manuel et al., 2014). Interestingly, carp CRF-BP inhibits CRF and urotensin-I (UI; see below) mediated actions on CRF receptors *in vitro*, and the extent of this inhibition is dependent on the receptor and ligand (Manuel et al., 2014)

Although CRF is considered to be the primary releasing-hormone for ACTH (Flik et al., 2006; Lederis et al., 1994), other neuropeptides and compounds may also contribute to ACTH release. A family of CRF-like peptides exists and includes UI, the teleost ortholog to the mammalian urocortin-I (UcnI) (Vaughan et al., 1995), as well as UcnII and UcnIII (Boorse et al., 2005; Brautigam et al., 2010; Huising et al., 2004). The

neurohypophysial hormones, arginine vasotocin (AVT) and isotosin (IST), homologous to mammalian arginine vasopressin (AVP) and oxytocin, respectively, increase ACTH release from the pituitary of rainbow trout (Baker et al., 1996; Pierson et al., 1996) and goldfish (Fryer et al., 1985). Both AVT and IST have lower ACTH-releasing efficacy than CRF but in rainbow trout, similar to mammals, AVT potentiates CRF-induced release of ACTH (Baker et al., 1996). Preoptic area AVT mRNA increases in response to a number of acute stressors, but evidence for stress-induced elevation in IST is limited in fish (reviewed by Bernier et al., 2009). Angiotensins I and II also stimulated ACTH secretion from superfused goldfish (*Carassius auratus*) pars distalis cells (Weld and Fryer, 1987, 1988) but whether angiotensins play a role in regulation of the stress response *in vivo* remains unclear (reviewed by Bernier et al., 2009). Although Rotllant et al. (2000b) observed ACTH secretion from gilthead seabream (*Sparus aurata*) pituitaries superfused with thyrotropin-releasing hormone (TRH), the role of TRH in pituitary release of ACTH as part of the stress response remains controversial (reviewed by Bernier et al., 2009; Cerdá-Reverter and Canosa, 2009). Together, these studies suggest other possible contributors to ACTH release in response to stress, but the extent of these contributions and species-specific effects require further investigation.

Teleost fish have a unique caudal neurosecretory system (CNSS) at the caudal end of the spinal cord that additionally produces CRF and urotensins (Bernier et al., 2008; Craig et al., 2005). The CNSS is thought to play a role in physiological processes such as osmoregulation and responses to stress (reviewed by McCrohan et al., 2007). Bernier et al. (2008) assessed changes in mRNA levels of POA and CNSS CRF and UI in response to a number of stressors (hyperammonemia, hypoxia, isolation, or social

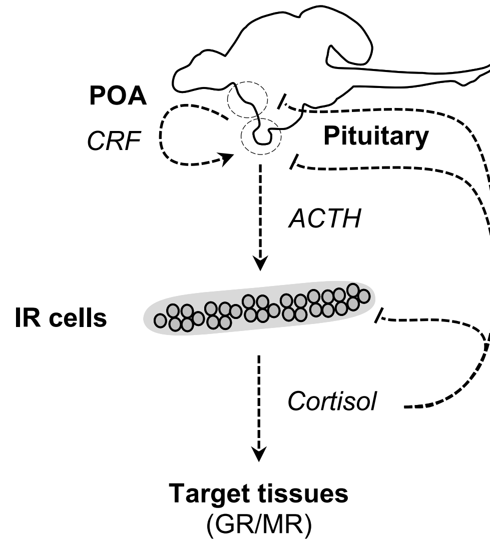
subordination) in rainbow trout and found stressor- and tissue-specific effects. Interestingly, although hyperammonemia, hypoxia, and isolation affected CNSS CRF and/or UI mRNA levels, social subordination (see below, section 1.3), which elicited the greatest increase in plasma cortisol, had no effect on CNSS transcript levels (Bernier et al., 2008). Thus, although the CNSS likely contributes to stress responses in a number of situations, it does not appear to be important in the response to social stress.

Adrenocorticotropic hormone is considered to be the main secretagogue of cortisol (Balm and Pottinger, 1995; Bernier et al., 2009; Wendelaar Bonga, 1997). Proopiomelanocortin (POMC) is cleaved by prohormone convertase 1 (PC1) in corticotropes of the pituitary to form ACTH and β-lipotrophic hormone (β-LPH), which is further processed to β-endorphin (β-END) by PC2. More extensive cleavage of POMC by PCs in melanotropes gives rise to α-melanocyte-stimulating hormone (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP) as well as a different form of β-ENDs (Kawauchi and Sower, 2006; Tanaka, 2003; Zhou et al., 1993). Once released into the circulation, ACTH binds to melanocortin 2 receptors (MC2R), G protein-coupled receptors, on the interrenal cells of the head kidney region (Fig. 1.1B) (Aluru and Vijayan, 2008). Teleosts lack the distinct adrenal gland observed in mammals, and instead, steroidogenic cells (i.e., interrenal cells) are found along the posterior cardinal vein, in close proximity to catecholamine-producing chromaffin cells (reviewed by Gallo and Civinini, 2003; Grassi Milano et al., 1997; Wendelaar Bonga, 1997). Binding of ACTH to MC2R results in activation of adenylyl cyclase (AC) to produce cyclic adenosine monophosphate (cAMP) (Gantz and Fong, 2003). Elevated intracellular levels of cAMP lead in turn to activation of protein kinase A (PKA), which initiates two

temporally distinct responses to increase cortisol biosynthesis (reviewed by Sewer and Waterman, 2003).

In the acute response, PKA phosphorylates and thus activates cholesterol ester hydroxylase; this enzyme catalyses the conversion of cholesterol ester (predominantly from low or high density lipoproteins; LDL or HDL) to free cholesterol, the precursor to cortisol (Jefcoate et al., 1992). Cholesterol is shuttled to the inner mitochondrial membrane, a process facilitated by steroidogenic acute regulatory protein (StAR) (Aluru and Vijayan, 2006; Hagen et al., 2006; Mommsen et al., 1999). Transcription of StAR is also increased during the acute response (Clark et al., 1994; Stocco, 2001b). Cholesterol is cleaved to pregnenolone by cytochrome P450 side chain cleavage enzyme (P450scc, also known as CYP11A1) in a process that involves three sequential oxidation reactions (reviewed by Mommsen et al., 1999; Payne and Hales, 2004). Both the movement of cholesterol from the outer to the inner mitochondrial membrane by StAR (Stocco, 2001a) and the cleavage of cholesterol by P450scc are considered rate-limiting in cortisol synthesis (Bernier et al., 2008; Mommsen et al., 1999); however, shuttling by StAR is likely the more important rate-limiting step in steroidogenesis because simple diffusion of hydrophobic cholesterol across the aqueous membrane space could not sustain acute synthesis of cortisol (reviewed by Stocco, 2001a). Pregnenolone is the substrate for subsequent synthesis of cortisol through a number of enzyme-catalyzed reactions, with the final step being the conversion of 11-deoxycortisol to cortisol catalyzed by 11β-hydroxylase (11β-hyd, also

Figure 1.1 Schematic representation of the hypothalamic-pituitary-interrenal (HPI) axis (A), and cortisol synthesis in the interrenal cell (B). Corticotropin-releasing factor (CRF) is released from the preoptic area (POA) of the brain to the pituitary to stimulate adrenocorticotropic hormone (ACTH) secretion. Melanocortin 2 receptors (MC2R), G protein-coupled receptors on the interrenal (IR) cells, are activated by ACTH leading to an increase in intracellular cAMP through activation of adenylyl cyclase (AC) to activate protein kinase A (PKA) that, in turn, initiates the synthesis of cortisol. Activation of PKA results in the mobilization of cholesterol, the precursor to cortisol, as well as transcriptional increases in steroidogenic genes. Cholesterol is moved to the inner-mitochondrial membrane facilitated by steroidogenic acute regulatory protein (StAR). Cholesterol is converted to pregnenolone in a step catalyzed by cytochrome P450 side chain cleavage enzyme (P450scc). Pregnenolone is moved to the endoplasmic reticulum and enters a series of enzyme-catalyzed reactions ending with the production of 11-deoxycortisol, which is converted to cortisol in a reaction catalyzed by 11β-hydroxylase (11β-hyd) located in the inner mitochondrial membrane. Cortisol acts on glucocorticoid or mineralocorticoid receptors (GR and MR) to elicit genomic effects. Activation of the HPI axis is regulated by negative feedback (flat-ended arrows) at multiple levels.



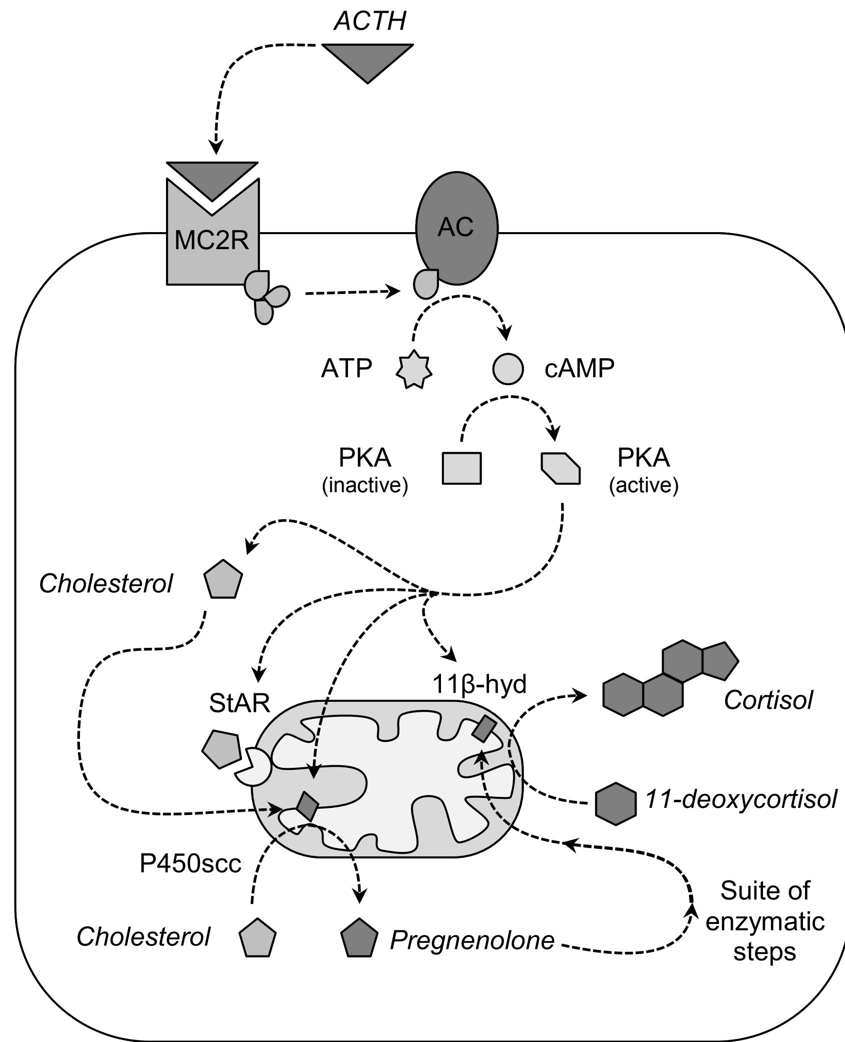


Figure 1.1

known as CYP11B1 or P450c11) (Aluru and Vijayan, 2006; Mommsen et al., 1999; Payne and Hales, 2004). In the chronic response, PKA activates transcription of all other steroidogenic genes (Sewer and Waterman, 2002), and although the mechanism of action remains unclear (i.e., targets for PKA phosphorylation are unknown), a number of transcription factors are likely involved (see Sewer and Waterman, 2003).

Synthesized cortisol is released from the head kidney tissue into the circulation. In mammals, the availability of cortisol to its target tissue receptors is regulated by plasma-binding proteins (corticosteroid-binding-globulin; CBG) (Breuner and Orchinik, 2002). Preliminary evidence suggests that a similar mechanism likely exists in fish (e.g., Idler and Freeman, 1968); however, identification of cortisol-binding proteins in fish requires further investigation (Iwama et al., 2006; Mommsen et al., 1999). Cortisol acts at target tissues through both genomic (i.e., corticosteroid receptors) and non-genomic modes. Recently, the occurrence of non-genomic signalling by cortisol in teleosts has become evident (Dindia et al., 2013; Dindia et al., 2012); however, much more is known about the genomic effects of cortisol. Corticosteroid receptors include both glucocorticoid (GR) and mineralocorticoid receptors (MR), which are ligand-activated transcription factors (Bamberger et al., 1996; Evans, 1988; Fuller et al., 2000; Schoneveld et al., 2004). In mammals, GR and MR are bound by glucocorticoids and aldosterone, respectively, and regulate, respectively, metabolism and stress (Evans, 1988; Fuller et al., 2000), as well as salt and water balance (Fuller et al., 2012; Johnson, 1992; Pascual-Le Tallec and Lonbès, 2005; Sheppard, 2002). Teleost fish, however, lack the ability to synthesize aldosterone (Balm et al., 1989; Jiang et al., 1998; Sangalang and Uthe, 1994) and thus cortisol is thought to elicit both glucocorticoid and

mineralocorticoid effects (Bury and Sturm, 2007; McCormick et al., 2008). Teleost GR is known to play roles in both metabolism and stress as well as salt and water balance (Aluru and Vijayan, 2007; Dean et al., 2003; Mommsen et al., 1999; Vijayan et al., 2003; Wendelaar Bonga, 1997), but a specific function for MR has yet to be determined (Bury and Sturm, 2007; Prunet et al., 2006; Takahashi and Sakamoto, 2013). Teleost fish synthesize the precursor to aldosterone, 11-deoxycorticosterone (DOC), which has been suggested as an alternative mineralocorticoid hormone in teleost fish but the potential roles of MR/DOC require further investigation (Stolte et al., 2008; Sturm et al., 2005).

Both GR and MR are widely distributed in the tissues of teleost fish (Bury et al., 2003; Greenwood et al., 2003; Sturm et al., 2005) and their role may vary with a fish's developmental stage. Like cortisol (see below, section 1.4.3), GR mRNA is transferred to embryos (Alsop and Vijayan, 2008) and developmental studies in teleost fish point to an important role of GR signalling during early embryogenesis. Morpholino knockdown of GR resulted in impaired mesoderm development, and reduced growth and post-hatch survival in zebrafish (Nesan et al., 2012; Pikulkaew et al., 2011). Embryonic GR signalling also likely mediates a number of other processes in developing zebrafish (reviewed by Nesan and Vijayan, 2013), such as osmoregulation (Kumai et al., 2012), myogenesis (Nesan et al., 2012), and cardiogenesis (Nesan and Vijayan, 2012). A study in rainbow trout also suggested that GR signalling regulates the expression of growth-related genes during early embryogenesis (Li et al., 2012b). Collectively, these results define a clear function for GR signalling during early

embryogenesis, but the role of MR remains less well understood (Alsop and Vijayan, 2008).

In most teleost fish studied to date, two distinct GRs, GR1 and GR2, have been identified but zebrafish have a single GR (Alsop and Vijayan, 2008) (Table 1.1). The two GRs are thought to be the result of a whole genome duplication event that occurred in ray-finned fish approximately 350 million years ago (Meyer and Van de Peer, 2005; Stolte et al., 2006; Vandepoele et al., 2004). Because duplicate GRs have been identified in two other cyprinid species, common carp (*Cyprinus carpio*) (Stolte et al., 2008) and fathead minnow (*Pimephales promelas*) (Alsop and Vijayan, 2009a; Filby and Tyler, 2007), Alsop and Vijayan (2009a) hypothesized that zebrafish lost one of the two GRs through a lack of sub-functionalization in the zebrafish ancestor. Although zebrafish possess only a single GR, two splice variants, GRα and GRβ, have been identified (Schaaf et al., 2008). However, GRα likely elicits the primary GR actions because GRβ does not bind cortisol and is not transcriptionally active. Instead, GRβ may play an inhibitory role (Schaaf et al., 2008).

The actions of cortisol can be further modulated at the target tissue level by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), the enzyme that catalyzes the conversion of cortisol to the inactive product, cortisone (Alderman and Vijayan, 2012). In mammals, 11β-HSD2 plays an important role in regulating glucocorticoid transfer to the fetus and is highly expressed in the placenta (Benediktsson et al., 1997; Krozowski, 1999; Wyrwoll et al., 2011). Furthermore, 11β-HSD2 often is co-localized with MR in mammals to prevent occupation of the receptor by cortisol and therefore encourage aldosterone binding to MR (Krozowski, 1999). Although teleost fish lack the capacity to

synthesize aldosterone and whether an alternative mineralocorticoid hormone exists is uncertain (see above) (McCormick et al., 2008), 11β-HSD2 is widely distributed, as are MRs (Greenwood et al., 2003; Sturm et al., 2005), and therefore 11β-HSD2 may prevent cortisol binding to MR (Jiang et al., 2003; Jiang et al., 1998; Kusakabe et al., 2003; Rasheeda et al., 2010).

Activity of the HPI axis is regulated by negative feedback at multiple levels in teleosts (Fig. 1.1A). Cortisol acts at the level of the brain to reduce synthesis and/or release of CRF. For example, Fryer and Peter (1977) reported that cortisol pellet implants placed near the nucleus lateral tuberis in the hypothalamus, as well as the near the nucleus preopticus (POA) and posterior telencephalon, suppressed the stress response in goldfish. Activity of the HPI axis also is regulated by the action of cortisol at the level of the pituitary to inhibit ACTH release. Using superfused goldfish anterior pituitary cells supplemented with cortisol, Fryer et al. (1984) found a reduction in both basal and stimulated (with UI, ovine CRF, and sauvagine) ACTH secretion levels. Cortisol further acts locally, at the level of the interrenal cells, to modulate ACTH-induced release of cortisol; ACTH-stimulated cortisol secretion of coho salmon (*O. kisutch*) interrenal cells *in vitro* was attenuated in cortisol-rich media (Bradford et al., 1992). Recently, Alderman and Vijayan (2012) also implicated 11β-HSD2 in negative feedback control of HPI axis functioning in the zebrafish brain, where brain 11β-HSD2 activity increased following acute stress exposure as well as an associated decrease in POA CRF mRNA. Alderman and Vijayan (2012) suggested that brain 11β-HSD2 activity rises post-exposure to a stressor and regulates HPI axis activity through negative feedback control of CRF transcription.

Table 1.1 Glucocorticoid receptors (GR) identified in teleost fish species.

Species	Glucocorticoid receptors (GR)	Reference
Astatotilapia burtoni	GR1 and GR2	Greenwood et al. (2003)
Cyprinus carpio	GR1 and GR2	Stolte et al. (2008)
Danio rerio	GR	Alsop and Vijayan (2008)
Dicentrarchus labrax	GR1 and GR2	Terova et al. (2005)
Gasterosteus aculeatus	GR1 and GR2	Alsop and Vijayan (2008)
Neolamprologus pulcher	GR1 and GR2	O'Connor et al. (2013)
Oncorhynchus mykiss	GR1 and GR2	Bury et al. (2003)
Opsanus beta	GR*	Rodela et al. (2011)
Oryzias dancena	GR1 and GR2	Kim et al. (2011)
Oryzias latipes	GR1 and GR2	Alsop and Vijayan (2008)
Porichthys notatus	GR*	Arterbery et al. (2010)
Takifugu rubripes	GR1 and GR2	Stolte et al. (2006)
Tetraodon nigroviridis	GR1 and GR2	Stolte et al. (2006)

* Authors suggest that the possibility of a second GR cannot be ruled out.

More recent work has revealed multi-level control of HPI axis function by GRs. Using mifepristone (RU486) to pharmacologically knock down GR protein levels (a new role for mifepristone, typically considered a GR antagonist), Alderman et al. (2012) found that POA CRF mRNA levels were diminished in rainbow trout. This result suggests a role for GR signalling in maintaining basal expression of CRF and that a separate, unidentified mechanism is responsible for CRF suppression by cortisol through negative feedback. In the same study, *in vivo* stress-induced and *in vitro* ACTH-stimulated cortisol production was reduced with GR knock down by mifepristone. In the case of the *in vitro* preparation, lower cortisol production was likely due to decreased levels of StAR mRNA in head kidney. Collectively, these results indicate the involvement of both central (effects on CRF) and peripheral (effects on interrenal cells) GR signalling in mediating HPI axis function (Alderman et al., 2012).

Serotonin or 5-hydroxytryptamine (5-HT) also seems to play a role in modulating activity of the stress axis. In mammals, 5-HT acts at multiple levels of the HPA axis, modulating CR-hormone (CRH) release from the paraventricular nucleus (PVN) (Herman et al., 2003; Liposits et al., 1987), ACTH release from the anterior pituitary (predominantly an indirect effect of PVN release of CRH) (Jørgensen et al., 2002; Jørgensen et al., 1998) and synthesis and release of corticosteroids from adrenocortical cells (Contesse et al., 2000). Similar, multi-level control of the HPI axis seems to exist in teleost fish, though the mechanisms of action remain unclear and vary across the species studied to date (Höglund et al., 2002; Lim et al., 2013; Medeiros et al., 2010). Treatment with 5-HT receptor agonists elicited mixed results in teleost fish. An increase in plasma cortisol levels was observed in goldfish (Lim et al., 2013), rainbow

trout (Winberg et al., 1997) and Arctic char (*Salvelinus alpinus*) (Höglund et al., 2002) treated with 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-OH-DPAT), a 5-$HT_{1A/7}$ receptor agonist, which contrasts with the gulf toadfish (*Opsanus beta*), where treatment with physiological levels of 8-OH-DPAT or α-methyl-5-HT, a 5-HT_2 receptor agonist, had no effect on circulating cortisol levels (McDonald, 2004). However, higher pharmacological doses of 8-OH-DPAT increased plasma cortisol levels in toadfish and Medeiros et al. (2010) suggested that a higher level of 8-OH-DPAT was necessary owing to desensitization of receptors by the unusually high circulating cortisol levels in this species. Serotonin-induced elevation in cortisol is regulated at the head kidney level in both toadfish and goldfish, where 5-HT stimulated cortisol secretion from head kidney preparations *in vitro*; however, selective 5-HT receptor agonists yielded mixed results and further investigation into the receptor subtypes regulating 5-HT-induced cortisol release is required (Lim et al., 2013; Medeiros and McDonald, 2012). Interestingly, Lim et al. (2013) localized 5-HT expression to chromaffin cells of the head kidney tissue, suggesting a possible paracrine mechanism for stimulation of cortisol synthesis.

Functioning of the HPI axis also can be affected by a number of other factors. For instance, cross-talk between the HPI axis and the thyroid or reproductive axes occurs (reviewed by Charmandari et al., 2005). Furthermore, anthropogenic factors, such as exposure to contaminants, also affect stress axis function (reviewed by Hontela and Vijayan, 2008). The purpose of this thesis was to determine the potential contributions of a series of what might be considered to be life history-related parameters, namely social stress, maternal stress, and parental investment, in modulating HPI axis function.

1.3 Social stress

Most teleost species produce a large number of offspring in environments where resources can be limited (e.g., structured environments such as coral reefs, streams) (reviewed by Johnsson et al. 2006). As juveniles, competition for food rich territory and thus monopolisation of food resources is high, during a period when growth and survival are important. As adults, competition over reproductive opportunities, which often also involve competing for reproductive territories, becomes more important. Thus, as both juveniles and adults, competition for limited resources, can lead to the formation of social hierarchies, with certain fish outcompeting their conspecifics to become dominant (reviewed by Johnsson et al. 2006).

1.3.1 Social hierarchies in salmonids

The causes and consequences of social status in salmonid fish have been studied extensively (reviewed by Gilmour et al., 2005; Johnsson et al., 2006). Salmonids form social hierarchies both in the wild and in a laboratory setting (e.g., Nakano, 1995; Noakes and Leatherland, 1977; Sloman et al., 2008; Sloman et al., 2000b), which allows for the study of an ecologically-relevant stressor within a controlled laboratory setting. Although social hierarchies form in fish held in small groups, these hierarchies are particularly strong in juvenile fish confined in pairs (Laidley and Leatherland, 1988; Sloman and Armstrong, 2002). Social contests begin with a period of aggressive interaction where catecholamine and cortisol levels increase in both fish (Øverli et al., 1999a; Thomas and Gilmour, 2006), but bouts of aggressive interactions become less frequent once the hierarchy has been established, which occurs within the first few

hours of interaction (Øverli et al., 1999a; Winberg and Lepage, 1998). In dominant fish, the acute increase in circulating cortisol is followed by a decrease to resting levels, whereas cortisol levels remain elevated in subordinate fish (Øverli et al., 1999a; Sloman et al., 2001). Subordinate fish also exhibit decreased growth (e.g., Abbott and Dill, 1989; DiBattista et al., 2006; Pottinger and Pickering, 1992; Sloman et al., 2000b) and increased instances of disease and mortality (e.g., Pottinger and Pickering, 1992), factors that are typically attributed to the prolonged elevation of circulating cortisol levels in subordinate fish. The sustained high circulating cortisol levels in subordinate fish likely reflect constant perception of a threat and a lack of social control rather than continual aggression from the dominant fish (reviewed by Johnsson et al., 2006; but also see Blanchard et al., 1993); however, the physiological mechanisms responsible for the prolonged elevation of plasma cortisol in subordinate salmonids remain unknown.

To address this knowledge gap, the first two data chapters of this thesis examined the effects of social status on HPI axis function in juvenile rainbow trout, attempting to identify both the mechanisms underlying chronic activation of the HPI axis and the consequences of social status on HPI axis activity. Chapter 2 tested the hypothesis that prolonged elevation of plasma cortisol levels in subordinate fish involves modulation of key proteins of the HPI axis and affects corticosteroid receptors in target tissues. Chronic activation of the HPI axis resulting in sustained elevation of circulating cortisol levels is expected to activate negative feedback mechanisms (see above). Thus, it was predicted that socially-subordinate fish would attenuate HPI axis function to reduce plasma cortisol levels and down-regulate corticosteroid receptors to protect against overstimulation of target tissues by cortisol. Furthermore,

chronic stress or administration of cortisol to mimic the effects of chronic stress on circulating cortisol levels diminishes the cortisol response to an acute stressor in fish (eg., Balm et al., 1994; Barton et al., 1987; Ings et al., 2011; Øverli et al., 1999b; Rotllant et al., 2000a; Wunderink et al., 2011), and rates of ACTH-stimulated cortisol production in an *in situ* perfused preparation were lower in subordinate compared to dominant trout (Sloman et al., 2002). Thus, Chapter 3 tested the hypothesis that the cortisol response to an acute stressor would be compromised by chronic social stress. It was predicted that subordinate fish would exhibit an attenuated cortisol response to an acute stressor, and this attenuation of the cortisol response would arise through modulation of HPI axis function at the level of the brain, pituitary, and/or interrenal cells.

1.3.2 Social hierarchies in zebrafish

Zebrafish have become the study organism of choice in a wide spectrum of research. Zebrafish spawn easily and frequently in a laboratory setting, and the rapid external development of their translucent embryos as well as their fully sequenced genome makes them a useful genetic model for studies in development and toxicology (see Ekker and Akimenko, 2010; Stegeman et al., 2010; Xu and Zon, 2010). More recently, zebrafish also have become recognized as a model organism for studying behaviour (Gerlai, 2003; Miklósi and Andrew, 2006; Norton and Bally-Cuif, 2010). Although zebrafish are social and prefer to swim in shoals (Pritchard, 2001), protection of finite resources such as food (Basquill and Grant, 1998; Grant and Kramer, 1992; Hamilton and Dill, 2002), territory (Spence et al., 2006; Spence and Smith, 2005), and

mates results in social ranking. In zebrafish, as in salmonids, social status is determined through aggressive interactions including fin erecting displays, dancing, undulating movements, as well as chasing and biting (Basquill and Grant, 1998; Gerlai, 2003; Spence et al., 2008). Within pairs, these intimidation tactics often result in one fish achieving dominance over the other (Dahlbom et al., 2011; Filby et al., 2010; Larson et al., 2006; Paull et al., 2010; Pavlidis et al., 2011; Sneddon et al., 2011).

The physiological consequences of social stress have been evaluated to some extent in zebrafish. In adult zebrafish, dominant fish tended to be larger (Filby et al., 2010; Paull et al., 2010), and dominant males exhibited a higher growth rate compared to subordinate fish (Filby et al., 2010). As in salmonids, subordinate zebrafish, regardless of sex, exhibited significantly elevated cortisol levels (Filby et al., 2010). In addition, subordinate males displayed higher telencephalic CRF and GR mRNA levels on the first day of interaction (Filby et al., 2010). Furthermore, Larson et al. (2006) found distinct differences in POA AVT expression, with dominant fish expressing AVT in 1 of 3 pairs of large cells in the magnocellular POA and subordinate fish expressing AVT in 7-11 pairs of cells in the parvocellular POA. In addition to its role in regulating HPI axis activity (see above), AVT and its mammalian homologue AVP, are recognized as mediators of social behaviour in a number of organisms including fish and mammals (see Greenwood et al., 2008). Together, these studies suggest regulation of HPI axis function by social status in adult zebrafish. Social status also appears to affect reproductive success and mate choice in zebrafish (Paull et al., 2010); however whether maternal social stress effects translate into consequences for offspring remains unknown.

1.4 Maternal stress and programing of the stress axis

Maternal experience, such as maternal exposure to stressors, has the potential to transfer important information to offspring. For example, maternal glucocorticoids transferred to developing eggs/embryos are thought to play an important role in programming offspring stress axis function across a number of taxa (reviewed by Love et al., 2013). Reproductive mode (placental *versus* egg-laying) and timing of stress axis development also vary across taxa and influence the role of maternal experience in programming offspring. For instance, in egg-laying vertebrates, offspring are predominantly sensitive to maternal glucocorticoids transferred during yolk formation (reviewed by Love et al., 2013), whereas in mammals, brain growth and maturation take place *in utero* in precocial young (e.g., sheep, guinea pigs, and hares), and also during the early postnatal period in altricial young (e.g., rats and rabbits) (Dobbing and Sands, 1979). For altricial offspring, the level of maternal care delivered during the early postnatal period can have long-lasting impacts on offspring stress axis programming (see below section 1.4.1).

Factors other than maternal glucocorticoids also impact egg quality. Mothers contribute a number of provisions to offspring such as nutrients, other hormones (e.g., estrogens and androgens), and mRNAs (Brooks et al., 1997). Provisioning of these resources to offspring can affect qualities of the offspring; most obviously by egg size but also egg number (Braun et al., 2013; Mousseau and Fox, 1998; Solemdal, 1997). For instance, adjusting egg size (i.e., laying larger eggs) can better prepare offspring for a resource-poor environment, but comes at a cost to the mother as fewer eggs are laid (i.e., trade-off between size and number of progeny) (Mousseau and Fox, 1998;

Solemdal, 1997). Laying order in birds and fish can also affect egg quality. Birds lay single eggs at daily or longer intervals. Thus, position in the laying order can affect egg viability because eggs likely are exposed to different environments over the laying interval (e.g., thermal effects before incubation starts) (Vleck and Vleck, 2011). Burton et al. (2012) found evidence for similar among-sibling differences in brown trout (*Salmo trutta*), where the phenotype of offspring (i.e., standard metabolic rate and size) was related to their location in the egg mass (i.e., front, middle, or rear third). In addition, the unpredictability of an environment can affect maternal provisioning, in that some mothers will differentially provision offspring within the same clutch, producing offspring that may perform better or worse than others, depending on the environment (i.e., bet-hedging) (Gagliano and McCormick, 2007; Love et al., 2005; Philippi and Seger, 1989). Sex ratio can also be manipulated by the mother in response to mate quality, such as in the case of female collared flycatchers (*Ficedula albicollis*), where male offspring are favoured (i.e., higher quality offspring) when mating occurs with males with large forehead patches (i.e., more attractive to the female) (Sheldon et al., 1997). Although not reviewed here to a large extent, paternal effects through parental care (see below for an example in smallmouth bass; section 1.5) and genetic contributions to offspring can further affect offspring phenotype. However, as long as female choice plays a role in male selection, these paternal consequences are considered a maternal effect (Mousseau and Fox, 1998). Clearly, there are a number of impacts that maternal experience can have on their offspring.

The following paragraphs will briefly introduce the effects of maternal stress on HPA axis programming in mammals and birds, as well as focusing on the existing research on HPI axis programming in teleost fish.

1.4.1 Maternal stress in mammals

Both prenatal and postnatal environments affect programming of HPA axis function in mammals. Maternal stress during the later stages of gestation results in sustained effects on HPA axis function in offspring (Cottrell and Seckl, 2009; de Kloet et al., 2005; Matthews et al., 2004; Meaney et al., 2007). Although glucocorticoids are necessary for normal brain development (Matthews, 1998), high levels of (or removal of) glucocorticoids can result in altered brain structure and function, influencing HPA axis function (Matthews, 2002; Muneoka et al., 1997; Sapolsky, 1987; Seckl, 2004). For instance, increased levels of fetal glucocorticoids reduce hippocampal GR and MR, resulting in a more active stress axis in adult offspring owing to reduced negative feedback (Emack et al., 2008; Kapoor et al., 2008; Welberg and Seckl, 2001; Welberg et al., 2001). Interestingly, mammals have a "natural barrier" to maternal glucocorticoid transfer, in that placental 11β-HSD2 (which converts glucocorticoids to their inactive forms; see above section 1.2) expression is high (Benediktsson et al., 1997). However, when maternal glucocorticoids increase in response to a stressor, 11β-HSD2 levels often are not sufficient to protect offspring from increased glucocorticoid exposure (Lesage et al., 2001; Lucassen et al., 2009). The mechanisms involved in prenatal programming of the stress axis by maternal glucocorticoids remain poorly understood, but epigenetic modification of brain GR and CRF-receptor promoter regions has been

suggested as a target for maternal glucocorticoid programming (McGowan and Szyf, 2010; Mueller and Bale, 2008; Szyf et al., 2008).

Programming of the HPA axis by the early postnatal environment has been a subject of great interest and thus extensive investigation (Francis and Meaney, 1999; Meaney, 2001; Meaney et al., 2007). The level of maternal care provided to rat pups affects HPA axis reactivity in the adult offspring, owing to differences in hippocampal GR expression resulting in altered negative feedback sensitivity (Liu et al., 1997). In this case, the mechanisms of action have been thoroughly investigated, and epigenetic programming by DNA methylation of the hippocampal GR promoter is responsible for changes in adult offspring HPA axis activity (Weaver et al., 2004; Weaver et al., 2005; Weaver et al., 2007). A high level of maternal care decreases the level of methylation at the GR promoter, allowing easier access to NGFI-A, a transcription factor that mediates GR expression. By contrast, a low level of maternal care results in an increased level of DNA methylation of the GR promoter, leading to a decreased level of GR and thus a hyperactive HPA axis owing to lower negative feedback sensitivity. Because cross-fostering experiments and manipulation of chromatin structure and methylation status eliminate these effects, they seem to be primarily the result of postnatal maternal care and are not genetically pre-determined (Francis et al., 1999; Weaver et al., 2004; Weaver et al., 2005).

1.4.2 Maternal stress in birds

As in mammals, prenatal maternal stress as well as the quality (i.e., provisioning rates) of the postnatal environment both influence responsiveness of the HPA axis in

birds. Elevated maternal glucocorticoid (corticosterone) levels translate into increased transfer of glucocorticoids to the yolk of developing eggs (Almasi et al., 2012; Hayward and Wingfield, 2004; Love et al., 2005; Saino et al., 2005). However, the story likely involves glucocorticoids as well as other factors. Experimental elevation of maternal glucocorticoid levels resulted in increased HPA axis activity in adult Japanese quail (*Coturnix coturnix japonica)* (Hayward and Wingfield, 2004), but experimental increases in yolk glucocorticoids led to decreased HPA axis activity in females but not males of the same species (Hayward et al., 2006). To further complicate this scenario, Marasco et al. (2012) reported the opposite effect; increasing yolk glucocorticoids increased the HPA axis response of Japanese quail. Additionally, experimentally-increased maternal glucocorticoid levels in domesticated chickens (*Gallus gallus domesticus*) had no effect on offspring HPA axis responsiveness, although yolk corticosterone was unaffected in this study (Henriksen et al., 2013). Clearly, differences exist in the effects of maternal stress and elevated yolk glucocorticoids between and within species in birds, and further investigation into the reasons for these differences is needed.

Many studies have been carried out on captive birds, whereas limited research has been performed on free-living species. However, Love and Williams (2008) assessed the effects of both prenatal (increased yolk corticosterone) and early postnatal environment (diminished maternal provisioning capacity by feather clipping) on HPA axis activity in European starlings (*Sturnus vulgaris*). Interestingly, fledglings treated with corticosterone prenatally exhibited an attenuated stress response, whereas decreasing the quality of the postnatal environment (limited provisioning

rates) increased activity of the HPA axis in fledglings. In this study, the authors suggested that prenatal glucocorticoid exposure resulted in a 'predictive adaptive response', a form of developmental plasticity where prenatal environmental cues confer an advantageous phenotype in a future life-history stage. By contrast, diminished quality of the postnatal environment was hypothesized to bring about a 'reactive adaptive response', where offspring adapt to an unpredictable change in the quality of their post-natal environment (Love and Williams 2008).

Although the mechanisms underlying programming of HPA axis function by glucocorticoids in birds largely remain unstudied, Ahmed et al. (2014) recently examined possible mechanisms in chickens. Prenatal exposure to a high dose (1 μg) of corticosterone resulted in offspring with higher baseline corticosterone levels compared to individuals exposed to a low corticosterone dose (0.2 μg) or control individuals. Although HPA responsiveness was not examined in this study, the authors found lower hippocampal CRH and AVT mRNA levels, as well as lower GR protein levels in individuals treated with the high corticosterone dose (Ahmed et al., 2014). Furthermore, a high corticosterone dose resulted in increased DNA methylation of both GR and CRH promoters, suggesting that epigenetic programming via glucocorticoids may be responsible for programming of the HPA axis during early development in birds (Ahmed et al. 2014), as in mammals.

1.4.3 Maternal stress in teleost fish

As in birds, corticosteroids transferred to eggs prior to fertilization seem to play an important role in programming of offspring in teleost fish (see Li et al., 2012b;

Schreck et al., 2001). The cortisol present in offspring in early development is of maternal origin, and cortisol levels then decrease until about the time of hatching, when teleost fish begin to synthesize cortisol *de novo* [Asian sea bass, *Lates calcarifer* (Sampath-Kumar et al., 1995); common carp (Flik et al., 2002); European sea bass, *Dicentrarchus labrax* (Tsalafouta et al., 2014); Japanese flounder, *Paralichthys olivaceus* (De Jesus et al., 1991); rainbow trout (Auperin and Geslin, 2008; Barry et al., 1995; Ghaedi et al., 2013); tilapia, *Oreochromis mossambicus* (Hwang et al., 1992); medaka, *Oryzias latipes* (Trayer et al., 2013); yellow perch, *Perca flavescens* (Jentoft et al., 2002); zebrafish (Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Wilson et al., 2013)]. The capacity to elevate cortisol levels in response to a stressor is further delayed until around the time of first feeding [Chinook salmon, *O. tshawytscha* (Feist and Schreck, 2002); common carp (Stouthart et al., 1998); European sea bass (Tsalafouta et al., 2014); yellow perch (Jentoft et al., 2002); zebrafish (Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Wilson et al., 2013)]. The delay in elevation of cortisol levels during early development (i.e., embryogenesis) points to a sensitive period when increased cortisol levels may impact offspring development and/or programming.

Increased transfer of cortisol to oocytes owing to maternal stress, or maternal cortisol treatment to mimic an effect of maternal stress, both resulted in increased egg/embryo levels of cortisol (Eriksen et al., 2006; Giesing et al., 2010; Kleppe et al., 2013; Stratholt et al., 1997). Studies that elevated embryo cortisol by exogenous approaches (i.e., egg/embryo bathing in a cortisol solution or microinjection) have reported wide-ranging and often deleterious effects of increased levels of cortisol on teleost development (Table 1.2). However, programming of offspring likely is more

complex than the actions of cortisol alone (see below), and exposure to elevated cortisol may not always be maladaptive (e.g., decreased stress responsiveness benefit offspring exposed to unfavourable conditions; see Table 1.2). Although maternal stress studies are not extensive in fish, a group of studies has examined offspring programming by maternal predator exposure in three-spined stickleback (*Gasterosteus aculeatus*). These studies have identified multiple impacts of maternal predation threat on offspring including increased egg size and cortisol levels, elevated oxygen consumption shortly after fertilization, and juveniles with altered predator avoidance strategies (Giesing et al., 2010; McGhee et al., 2012), as well as an altered stress response (the extent of this difference was unclear but suggests increased stress responsiveness) (Mommer and Bell, 2013). Additional studies identified whole-transcriptome differences (e.g., metabolic processes and epigenetic inheritance) between 3 dpf offspring of predator-exposed and unexposed females (Mommer and Bell, 2014), as well as differences in learning, where adult offspring of predator-exposed mothers located food more slowly than offspring of unexposed mothers (Roche et al., 2012). Together, these studies support the idea that programming of offspring by maternal stress can have both early and long-term effects.

Elevation of cortisol in the female may not always translate into elevated egg/embryo cortisol levels. Teleost fish, like mammals, may have a protective mechanism to buffer developing eggs from high maternal glucocorticoid levels. Recently, Li et al. (2012a) reported that rainbow trout ovarian follicles, oocytes in ovarian fluid, and early embryos had the capacity to metabolize cortisol to cortisone (catalyzed by 11β-HSD2) and then cortisone sulphates (catalyzed by glucocorticoid

sulphotransferase). In particular, ovarian follicles are likely the most effective barrier to cortisol transfer owing to thecal and granulosa cells (Li et al., 2012a); two layers of steroidogenic cells that envelope the oocytes and provide a barrier between the oocytes and maternal extracellular fluid, as well as express 11β-HSD2 (Kusakabe et al., 2003; Leatherland et al., 2010). However, the authors postulated that the protective actions of 11β-HSD2 and glucocorticoid sulphotransferase are limited and dependent on the severity of maternal stress (Li et al., 2012a). A similar situation occurs in mammals (see above) where 11β-HSD2 levels are not sufficient to prevent stress-induced levels of cortisol from breaking through the placental barrier (Lesage et al., 2001; Lucassen et al., 2009). Interestingly, even in the absence of increased embryo cortisol levels, effects of maternal stress have been observed in two teleost species [a cichlid, *Neolamprologus pulcher* (Mileva et al., 2011) and sockeye salmon, *O. nerka* (Sopinka et al., 2014)]. Mileva et al. (2011) reported that maternal stress reduced clutch and egg size in *N. pulcher*, and Sopinka et al. (2014) observed effects of maternal stress on offspring swim performance in sockeye salmon.

Chapter 4 of this thesis examined the effects of maternal social stress on development of the stress axis in zebrafish. This chapter tested the hypothesis that maternal social status would affect stress axis development and activity in offspring. Because maternal stress can result in increased embryo cortisol levels (e.g., Giesing et al., 2010; Stratholt et al., 1997), it was predicted that maternal social stress would increase egg/embryo cortisol levels. It was further predicted that maternal social stress would consequently affect mRNA expression profiles of genes associated with the HPI axis over development. Lastly, because maternal stress or exposure to elevated

egg glucocorticoid levels has been linked most often to dampening of offspring stress responsiveness (see above for examples), it was predicted that maternal social stress would attenuate the larval stress response.

Table 1.2 Reported impacts of experimentally-elevated levels of glucocorticoids during early development in teleost fish.

Species	Cortisol exposure	Effect	Response	Reference
Danio rerio	microinjection (32 pg nl^{-1})	cardiac performance	↑ heart deformities; ↓ key cardiac genes	Nesan and Vijayan (2012)
Oncorhynchus mykiss	1 h embryo exposure (1000 ng ml^{-1})[a]	stress response	↓ fingerling stress responsiveness	Auperin and Geslin (2008)
O. mykiss	3 h egg exposure (1000 ng ml^{-1})[b]	hatching and growth	↓ hatching rate; affected growth related genes	Li et al. (2010)
Oryzias latipes	embryo exposure (50 mg L^{-1})	ionocyte development	earlier expansion of ionocyte population	Trayer et al. (2013)
Pomacentrus amboinensis	embryo exposure (10 µg L^{-1})	hatching and growth	↓ delayed hatching; ↑ mortality & asymmetry	Gagliano and McCormick (2009)
Salmo trutta	3 h egg exposure (500 µg L^{-1})	O_2 consumption; ammonia excretion; behaviour	↑ juvenile O_2 consumption & ammonia excretion; ↑ aggression; altered maze performance	Sloman (2010)
S. trutta	2 h egg exposure (200 µg L^{-1})	behaviour	↓ aggression; ↑ chance of subordinance	Burton et al. (2011)

[a] Auperin and Geslin (2008) also used a 200 ng ml^{-1} cortisol dose that resulted in lower baseline cortisol but not stress-induced levels

[b] Li et al. (2010) also used 100 ng ml^{-1} cortisol dose that resulted in enhanced juvenile growth

1.5 Parental care in smallmouth bass

Among species that provide parental care to offspring, which parent, male and/or female, provides the care varies considerably across vertebrate taxa (reviewed by Gross, 2005). For instance, across mammalian species care is predominantly female-only, whereas birds provide mostly biparental care (90% in each case). Birds and mammals rarely exhibit male-only parental care (< 2% of species), but this strategy is common (about 50% of families) among the 20% of teleost fish families that provide parental care. Teleost fish therefore are an interesting system in which to study paternal care.

Smallmouth bass is a centrarchid fish that provides paternal care over a period of 4-6 weeks in the late spring or early summer. Males dig out a nest in the substrate, and court a female that leaves the nest once her eggs have been deposited and fertilized. Males continue to guard the nest until the offspring have reached independence (i.e., feed exogenously and can evade predators) (Brown, 1984). Parental care behaviours include chasing away nest predators, fanning the eggs to aerate them and keep the nest free of silt, and the removal of debris from the nest (Brown, 1984). In addition to the considerable energy that males expend through nest defence, parental bass also limit foraging opportunities, increasing the energy burden during the parental care period (Cooke et al., 2006; Cooke et al., 2002; Ridgway et al., 1991). For bass at the northern limit of their range, the energetic demands of reproduction are further compounded by spawning occurring immediately following the quiescent overwinter period (Adams et al., 1982); thus, bass spawn prior to repaying their overwintering energy debt. The energy exerted during the period of

parental care can negatively impact adult growth and survival over the following winter (Ridgway et al., 1991). Thus, a life-history trade-off exists between investing in the defence of a current brood (i.e., current reproductive outcome) *versus* adult growth and survival (i.e., future reproductive outcome) (Williams, 1966).

Hormones, particularly androgens and glucocorticoids, are thought to mediate both parental care and life-history trade-offs in the allocation of resources between current and future reproduction (Ricklefs and Wikelski, 2002). Recently, androgens and glucocorticoids were measured and manipulated over the parental care period in smallmouth bass. Androgens decreased over the parental care period (O'Connor et al., 2011b), but increased in response to a nest predator and were correlated with the level of nest defense (O'Connor et al., 2011a). Furthermore, treatment with the androgen receptor antagonist, cyproterone acetate, decreased nest defence but not nest success in smallmouth bass (Dey et al., 2010). Collectively, these data support a role for androgens in regulating the nest defense aspect of parental care. Interestingly, treatment with cortisol had no effect on nest defense, but increased nest abandonment in smallmouth bass (Dey et al., 2010) and the congeneric largemouth bass (*M. salmoides*) (O'Connor et al., 2009). Although baseline cortisol levels did not change significantly over the parental care period in smallmouth bass, stress-induced cortisol levels were attenuated during early parental care (when males were on fresh eggs) but were re-established toward the end of the parental care period (when males were caring for free-swimming fry) (O'Connor et al., 2011b). Attenuation of the stress response during the parental care period in smallmouth bass may represent an effort to minimize the negative impacts of elevated cortisol on reproduction (Fuzzen et al.,

2011a; Schreck, 2010; Schreck et al., 2001) and allow for successful reproduction in fish invested in a current brood. The mechanisms for this attenuation of the stress response are as yet unknown.

This thesis examined possible mechanisms underlying reduction of stress-induced cortisol levels in parental male smallmouth bass. Chapter 5 tested the hypothesis that attenuation of the cortisol response in male smallmouth bass during early parental care reflected modulation of HPI axis activity. Because key mediators of cortisol synthesis such as CRF, ACTH, MC2R, StAR, and P450scc are expected to increase in response to a stressor (Aluru and Vijayan, 2006, 2008; Fuzzen et al., 2010), it was predicted that parental male smallmouth bass would exhibit reduced stress-induced ACTH levels and mRNA abundance of HPI axis genes including CRF, MC2R, StAR, and P450scc during early but not late parental care.

1.6 Goals of this thesis

In this thesis, a combination of behavioural, physiological, and molecular techniques was used to assess the impacts of social stress, maternal social stress, and parental investment on HPI axis function in teleost fish. Chapter 2 tested the hypothesis that prolonged elevation of plasma cortisol levels in subordinate fish modulates key proteins of the HPI axis and affects corticosteroid receptors in target tissues in juvenile rainbow trout. Chapter 3 further examined effects of social status on HPI axis function in rainbow trout by testing the hypothesis that the cortisol response to an acute stressor is compromised by chronic social stress. Juvenile rainbow trout readily form dominance hierarchies when placed in pairs, and the considerable

research on salmonid social hierarchies (reviewed by Gilmour et al., 2005; Johnsson et al., 2006) made them an attractive study species for this work. Although several causes and impacts of social status are well understood in salmonids, there is a general lack of information on how genes associated with the HPI axis are regulated during social stress, a knowledge gap addressed by this thesis. Chapter 4 continued to focus on the effects of social status, but in this case on the next generation, by testing the hypothesis that maternal social status affects stress axis development and activity in offspring in zebrafish. Zebrafish were used as a study species for this work to take advantage of the reproductive and genetic benefits of working with this species. As noted above (section 1.3.2), zebrafish spawn easily and frequently in a laboratory setting which, in addition to their sequenced genome and the existing thorough understanding of the ontogeny of their HPI axis, made them a good fit for this work. Only a small number of studies have assessed the impacts of maternal stress on fish offspring and fewer have assessed consequences of maternal stress on HPI functioning in teleost fish (see section 1.4.3). The goal of Chapter 4 was therefore to assess both intergenerational impacts of maternal social status and to add to the current, limited literature on maternal stress in teleost fish. The final chapter of this thesis (Chapter 5) assessed additional effects of behaviour on HPI axis functioning, testing the hypothesis that attenuation of the cortisol response in male smallmouth bass during early parental care reflects modulation of HPI axis activity. Neither rainbow trout nor zebrafish care for their offspring, whereas smallmouth bass provided a study species that both invests extensively in care and was known to transiently modulate responsiveness of the stress axis during the parental care period (see section 1.5). Few studies have assessed

physiological mechanisms underlying regulation of the stress axis during parental care; the goal of this thesis was to shed light on such mechanisms. Together, the four data chapters of this thesis provide a diverse, detailed examination of three different situations in which HPI axis function in teleost fish is modulated. All three cases contribute to a common theme of understanding how behaviour modulates HPI axis function at multiple levels.

CHAPTER 2

Modulation of hypothalamic-pituitary-interrenal axis function by social status in rainbow trout

Notes on Chapter 2

Chapter 2 was published in *General and Comparative Endocrinology* as per the following citation:

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Real-time RT-PCR analysis of GR and MR mRNA levels was carried out as part of the honours undergraduate thesis of J.D. Jeffrey (2008), which was mentored by A.J. Esbaugh, a postdoctoral fellow in the Gilmour lab at the time. M.M. Vijayan was a collaborator at the University of Waterloo, where GR and MR western analysis was performed by J.D. Jeffrey.

2.1 Abstract

Juvenile rainbow trout form stable dominance hierarchies when confined in pairs. These hierarchies are driven by aggressive competition over limited resources and result in one fish becoming dominant over the other. An important indicator of low social status is sustained elevation of circulating cortisol levels as a result of chronic activation of the HPI axis. In the present study it was hypothesized that social status modulates the expression of key proteins involved in the functioning of the HPI axis. Cortisol treatment and fasting were used to assess whether these characteristics seen in subordinate fish also affected HPI axis function. Social status modulated plasma ACTH levels, cortisol synthesis, and liver GR expression. Plasma ACTH levels were lower by approximately 2-fold in subordinate and cortisol-treated fish, consistent with a negative feedback role for cortisol in modulating HPI axis function. Although cortisol-treated fish exhibited differences in CRF and CRF-BP mRNA relative abundances in the preoptic area and telencephalon, respectively, no effect of social status on CRF or CRF-BP was detected. Head kidney MC2R mRNA relative levels were unaffected by social status, while mRNA relative abundances of StAR and P450scc enzyme were elevated in dominant fish. Liver GR2 mRNA and total GR protein levels in subordinate fish were lower than control values by approximately 2-fold. In conclusion, social status modulated the functioning of the HPI axis in rainbow trout. Our results suggest altered cortisol dynamics and reduced target tissue response to this steroid in subordinate fish, while the higher transcript levels for steroid biosynthesis in dominant fish leads us to propose an adaptive role for responding to subsequent stressors.

2.2 Introduction

Among juvenile salmonid fish, social hierarchies readily form in small groups of fish that compete over resources that are limited in the environment, such as food. Fish that outcompete their conspecifics for these resources achieve dominance (Adams et al., 1998; Kadri et al., 1996; McCarthy et al., 1992; Metcalfe et al., 1989). The interactions between dominant and subordinate fish often are aggressive; dominant fish may chase and/or bite subordinate fish to attain and maintain high social rank (Abbott and Dill, 1985; Adams et al., 1998; Oliveira et al., 2011). Low social status can be disadvantageous for subordinate fish in that they reduce activity and feeding (Abbott and Dill, 1985; McCarthy et al., 1992), and experience prolonged elevation of cortisol, the main stress hormone in fish, owing to continued activation of the HPI axis (reviewed by Gilmour et al., 2005).

Activation of the HPI axis results in the synthesis and release of cortisol (reviewed by Mommsen et al., 1999; Wendelaar Bonga, 1997). Corticotropin-releasing factor is released directly into the pituitary of teleost fish by neurons that have their cell bodies in the POA of the brain (Wendelaar Bonga, 1997). Corticotropin-releasing factor-BP is highly localized to the central nervous system in fish and is thought to play a role in regulating CRF effects (Alderman et al., 2008; Seasholtz et al., 2002). Adrenocorticotropic hormone, a peptide derived from POMC, and the primary hormone that stimulates cortisol synthesis (Wendelaar Bonga, 1997), is then released as a result of activation of corticotropes by CRF. Interrenal cells in the head kidney are stimulated by binding of ACTH to the MC2R, a G protein-coupled receptor (Aluru and Vijayan, 2008). Binding of ACTH activates AC to initiate a cAMP-signaling cascade resulting in

the movement of cholesterol to the inner mitochondrial membrane with the help of the transport protein, StAR (Stocco, 2000). In the mitochondrion, P450scc then cleaves cholesterol to pregnenolone in the first and rate-limiting step of cortisol synthesis (Payne and Hales, 2004). Secreted cortisol acts on corticosteroid receptors, which are ligand-activated transcription factors (Prunet et al., 2006). The corticosteroid receptors include GR and MR. Most teleost species studied to date, with the exception of zebrafish, have multiple GR isoforms (Alsop and Vijayan, 2009a; Alsop and Vijayan, 2008; Bury et al., 2003; Greenwood et al., 2003; Prunet et al., 2006). In addition, teleost fish do not produce aldosterone as a mineralocorticoid (reviewed by Prunet et al., 2006), and, therefore, cortisol and potentially other corticosteroids (e.g., DOC) are thought to act as MR ligands (Colombe et al., 2000; Kiilerich et al., 2011a; 2011b; McCormick et al., 2008; Prunet et al., 2006; Sturm et al., 2005).

Several studies have investigated the physiological causes and consequences of social status in salmonid fish (reviewed by Gilmour et al., 2005). However, how chronic social stress modifies HPI axis functioning is not well understood. Filling this information gap is important in understanding the potential impact of chronic stress on a fish's ability to respond to additional environmental stressors (i.e., acute stress response). Thus, the present study investigated the effects of social status on the functioning of the HPI axis using pairs of juvenile rainbow trout. It was hypothesized that prolonged elevation of plasma cortisol levels in subordinate fish involves modulation of key proteins of the HPI axis and affects corticosteroid receptors in target tissues. During periods of chronic stress such as social stress, fish experience two conflicting demands: continual activation of the HPI axis resulting in elevated plasma

cortisol levels and the associated activation of the negative feedback mechanism to regulate this steroid level (Balm and Pottinger, 1995). As such, we predicted that socially-subordinate fish would attenuate HPI axis functioning to regulate plasma cortisol levels. Furthermore, social subordinance also was predicted to down-regulate corticosteroid receptors to protect against overstimulation of target tissues by cortisol. To test this hypothesis, we used semi-quantitative real-time RT-PCR (S-QPCR) and immunodetection to examine gene and protein expressions, respectively, at multiple levels of the HPI axis in control, fasted, cortisol-treated, dominant, and subordinate fish. Fasted and cortisol-treated groups were included in the experimental design to tease out the roles of specific characteristics of subordinate fish; subordinates often do not feed (McCarthy et al., 1992; Øverli et al., 1998), and have elevated circulating plasma cortisol levels (reviewed by Gilmour et al., 2005).

2.3 Materials and Methods

2.3.1 Experimental animals

Juvenile female freshwater rainbow trout (85.0 ± 1.9 g, mean ± standard error; SEM; N = 123) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and were kept at the University of Ottawa Aquatic Facility. Fish were housed in 1275 L fiberglass stock tanks under a 12L:12D photoperiod; the tanks were supplied with 13°C dechloraminated, aerated city of Ottawa tap water. Fish were fed every second day to satiation by scattering commercial trout pellets on the water's surface. Trout were allowed to acclimate to these holding conditions for at least 2 weeks prior to experimentation. Fish were chosen from the stock tanks at random and placed into

appropriate experimental tanks for a 5 day-period (see below) with the exception of control fish that were sampled directly from stock tanks. Fish masses did not differ significantly among treatment groups (P = 0.342, one-way analysis of variance; ANOVA; data not shown). The holding conditions (e.g., use of scatter feeding, homogenous tanks with a mild current) tended to minimize hierarchy formation.

Social pairing experiments were performed as described by DiBattista et al. (2005; 2006). Briefly, rainbow trout were lightly anesthetized (to the point of losing equilibrium) in a solution of benzocaine (0.05 g L^{-1} ethyl *p*-aminobenzoate) to assess fish weight, fork length, and fin damage. Each fish was paired with a conspecific that was no more than 0.1 ± 0.3% (mean ± SEM) different in fork length (Abbott et al., 1985; DiBattista et al., 2005). The fish in a pair were placed individually on either side of an opaque divider in a 40 L flow-through observation tank for an overnight recovery period. The divider was removed the following morning (day 1), and fish were allowed to interact for a period of 5 days (representing a chronic stress scenario). A PVC tube (t-shape, 13 x 11 cm long, 6 cm diameter) was added on the afternoon of day 1 to provide a shelter.

Fish were observed twice daily for 5 min per observation period and were scored on the occurrence of acts of aggression, position within the tank (e.g., patrolling the water column *versus* at the surface, bottom or in the shelter), and feeding; the scoring system awarded more points for more dominant behaviours such as initiating aggressive attacks, patrolling the water column, and taking food. Fin damage was assessed on the final day and compared to initial fin damage observed. This method of scoring behaviour is similar to methods used previously to assign social status in

salmonid fish (for examples see DiBattista et al., 2005; DiBattista et al., 2006; Metcalfe et al., 1989; Sloman et al., 2000a; Sloman et al., 2000b; Sloman et al., 2001). Principal component analysis (SPSS, v 16.0) was used to generate an overall behaviour score for each fish. Within a pair, the fish with the higher score was assigned dominant status. Only pairs for which the behaviour scores differed by at least 0.5 were used for subsequent analysis.

To generate fasted and cortisol-treated groups, fish were placed in 115-230 L holding tanks in groups of 10; this group size (together with the use of scatter-feeding, where appropriate, and homogenous tanks with a mild current) was sufficient to minimize unwanted hierarchy formation. Food was withheld from fasted fish for a period of 5 days. Cortisol-treated fish were lightly anaesthetized as above, and given an intraperitoneal injection of 0.005 ml g^{-1} body mass cocoa butter (Now Personal Care) containing 110 mg kg^{-1} body mass hydrocortisone 21-hemisuccinate (Sigma-Aldrich). This dose was chosen on the basis of previous work in which the circulating cortisol levels achieved were similar to those in subordinate rainbow trout (DiBattista et al., 2005); also as in previous work, sham-injected fish (i.e., fish injected with vehicle alone) were not used because the injection itself raises circulating cortisol levels in an unpredictable fashion, confounding interpretation of the effects of cortisol elevation (DiBattista et al., 2005).

2.3.2 Collection of tissue samples

Fish in all experimental groups were terminally anaesthetized with an overdose of benzocaine (0.5 g L^{-1} ethyl *p*-aminobenzoate) on day 5 and tissue samples were

collected. Gill, liver, and white muscle were collected for analysis of corticosteroid receptor mRNA expression and protein levels. Liver was chosen because of the well documented role of cortisol in regulating metabolism (reviewed by Mommsen et al., 1999; Wendelaar Bonga, 1997), white muscle because of the impact of social status on growth (DiBattista et al., 2006), and gill because it is a key site of ionic regulation, which is also regulated by cortisol in fish (reviewed by Mommsen et al., 1999; Wendelaar Bonga, 1997). Head kidney tissue was collected for MC2R, P450scc, and StAR mRNA analysis. Brain tissue, specifically POA and telencephalon, was collected for analysis of CRF and/or CRF-BP mRNA expression. The POA is the major site of CRF production for regulation of pituitary ACTH secretion (Alderman et al., 2008). Telencephalon was isolated in addition to POA for CRF-BP analysis owing to the possible regulation of CRF-BP by social stress in these tissues (Alderman et al., 2008). In addition, blood samples were collected by caudal puncture. Blood samples were centrifuged (10,000 *g* for 2 min) and plasma was extracted for later analysis of ACTH and/or cortisol concentrations using radioimmunoassay (RIA) kits (MP Biomedical) previously validated for analysis of trout plasma samples (Gamperl et al., 1994). A heparinized (2500 IU ml^{-1} heparin sodium salt from porcine intestinal mucosa, Sigma-Aldrich) syringe and a syringe rinsed with 0.5 M Na_2-ethylenediaminetetraacetic acid (EDTA), were used to collect blood samples for cortisol and ACTH analysis, respectively. Plasma and tissue samples were flash frozen in liquid nitrogen and stored at -80°C for later analysis.

2.3.3 Analysis of mRNA relative abundance

Total RNA was extracted from 10 to 100 mg of tissue using TRIzol reagent (Invitrogen) following the manufacturer's protocol. Tissues were homogenized by forcing the solution of TRIzol and tissue through 18 G and 23 G needles using a syringe until the solution passed easily through the needle. To facilitate homogenization, gill, liver, and white muscle samples were ground to a powder in liquid nitrogen using a mortar and pestle prior to homogenization. Extracted RNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer. To generate cDNA, 1 µg (for POA and telencephalon) or 2 µg (for gill, liver, white muscle, and head kidney) of RNA was first treated with deoxyribonuclease I (amplification grade, DNase; Invitrogen) according to the manufacturer's protocol. The RNA was then reverse transcribed using 100 U of RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) and 2 µg of random hexamer primer (IDT ReadyMade Primer) according to the manufacturer's protocol. The resultant cDNA was diluted with molecular grade water from the initial volume (20 µl) to a final volume of 40 µl, with the exception of the POA and telencephalon samples. To improve the efficiency of gene specific S-QPCR reactions, gill, liver, white muscle, and head kidney cDNA was purified using QIAquick PCR Purification kit (Qiagen) following the manufacturer's protocol to obtain an eluted volume of 40 µl.

Semi-quantitative RT-PCR was used to assess changes in mRNA relative abundances at various levels of the HPI axis. Target genes included: POA CRF; POA and telencephalon CRF-BP; head kidney MC2R, StAR, and P450scc; as well as gill, liver, and white muscle GR1, GR2, and MR. Gene-specific primers (Table 2.1) were generated using GeneTool software (BioTools Inc.). The ribosomal 18S subunit (AF309412.1) was

used as a reference gene and three sets of primers were designed to best suit specific primer sets: ''18S short'' was designed to suit the shorter products of CRF and CRF-BP primer sets; ''18S high'' was designed to suit a real-time RT-PCR cycle with a higher annealing temperature; and finally ''18S long'' was designed to suit the corticosteroid receptor primer sets (Table 1). The specificity of each primer pair was verified by sequencing the amplicons.

All S-QPCR reactions were performed in a similar manner using SYBR green mastermix kit (Stratagene) and an Mx3000P real-time PCR system (Stratagene). The manufacturer's instructions were followed with the exception that the total reaction volume was scaled to 12.5 μl instead of 25 μl. In addition, cDNA was diluted by 25-fold for CRF, CRF-BP, MC2R, and P450scc, as well as 1000-fold for the reference gene 18S. The annealing temperature of 58 °C (for GR1, GR2, and MR; 60 s) or 60 °C (for CRF, CRF-BP, MC2R, StAR, and P450scc; 60 s) and extension temperature of 72 °C (60 s) were used over 40 cycles. Standard curves were developed for each primer set (Table 2.1) using pooled cDNA from control, fasted, cortisol-treated, dominant, and subordinate fish, and reaction compositions were adjusted to optimize the efficiency of the reaction; the efficiency for each primer set was between 0.96 and 1.16 with the exception of GR2 that was 1.38. Following the completion of 40 cycles, SYBR green dissociation curves were used to confirm single amplicons for each primer pair. To ensure the absence of genomic DNA contamination, samples from which reverse transcriptase was omitted during cDNA synthesis were included in the analysis. For comparisons among treatment groups (control, fasted, cortisol-treated, dominant, and subordinate), the mRNA expression of each gene was calculated relative to the control

group using the modified delta-delta Ct method (Pfaffl, 2001) with 18S mRNA expression as a normalizing gene (Gilmour et al., 2010).

2.3.4 SDS–PAGE and Western blotting

Gill, liver, and white muscle samples were homogenized, and then sonicated in 50 mM Tris buffer (pH 7.5) supplemented with protease inhibitors (Roche, Mannheim, Germany) according to the basic protocol of Vijayan et al. (2006). Total protein concentration was determined using the bicinchoninic acid method with bovine serum albumin (BSA) as a standard. SDS–PAGE and Western blotting were performed according to established protocols (Boone et al., 2002; Sathiyaa and Vijayan, 2003). Briefly, samples in Laemmli's buffer (60 μg total protein for gill; 40 μg total protein for liver and white muscle) were separated by size on 8% polyacrylamide gels. Proteins were then transferred onto nitrocellulose membranes using a semidry transfer unit (Bio-Rad) at 20 V for 26 min with transfer buffer (25 mM Tris, 192 mM glycine, and 20% vol/vol methanol, pH 8.3). Membranes were blocked with 5% skimmed milk in TBS-T (20mM Tris, 300 mM NaCl and 0.1% Tween 20 with 0.02% sodium azide, pH 7.5) for 60 min at room temperature followed by an overnight incubation at 4°C in primary antibody for GR [polyclonal rabbit anti-trout GR, 1:1500; (polyclonal rabbit anti-trout GR, 1:1500; (Sathiyaa and Vijayan, 2003)] or 1 h 45 min at room temperature for MR (polyclonal rabbit anti-zebrafish MR, 1:1000; custom made using antigenic peptide sequence of zebrafish MR from (Alsop and Vijayan, 2008); MR 63-79, Ac-FKNTEAT-MIRVNQNQPLC-amide; and the affinity purified serum was used; 21st century Biochemicals, Inc.). The specificity was confirmed by pre-absorbing the antibody with

the specific peptide, as well as serial dilution of the sample (Sandhu et al., 2014). Membranes were washed with TBS-T (3-10min) and TBS (1-10 min) and incubated in secondary antibody (horseradish-peroxidase goat anti-rabbit IgG, 1:3636; Bio-Rad) for 60 min at room temperature and subsequently washed as before. Band detection was carried out with the ECL-Plus Western blotting detection system (GE Healthcare Life Sciences) and scanned by Typhoon imager using Cy2 blue laser. Bands were quantified using Alpha-Ease software (Alpha Innotech). Equal loading of samples was confirmed by probing the blots with β-actin (mouse Cy3-coupled monoclonal primary antibody, 1:1000; Sigma). Samples were expressed relative to β-actin as well as relative to a reference sample that was run on each blot.

2.3.5 Statistical analysis

Data are expressed as mean values ± 1 SEM. One-way ANOVA followed by post hoc multiple comparisons tests, as appropriate, was used to assess the statistical significance of differences among treatment groups. In cases where data did not meet the assumptions of normality and/or equal variance, non-parametric tests were used (one-way ANOVA on ranks) or data were transformed. The fiducial limit of significance in all tests was 0.05, and tests were carried out using a commercial package (SigmaStat v3.5 in SigmaPlot v11; SPSS).

Table 2.1 Oligonucleotide primer pairs used for semi-quantitative real-time RT-PCR in rainbow trout (*Oncorhynchus mykiss*).

Gene	Primers	Accession number	Reference
18S "short"	F – cgg cgg cgt tat tcc cat gac R – ccc ccc gga acc caa aga ctt	AF309412.1	
CRF	F – aca acg actc aac tga aga tct cg R – agg aaa ttg agc ttc atg tca gg	AF296672.1	Bernier et al. (2008)
CRF-BP	F – gga gga gac ttc atc aag gtg tt R – ctt ctc tcc ctt cat cac cca g	AY363677.1	Alderman et al. (2008)
18S "high"	F – ggc ggc gtt att ccc atg a R – tgc cct tcc gtc aat tcc ttt a	AF309412.1	
MC2R	F – gag aac ctg ttg gtg gtg gt R – gag gga gga gat ggt gtt ga	EU119870.1	Aluru and Vijayan (2008)
StAR	F – tgg gga agg tgt tta agc tg R – agg gtt cca gtc tcc cat ct	AB047032.2	Aluru and Vijayan (2008)
P450scc	F – gct tca tcc agt tgc agt ca R – cag gtc tgg gga aca cat ct	S57305.1	Aluru and Vijayan (2008)
18S "long"	F – ggc ggc gtt att ccc atg acc R – ggt ggt gcc ctt ccg tca att c	AF309412.1	
GR1	F – ccg aac cct ccc cca cct gac R – att ccc gac acc tcc acc cgc	NM001124730.1	
GR2	F – ggg gca agg ggg caa cac aac R – acc act gcc gag ctg acc ccc	NM001124482.1	
MR	F – caa cgt agg cct gga cca cat R – gtc tct tcc agt cag gtc cgt cc	AY495584.1	

Primer sequences are given in the 5' to 3' direction. CRF, corticotropin-releasing factor; CRF-BP, CRF-binding protein; MC2R, melanocortin 2 receptor; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 side chain cleavage enzyme; GR, glucocorticoid receptor; MR, mineralocorticoid receptor.

2.4 Results

Juvenile rainbow trout paired for 5 days formed social hierarchies as indicated by the strongly polarized behaviour scores determined for dominant (0.98 ± 0.07, $N = 23$) *versus* subordinate (-0.98 ± 0.06, $N = 23$) fish.

Activation of the stress axis elevates circulating cortisol levels, and ACTH is a proximate stimulus for cortisol synthesis. Plasma cortisol concentrations were significantly higher in subordinate fish than in control, fasted, or dominant fish (Fig. 2.1A; ANOVA on ranks; $P < 0.001$). Cortisol-treated fish exhibited circulating cortisol levels comparable to those of subordinate fish. Correspondingly, plasma ACTH concentrations in subordinate and cortisol-treated fish were significantly lower than those in all other treatment groups (Fig. 2.1B; ANOVA on inverse transformed data; $P = 0004$).

The stress axis is activated at the level of the brain where CRF, thought to be regulated at least in part by CRF-BP, acts on corticotropes to stimulate ACTH release. Preoptic area CRF relative mRNA levels were significantly elevated in cortisol-treated fish compared to all other treatment groups (Fig. 2.2A; ANOVA; $P = 0.006$). By contrast, CRF-BP exhibited essentially the opposite pattern of relative mRNA expression. In the telencephalon, CRF-BP relative mRNA levels were lower in cortisol-treated fish compared to control and fasted fish, although not significantly lower than in dominant or subordinate fish (Fig. 2.2B; ANOVA on ranks; $P = 0.014$). A similar trend was observed in POA CRF-BP relative mRNA levels but did not quite reach statistical significance (Fig. 2.2C; ANOVA; $P = 0.080$). In no case were significant differences between dominant and subordinate fish detected.

Social status may also exert modulatory effects on the interrenal tissue, the site of cortisol synthesis. Although MC2R relative mRNA expression did not differ significantly among treatment groups (Table 2.2; ANOVA; $P = 0.679$), differences were observed in the relative mRNA expression of key players in cortisol biosynthesis. Specifically, dominant fish exhibited significantly higher StAR and P450scc relative mRNA levels than all other treatment groups (Fig. 2.3; ANOVA on ranks; $P = 0.034$ and 0.017, respectively).

Finally, in addition to changes in the stress axis itself, modulation may occur at the level of target tissues through changes in corticosteroid receptor expression. The liver plays a critical role in metabolism and the regulation of metabolism by cortisol through GRs. Liver GR1 and GR2 relative mRNA levels differed significantly among treatment groups (ANOVA on ranks; $P = 0.037$ and ANOVA; $P = 0.013$, respectively), but in the case of liver GR1, post hoc tests were not able to identify the origin of these differences (Fig. 2.4A). For GR2, subordinate fish exhibited significantly lower relative mRNA expression than all other treatment groups (Fig. 2.4B). The GR antibody used in this study is unable to distinguish between GR1 and GR2 and therefore total GR protein levels were measured. Liver GR relative protein expression was significantly lower in subordinate fish than in control fish, as was also the case for cortisol-treated fish (Fig. 2.4C and 2.5; ANOVA on log transformed data; $P = 0.021$), despite the absence of an effect of cortisol treatment on liver GR mRNA relative levels. Interestingly, the relative mRNA expression of MR in liver was significantly lower in both dominant and subordinate fish than in control or fasted fish (Fig. 2.6A; ANOVA; $P < 0.001$). However,

no significant differences among treatment groups were detected in liver MR protein levels (Fig. 2.6B and 2.7; ANOVA; $P = 0.399$).

The gill is central to ionic regulation, in which cortisol plays a regulatory role, and in addition, social status impacts growth, suggesting that effects may arise in white muscle. Significant treatment effects were found in gill and white muscle GR2 relative mRNA expression. Glucocorticoid receptor 2 mRNA relative abundance was significantly higher in the gill of fasted fish than in subordinate fish (Fig. 2.8A; ANOVA; $P = 0.020$), and significantly lower in white muscle of dominant fish than in all other treatment groups (Fig. 2.8B; ANOVA on ranks; $P = 0.019$). However, no other significant differences in GR or MR mRNA or protein expression among treatment groups were detected in gill and white muscle tissue (Table 2.2). Gill and white muscle MR protein expression was below the level of detection of the Western blot analysis.

Figure 2.1 Plasma cortisol (A) and adrenocorticotropic hormone (ACTH; B) levels in control (con; *N* = 12 and 8 for A and B, respectively), fasted (fast; *N* = 15 and 6), cortisol-treated (cort; *N* = 15 and 8), dominant (dom; *N* = 16 and 7), and subordinate (sub; *N* = 16 and 7) rainbow trout (*Oncorhynchus mykiss*). Values are presented as means + SEM. Treatment groups that share a letter are not significantly different from one another (see text for details).

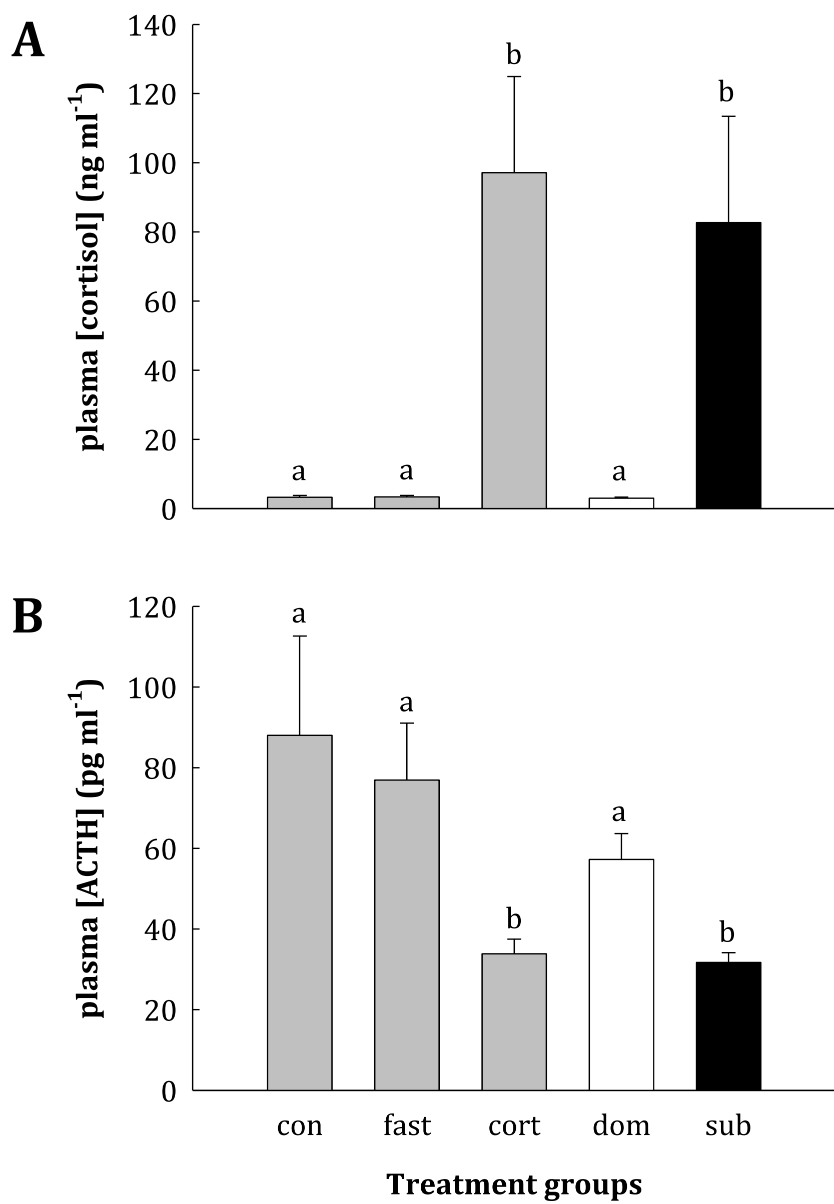

Figure 2.1

Figure 2.2 Relative mRNA levels of corticotropin-releasing factor (CRF) in the preoptic area (POA; A), and CRF-binding protein (CRF-BP) in the telencephalon (Tel; B) and POA (C) of control (con), fasted (fast), cortisol-treated (cort), dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*). In each case, *N* = 6 for all treatment groups except for dominant fish where *N* = 4-6. All data are expressed relative to mRNA expression of the normalizing 18S gene, and to the control group. Values are presented as means + SEM. Treatment groups that share a letter are not significantly different from one another (see text for details).

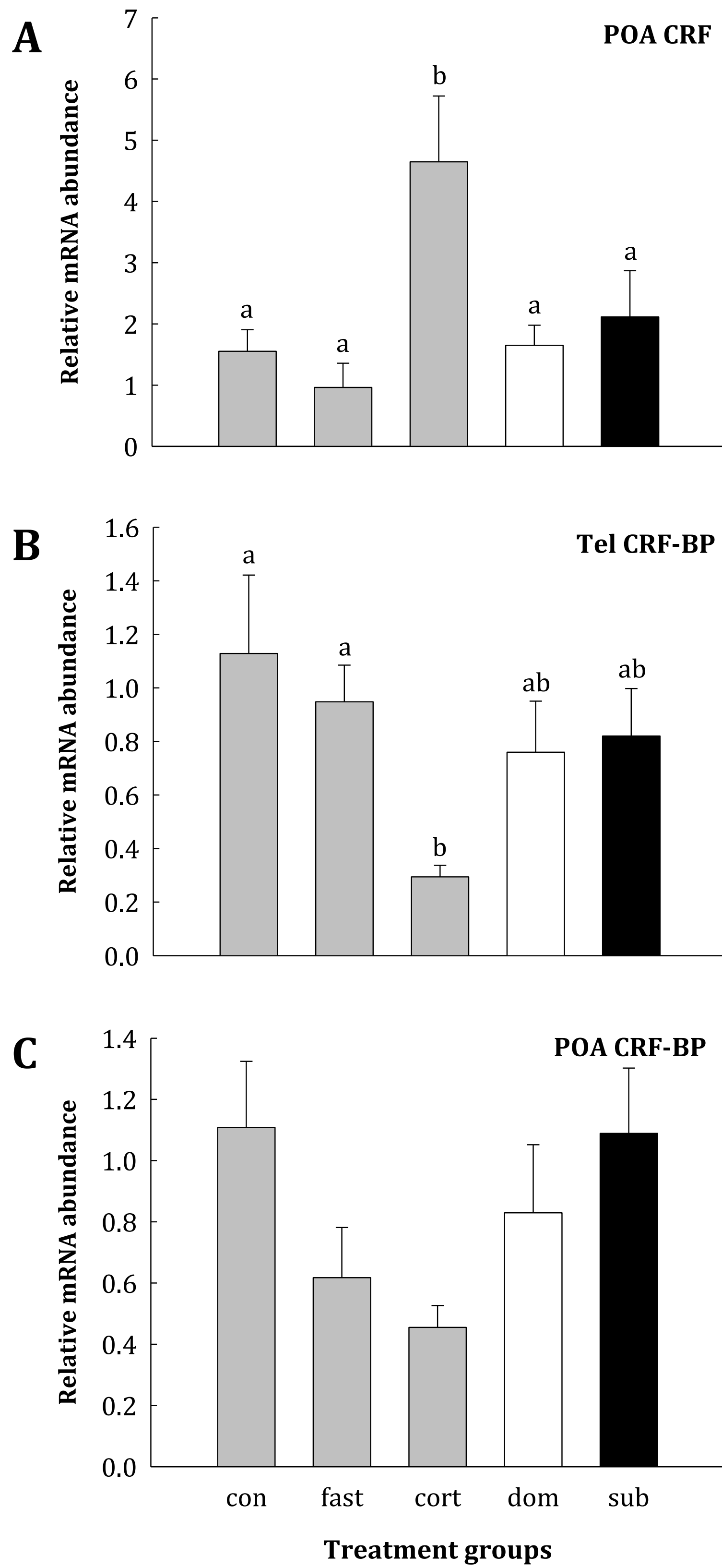

Figure 2.2

Figure 2.3 Relative mRNA levels of steroidogenic acute regulatory protein (StAR; A) and cytochrome P450 side chain cleavage enzyme (P450scc; B) in head kidney of control (con), fasted (fast), cortisol-treated (cort), dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*). For all treatment groups, N = 6. All data are expressed relative to mRNA expression of the normalizing 18S gene, and to the control group. Values are presented as means + SEM. Treatment groups that share a letter are not significantly different from one another (see text for details).

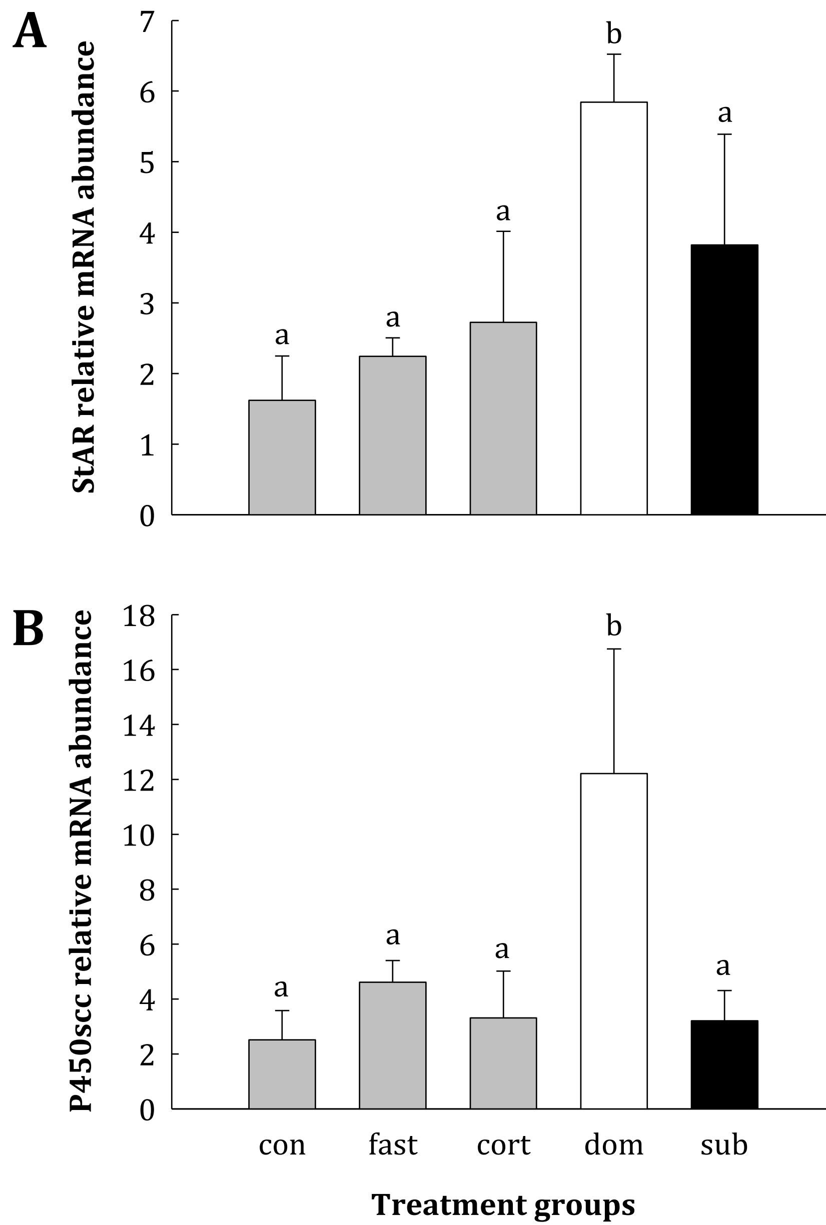

Figure 2.3

Figure 2.4 Relative mRNA levels of glucocorticoid receptor (GR) 1 (A) and GR2 (B), as well as relative protein levels of GR (C) in liver of control (con; *N* = 6-7), fasted (fast; *N* = 5-7), cortisol-treated (cort; *N* = 5-7), dominant (dom; *N* = 6-7), and subordinate (sub; *N* = 6-7) rainbow trout (*Oncorhynchus mykiss*). In panels A and B, data are expressed relative to mRNA expression of the normalizing 18S gene, and to the control group. In panel C, data are expressed relative to protein levels of β-actin and to a blot reference sample. Values are presented as means + SEM. Treatment groups that share a letter are not significantly different from one another (see text for details).

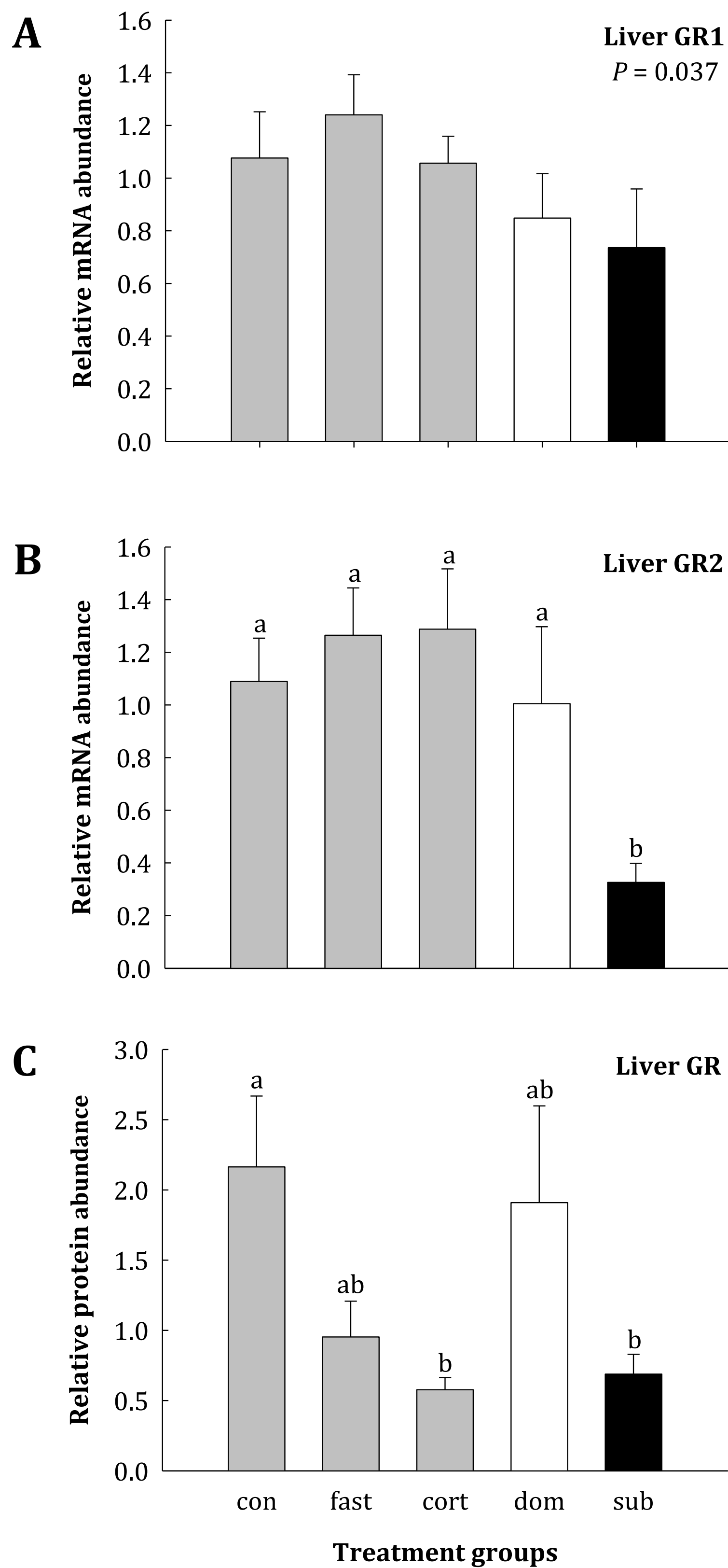

Figure 2.4

Figure 2.5 Representative western blots for liver total glucocorticoid receptor (GR; A) and β-actin (B) protein levels of control (con), fasted (fast), cortisol-treated (cort), dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*). A reference (ref) sample was included in every blot as a cross-blot comparison. Molecular weight marker (mm) sizes are in kilodaltons. Lane (x) was not used in the analysis.

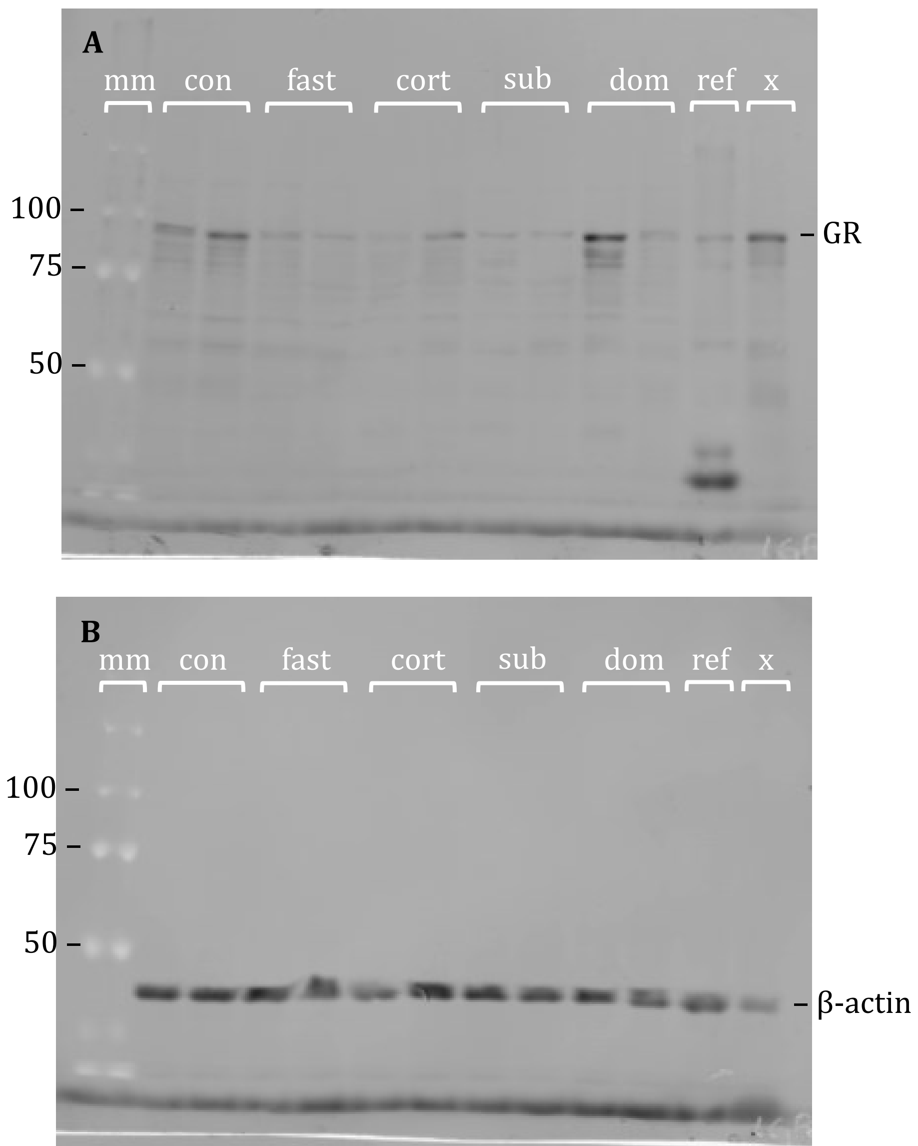

Figure 2.5

Figure 2.6 Relative mRNA (A) and protein levels (B) of mineralocorticoid receptor (MR) in liver of control (con; N = 6-7), fasted (fast; N = 6-7), cortisol-treated (cort; N = 5-7), dominant (dom; N = 6-7), and subordinate (sub; N = 6-7) rainbow trout (*Oncorhynchus mykiss*). In panel A, data are expressed relative to mRNA expression of the normalizing 18S gene, and to the control group. In panel B, data are expressed relative to protein levels of β-actin and to a blot reference sample. Values are presented as means + SEM. Treatment groups that share a letter are not significantly different from one another (see text for details).

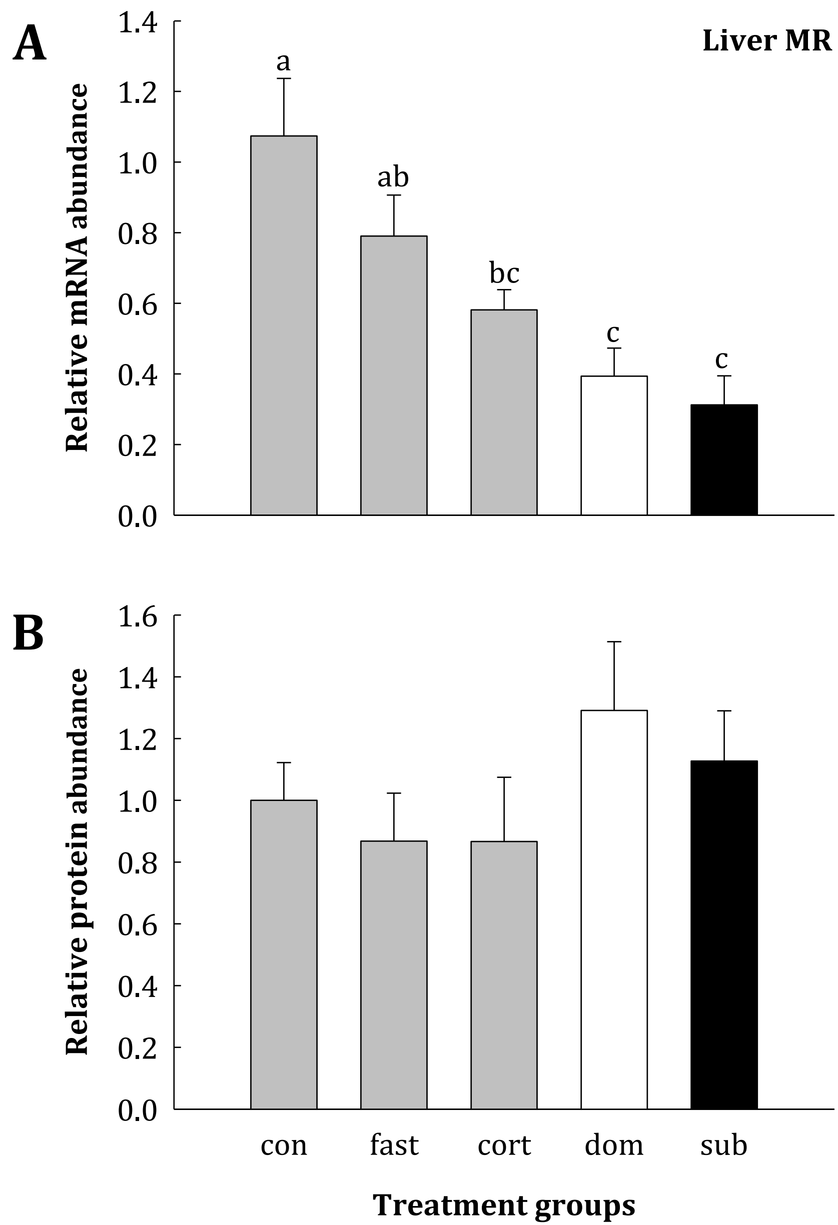

Figure 2.6

Figure 2.7 Representative western blots for liver mineralocorticoid receptor (MR; A) and β-actin (B) protein levels of control (con), fasted (fast), cortisol-treated (cort), dominant (dom), and subordinate (sub) rainbow trout (*Oncorhynchus mykiss*). A reference (ref) sample was included in every blot as a cross-blot comparison. Molecular weight marker (mm) sizes are in kilodaltons.

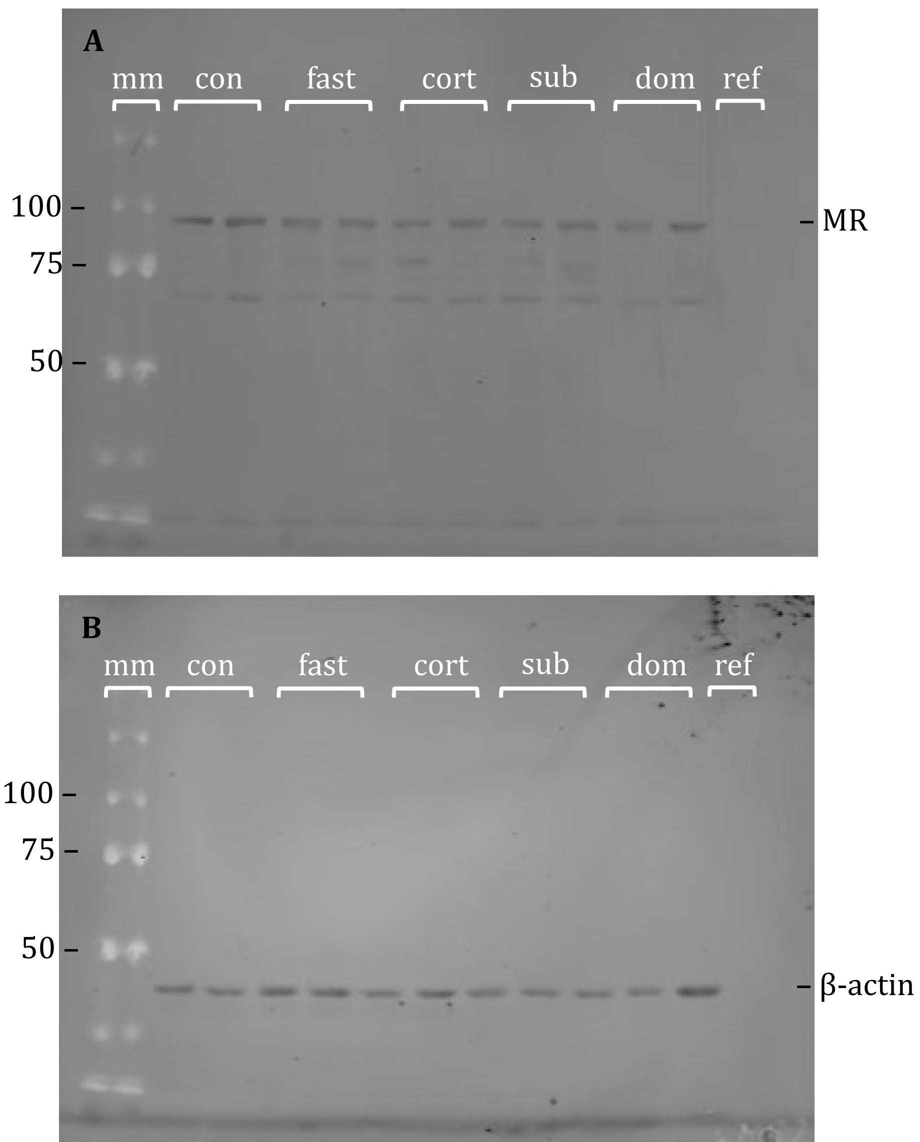

Figure 2.7

Figure 2.8 Relative mRNA levels of glucocorticoid receptor (GR) 2 in gill (A) and white muscle (B) of control (con; *N* = 6-7), fasted (fast; *N* = 6-7), cortisol-treated (cort; *N* = 5-7), dominant (dom; *N* = 6-7), and subordinate (sub; *N* = 6-7) rainbow trout (*Oncorhynchus mykiss*). All data are expressed relative to mRNA expression of the normalizing 18S gene, and to the control group. Values are presented as means + SEM. Treatment groups that share a letter are not significantly different from one another (see text for details).

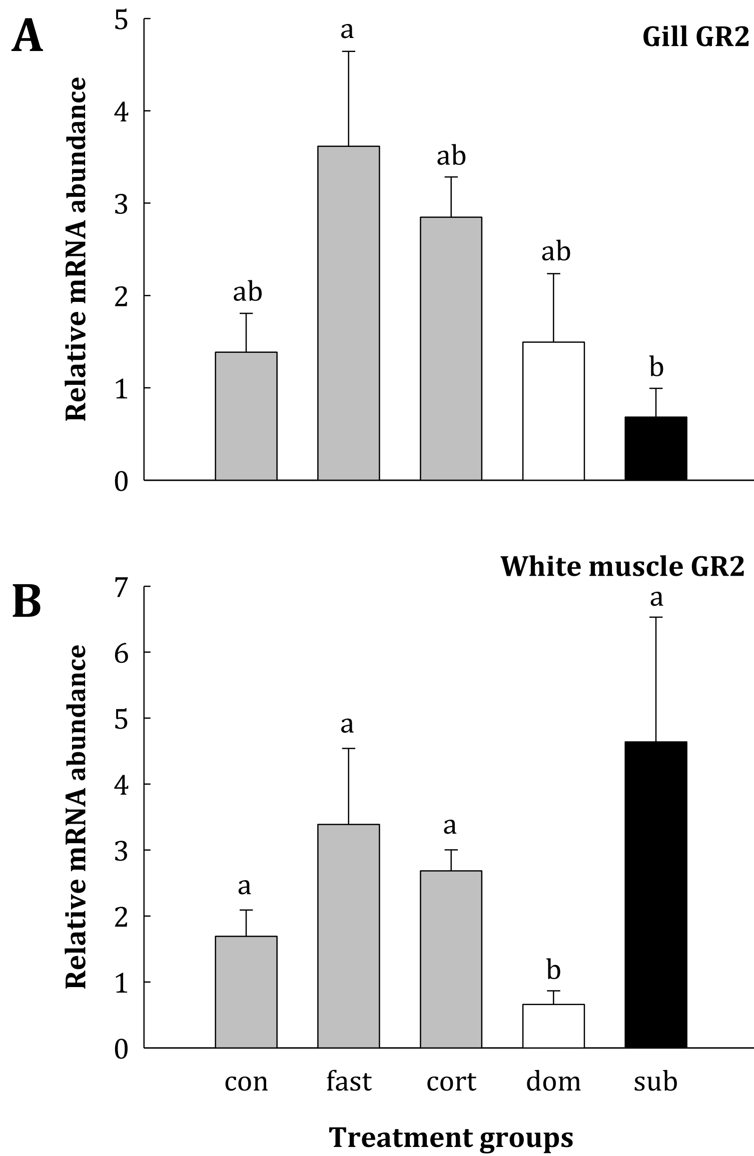

Figure 2.8

Table 2.2 Relative mRNA expression and/or protein levels of head kidney melanocortin 2 receptor, and gill and white muscle corticosteroid receptors in rainbow trout (*Oncorhynchus mykiss*).

Tissue	Gene/protein	Treatment group					*P* value
		Control	Fasted	Cortisol-treated	Dominant	Subordinate	
Head kidney	MC2R mRNA	1.73 ± 0.64	2.55 ± 0.32	3.17 ± 1.00	3.37 ± 0.71	2.64 ± 1.22	0.679
Gill	GR1 mRNA	1.24 ± 0.33	1.67 ± 0.36	1.38 ± 0.20	2.03 ± 0.82	0.61 ± 0.25	0.263
	MR mRNA	1.18 ± 0.25	1.44 ± 0.33	1.55 ± 0.16	1.33 ± 0.59	0.51 ± 0.23	*0.170*
	GR protein	0.21 ± 0.02	0.36 ± 0.10	0.45 ± 0.09	0.23 ± 0.04	0.25 ± 0.04	0.079
White muscle	GR1 mRNA	1.24 ± 0.33	1.67 ± 0.36	1.38 ± 0.20	2.03 ± 0.82	0.61 ± 0.26	*0.263*
	MR mRNA	1.61 ± 0.41	1.35 ± 0.51	1.61 ± 0.29	1.69 ± 0.38	1.25 ± 0.20	0.893
	GR protein	1.00 ± 0.16	1.10 ± 0.35	1.64 ± 0.44	2.08 ± 0.76	0.91 ± 0.14	*0.428*

Values are means ± SEM; *N* = 7 in all cases except MC2R and gill GR protein, where *N* = 6. All "mRNA" data are expressed relative to mRNA expression of the normalizing 18S gene, and to the control group. "Protein" data are expressed as relative to protein levels of β-actin and to a blot reference sample. Data were analyzed by one-way ANOVA (on ranks indicated by italics); in no case did the differences reach statistical significance. MC2R, melanocortin 2 receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor.

2.5 Discussion

This study provides evidence that social status modulates HPI axis function in rainbow trout. As expected from previous studies (e.g., DiBattista et al., 2005; DiBattista et al., 2006; Doyon et al., 2003; Øverli et al., 1999a; Sloman et al., 2001), circulating cortisol levels were elevated in subordinate trout. The impact of low social status on food intake has also been well documented, (e.g., DiBattista et al., 2006; McCarthy et al., 1992; Øverli et al., 1998; Winberg et al., 1993), and activation of the HPI axis is associated with fasting during periods of stress (reviewed by Bernier, 2006). Thus, cortisol-treated and fasted fish were utilized to understand whether observed changes in steroidogenesis and corticosteroid receptor dynamics in the subordinate group were related to high plasma cortisol levels and/or fasting. The results suggest that persistent steroid elevation in subordinate fish may explain some differences in HPI axis function between dominant and subordinate fish, whereas fasting does not, leaving open the possibility that other mechanisms may mediate responses in subordinate fish.

2.5.1 Effects of social status on HP axis function

Adrenocorticotropic hormone is the primary secretagogue for cortisol in fish (reviewed by Mommsen et al., 1999; Wendelaar Bonga, 1997); exposure to an acute stressor (e.g., handling, confinement, or thermal shock) typically causes elevation of circulating ACTH concentrations in conjunction with a rise in plasma cortisol levels (e.g., Aluru and Vijayan, 2008; Balm and Pottinger, 1995; Pickering et al., 1986; Sumpter et al., 1986). However, much less is known about plasma ACTH levels in

chronically-stressed animals (Balm and Pottinger, 1995; Höglund et al., 2000). In the current study, plasma cortisol levels in subordinate fish (representing a chronic stress scenario) were significantly higher than those in control or dominant fish, despite lower plasma ACTH levels, a situation that may point to alterations in cortisol clearance (Mommsen et al., 1999). Low cortisol clearance rates in subordinate fish could explain persistent elevation of plasma cortisol concentrations in the face of low ACTH levels and is a possibility that warrants investigation. By contrast, plasma levels of both cortisol and ACTH were elevated in subordinate Arctic charr (Höglund et al., 2000); species differences and/or effects of group size (groups of 3 *versus* pairs) could account for this difference. In the present study, cortisol-treated fish also exhibited high cortisol and low ACTH levels, suggesting a role for cortisol in modulating ACTH levels through negative feedback in both subordinate and cortisol-treated fish. These findings are in agreement with previous work in rainbow trout in which cortisol-treated fish exhibited lower levels of ACTH than control fish (Balm and Pottinger, 1995). The same study reported that 96 h after the onset of confinement, ACTH levels that were initially high began to decrease back to control levels despite sustained high cortisol levels (Balm and Pottinger, 1995). Regulation of ACTH levels in fish chronically stressed by subordinate social status (this study) or confinement (Balm and Pottinger, 1995) may provide a mechanism to down-regulate HPI axis function.

How is the reduction in plasma ACTH levels in chronically-stressed trout achieved? CRF is a key neuropeptide in the stress response, specifically in stimulating the release of ACTH from the pituitary. In subordinate fish of the present study, POA CRF relative mRNA levels did not differ from those of control or dominant fish.

However, previous studies have reported significant elevation of POA CRF mRNA levels in subordinate rainbow trout at 8 h (Bernier et al., 2008) and 3 days (Doyon et al., 2003) of social interaction. Thus, the absence of a significant difference between dominant and subordinate trout in the present study suggests that decreases in CRF may contribute to the lowering of plasma ACTH levels in subordinate fish after 5 days of social interaction. This response may be independent of cortisol, because POA CRF mRNA levels were significantly elevated in cortisol-treated fish relative to the other treatment groups. These findings are consistent with the observation of Bernier et al. (2008) that POA CRF relative mRNA expression was not correlated in magnitude or duration with the plasma cortisol levels of rainbow trout exposed to stressors of different intensities, and emphasize the complexity of HPI axis regulation that likely exists (Wendelaar Bonga, 1997).

The CRF-BP provides a good example of this complexity. This highly conserved protein is broadly expressed in the brain of rainbow trout and its distribution is consistent with a role in regulating the functions of CRF, although its actions remain to be defined. Stress-induced changes in CRF-BP mRNA abundance suggest a role for this protein in the regulation of the HPI axis and/or stress responses (Alderman et al., 2008; Doyon et al., 2005). In particular, Alderman et al. (2008) reported differences in telencephalon and hypothalamic, but not POA mRNA expression of CRF-BP between dominant and subordinate trout after 24 h but not 8 h of social interaction. Similarly, we found that POA CRF-BP mRNA was unaffected by social status. Telencephalon CRF-BP relative mRNA abundance was also unaffected by social status at 5 days of interaction in the present study, a result that emphasizes the time-dependent nature of

the changes in this protein. Interestingly, cortisol-treated fish exhibited telencephalon CRF-BP mRNA expression that was significantly lower than that of the other treatment groups. As with CRF, differences in the level of CRF-BP mRNA between subordinate and cortisol-treated fish indicate that a factor other than cortisol modulates telencephalon CRF-BP in subordinate fish.

2.5.2 Effects of social status on interrenal steroidogenic capacity

Modulation of the stress axis by social status may also occur at the site of cortisol synthesis in the interrenal tissues. Previous studies have reported desensitization of the acute cortisol response by chronic stressors such as confinement (e.g., Balm and Pottinger, 1995), high density stocking (e.g., Rotllant et al., 2000b), exposure to pollutants (e.g., Hontela, 1998), and social status (e.g., Sloman et al., 2002). The present study provides insight into the possible mechanisms underlying such effects.

Cortisol synthesis is stimulated by the action of ACTH on MC2R, and exposure of rainbow trout to an acute netting/chasing stressor resulted in a significant, transient increase in MC2R mRNA relative abundance (Aluru and Vijayan, 2008). In the present study, MC2R mRNA relative levels did not differ significantly among treatment groups. A similar absence of change in MC2R transcript levels was reported following a 14-day exposure to wastewater effluent (Ings et al., 2011). These results suggest that MC2R gene regulation may be transient and more apparent with an acute stressor than with chronic stress. However, we cannot exclude the possibility of differences in protein levels or other factors regulating MC2R activation, including accessory proteins.

The signaling cascade initiated by the action of ACTH on MC2R leads to the transport of cholesterol from the outer to the inner mitochondrial membrane, a step in which StAR is thought to play a key role (Stocco, 2000), followed by the enzymatic conversion of cholesterol to pregnenolone and ultimately, cortisol. The cleavage of cholesterol to yield pregnenolone is the first and rate-limiting step in cortisol biosynthesis and is catalyzed by P450scc (Payne and Hales, 2004; Stocco, 2000). Transcriptional regulation of StAR and P450scc has been reported in trout head kidney tissue following ACTH stimulation or acute stress (Aluru and Vijayan, 2006; Geslin and Auperin, 2004; Hagen et al., 2006). Less is known about effects of chronic stress, although high density stocking in gilthead seabream elevated StAR transcript levels compared to controls (Castillo et al., 2008). Social subordinance in the present study did not affect StAR or P450scc mRNA, suggesting that transcriptional regulation of the steroid biosynthetic machinery does not contribute to the high cortisol levels of these fish. The relative mRNA expression of StAR and P450scc were, however, significantly elevated in dominant fish. Dominant fish experience acute stress during the early stages of hierarchy formation, as evidenced by elevation of cortisol and catecholamine levels (Øverli et al., 1999a; Thomas and Gilmour, 2006). Elevation of transcripts (and presumably protein) critical to cortisol synthesis several days following acute stress is suggestive of a 'pre-adaptation' effect, that is, that the acute stress of hierarchy formation prepares the stress axis for subsequent responses. Future studies are required to better elucidate the immediate and longer-term impacts of both acute and chronic stress on the modulation of cortisol biosynthetic capacity and the cortisol response of fish exposed to multiple stressors.

2.5.3 Effects of social status on target tissue corticosteroid receptor expression

A key component of stress axis activation is the target tissue response to corticosteroids and this is mediated in part by the regulation of corticosteroid receptors (Aluru and Vijayan, 2007; Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). Although most fish, including trout, possess multiple GRs, the distinct roles of these various isoforms remain largely unknown (e.g., Alsop and Vijayan, 2008; Bury et al., 2003; Greenwood et al., 2003). In the present study, there was little evidence of differential regulation of corticosteroid receptor transcript or protein levels by social status in any tissue but liver. Previous studies in rainbow trout found that liver (a well-established site of cortisol-mediated control of metabolism) GR mRNA expression (including both GR1 and GR2 isoforms) was elevated, and GR protein levels were depressed, in fish treated with a cortisol implant or in hepatocytes exposed to cortisol *in vitro* (Aluru and Vijayan, 2007; Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). These results suggested the presence in hepatocytes of a negative feedback loop for GR gene regulation by cortisol. As in previous work, cortisol-treated fish in the present study exhibited a decrease in liver GR protein levels, but this decrease occurred in the absence of any change in mRNA expression of either GR1 or GR2 relative to control fish. This difference may reflect differences in GR mRNA stability between the two studies, perhaps mediated by changes in circulating cortisol levels [approximately 400 ng ml^{-1} (Vijayan et al., 2003) *versus* less than 100 ng ml^{-1} (present study)]. Subordinate fish also exhibited significantly lower liver GR protein levels than control fish, accompanied by significantly lower GR2 relative mRNA abundance. Vijayan et al. (2003) hypothesized that GR auto-regulation may serve to offset decreased tissue

responsiveness resulting from GR down-regulation by chronic cortisol stimulation. The findings of the current study suggest that aspects of social stress override GR auto-regulation, perhaps as a protective mechanism to minimize liver GR stimulation by the persistent steroid elevation elicited by chronic social stress.

2.5.4 Conclusion

In summary, low social status resulted in modulation of HPI axis function. Plasma cortisol levels of subordinates were higher than those of dominant fish despite low ACTH levels, perhaps owing to altered rates of cortisol clearance. Low plasma ACTH levels in subordinate fish may be a direct result of high plasma cortisol levels; this response may serve to attenuate activity of the HPI axis in a situation of continued stimulation by the presence of the dominant fish, a stressor. Furthermore, target tissue responses in liver are likely to be affected in subordinate fish owing to GR down-regulation. In dominant fish, transcripts (and presumably protein) for steroid biosynthesis were increased, a response that may result from transient elevation of plasma cortisol levels during hierarchy formation and may have adaptive value in responding to subsequent stressors. This study is the first to examine the effects of social status on regulation of HPI axis functioning by targeting multiple components of the stress axis. The results support not only the down-regulation of key players at multiple levels of the HPI axis in subordinate fish that was predicted to occur, but also that dominant social status may affect cortisol biosynthesis. Further studies are required to elucidate the consequences of these differences in HPI axis function between dominant and subordinate fish.

2.6 Acknowledgments

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CHAPTER 3

Social stress modulates the cortisol response to an acute stressor in rainbow trout (*Oncorhynchus mykiss*)

Notes on Chapter 3

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M.J. Gollock was a postdoctoral fellow in the Gilmour lab and carried out the “series 1, *in vivo* cortisol response” and “series 2, cortisol production *in vitro*” experiments, see sections 3.3.2.1 and 3.3.2.2 in the Materials and Methods section.

3.1 Abstract

In rainbow trout of subordinate social status, circulating cortisol concentrations were elevated under resting conditions but the plasma cortisol and glucose responses to an acute stressor (confinement in a net) were attenuated relative to those of dominant trout. An *in vitro* head kidney preparation, and analysis of the expression of key genes in the stress axis prior to and following confinement in a net were then used to examine the mechanisms underlying suppression of the acute cortisol stress response in trout experiencing chronic social stress. With porcine ACTH as the secretagogue, ACTH-stimulated cortisol production was significantly lower for head kidney preparations from subordinate trout than for those from dominant trout. Dominant and subordinate fish did not, however, differ in the relative mRNA abundance of MC2R, StAR, or P450scc within the head kidney, although the relative mRNA abundance of these genes was significantly higher in both dominant and subordinate fish than in sham trout (trout that did not experience social interactions but were otherwise treated identically to the dominant and subordinate fish). The relative mRNA abundance of all three genes was significantly higher in trout exposed to an acute net stressor than under control conditions. Upstream of cortisol production in the stress axis, plasma ACTH concentrations were not affected by social stress, nor was the relative mRNA abundance of the binding protein for CRF. The relative mRNA abundance of CRF in the POA of subordinate fish was significantly higher than that of dominant or sham fish 1 h after exposure to the stressor. Collectively, the results indicate that chronic social stress modulates cortisol production at the level of the interrenal cells, resulting in an attenuated cortisol response to an acute stressor.

3.2 Introduction

In teleost fish, as in other vertebrates, the endocrine response to a stressor encompasses both an adrenergic response, the mobilization of catecholamines, and a HPI (HPA, in other vertebrates) response, the synthesis and secretion of glucocorticoid hormones (reviewed by Wendelaar Bonga, 1997). In teleost fish, cortisol is the main glucocorticoid. Cortisol production in response to a stressor is initiated by activation of MC2R (Aluru and Vijayan, 2008) by ACTH released from the pituitary (Bernier et al., 2009; Wendelaar Bonga, 1997). Release of ACTH, in turn, reflects the action of CRF on the corticotropes of the pituitary (reviewed by Bernier et al., 2009; Flik et al., 2006). Corticotropin-releasing factor is synthesized in the POA of the brain as well as in several hypothalamic areas (Bernier et al., 2009), and its actions are thought to be regulated at least in part by CRF-BP (Alderman et al., 2008; Seasholtz et al., 2002). Binding of ACTH to MC2R, a G protein-coupled receptor, activates AC to initiate a cAMP-signalling cascade resulting in the movement of cholesterol to the inner mitochondrial membrane with the help of StAR (Aluru and Vijayan, 2008; Hagen et al., 2006). Subsequent conversion of cholesterol to cortisol begins with the cleavage of cholesterol to pregnenolone by P450scc; this step is the first and rate-limiting step in cortisol biosynthesis (Aluru and Vijayan, 2008; Mommsen et al., 1999).

Although the cortisol response to an acute stressor is thought to benefit the fish by, for example, mobilizing energy reserves, prolonged elevation of circulating cortisol levels during exposure to a chronic stressor may result in negative effects (Wendelaar Bonga, 1997). For example, both chronic stress and the administration of cortisol to mimic chronic stress have been reported to diminish the cortisol response to an acute

stressor (Balm et al., 1994; Barton et al., 1987; Ings et al., 2011; Øverli et al., 1999b; Rotllant et al., 2000a; Wunderink et al., 2011). The mechanisms underlying attenuation of the acute cortisol response in chronically-stressed fish remain poorly understood. Decreased interrenal cell-sensitivity to ACTH has been documented in several studies (Rotllant et al., 2000b; Sloman et al., 2002; Vijayan and Leatherland, 1990), whereas other studies failed to find an effect of cortisol administration or chronic stress on the response of interrenal cells to ACTH and pointed instead to possible effects upstream in the HPI axis, at the level of the pituitary or brain (Rotllant et al., 2000a). Thus, the objective of the present study was to investigate mechanisms at the level of the brain, pituitary, and interrenal cells through which chronic stress could suppress the cortisol response to an acute stressor.

Juvenile salmonid fish confined in pairs form social hierarchies in which subordinate fish experience a sustained elevation of circulating cortisol concentrations characteristic of chronic stress (reviewed by Gilmour et al., 2005; e.g., Sloman et al., 2001). Subordinate rainbow trout exhibited (unstressed) plasma ACTH concentrations that were significantly lower than control values (Jeffrey et al., 2012), and rates of ACTH-stimulated cortisol production for *in situ* preparations from subordinate trout were lower than those for dominant trout (Sloman et al., 2002). These observations suggest that the cortisol response to an acute stressor will be compromised in subordinate trout. To test this hypothesis, the cortisol response to an acute netting stressor was evaluated in dominant *versus* subordinate trout. In addition, mechanisms that could attenuate the cortisol response to an acute stressor in fish experiencing chronic social stress were investigated at the level of the brain (CRF and CRF-BP mRNA

abundance), pituitary (circulating ACTH levels), and interrenal cells (cortisol production *in vitro*; MC2R, StAR, and P450scc mRNA abundance).

3.3 Materials and methods

3.3.1 Experimental animals

Female juvenile rainbow trout (mass = 83.6 ± 1.3 g, fork length = 20.2 ± 0.1 cm, mean ± SEM, N = 167) obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) were transported to the University of Ottawa aquatics facility and housed in 1275 L fibreglass stock tanks. Tanks were supplied with flowing, dechloraminated, aerated city of Ottawa tap water at 13°C. Fish were held under a 12:12 h L:D photoperiod and were fed every second day to satiation by scattering commercial trout pellets on the surface of the water. Trout were allowed to acclimate to these holding conditions, which tended to minimize hierarchy formation (e.g., use of scatter feeding, homogeneous tanks with a mild current), for at least 2 weeks prior to experimentation. All experiments complied with University of Ottawa institutional guidelines, were approved by the Animal Care Committee (protocol BL-228), and were in accordance with guidelines of the Canadian Council on Animal Care for the use of animals in research and teaching.

Social hierarchies were established in fork-length matched pairs of rainbow trout (fork-length difference averaged 2 ± 0.2 mm or 1.1% of fork length for 71 pairs in total) that were confined together for 3 to 5 d. Fish in a “sham” treatment group were handled identically but housed individually rather than with a conspecific. Rainbow trout were lightly anesthetized (to the point of losing equilibrium) in a solution of

benzocaine (0.05 g L^{-1} ethyl *p*-aminobenzoate) and fish mass, fork length, and fin damage were assessed. Fork length-matched fish were placed individually on either side of an opaque divider in a 40 L flow-through observation tank. The divider was removed after an overnight recovery period. Fish were observed twice daily for 5 min and scored for position in the tank, acts of aggression, and feeding. Fin damage was re-assessed at the end of the interaction period, and points awarded for the extent of fin damage accumulated during the interaction period as well as position, aggression, and feeding were combined by principal components analysis (SPSS v16.0) to generate an overall behaviour score for each fish. The point system used was similar to that of previous studies (e.g., Jeffrey et al., 2012; Metcalfe et al., 1989; Sloman et al., 2001), and associates higher scores with more dominant traits and behaviours such as less damage to the dorsal and caudal fins (Moutou et al., 1998), occupying the preferred position in the environment, carrying out aggressive acts, and monopolizing food resources (Abbott and Dill, 1989; Abbott et al., 1985; McCarthy et al., 1992; Metcalfe, 1986; Metcalfe et al., 1989). Behaviour scores typically ranged from -1 (indicative of subordinate behaviour) to +1 (indicative of dominant behaviour), and the fish within a pair that received the higher behaviour score was assigned dominant social status. Pairs in which behaviour scores differed by less than 0.5 (2% of pairs) were excluded from subsequent experimentation.

3.3.2 Experimental protocols

Three experimental series were conducted with the objectives of evaluating the cortisol response to an acute stressor *in vivo* (Series 1), measuring cortisol production

in vitro (Series 2), and investigating the mRNA abundance of key genes in the HPI axis (Series 3).

3.3.2.1 Series 1: *In vivo* cortisol response

A set of 28 pairs of trout was used for this experiment (mass = 82.1 ± 2.2 g, fork length = 19.6 ± 0.1 cm, *N* = 56 fish; for pairs, fork-length difference = 0.8 ± 0.1%, behaviour score difference = 1.75 ± 0.13, *N* = 28 pairs). After 3 d of confinement in pairs, trout were lightly anaesthetized (as above) and a 250 µl blood sample was withdrawn by caudal puncture. Fish were then returned to their tank for a 24 h recovery period, following which a second blood sample was withdrawn by caudal puncture but following terminal anaesthesia (0.5 g l^{-1} ethyl *p*-aminobenzoate), either immediately upon removal from the tank ('control' fish), or at the end of 1 h of confinement in a net ('netting stressor'). The members of a pair were always removed from and returned to the tank at the same time, but were confined individually in nets for the netting stressor. Blood samples were collected using a syringe rinsed with 0.5 M Na_2-EDTA as an anti-coagulant, and were immediately centrifuged (13,200 *g* for 3 min) to yield plasma that was flash frozen in liquid N_2 and stored at -80°C for later analysis of cortisol, ACTH, and/or glucose concentrations. Cortisol and ACTH concentrations were measured using commercial RIA kits (MP Biomedical) previously validated for analysis of trout plasma samples (Doyon et al., 2006; Gamperl et al., 1994). For cortisol, intra-assay variation (% CV) was 7.3% and inter-assay variation was 6.9%. For ACTH, values for intra- and inter-assay variation were 6.3% and 8.2%, respectively. Plasma glucose concentrations were assayed according to the method of Bergmeyer (1974).

3.3.2.2 Series 2: Cortisol production *in vitro*

A set of 10 pairs of trout was used for this experiment (mass = 75.6 ± 1.9 g, fork length = 19.8 ± 0.1 cm, *N* = 20 fish; for pairs, fork-length difference = 0.7 ± 0.3%, behaviour score difference = 1.78 ± 0.28, *N* = 10 pairs). After 4 d of confinement in pairs (to match the terminal sampling point of Series 1), trout were euthanized by terminal anaesthesia and a blood sample (250 μl) was withdrawn by caudal puncture as described above for the analysis of plasma cortisol concentrations. Chilled saline was then injected into the caudal vein to clear the vessel of blood, the kidney was exposed by a ventral mid-line incision, and head kidney tissue was removed for *in vitro* incubation according to the protocol of Aluru et al. (2005). In brief, the excised head kidney tissue was minced using a razor blade and divided into three equal portions by weight (weights were recorded). Each portion was incubated with gentle shaking in a 24-well microplate at 13°C in 300 μl of minimum essential medium (MEM; Sigma) supplemented with 26 mM $NaHCO_3$ and BSA (0.1%; Sigma). After 2 h, 250 μl of medium were removed and replaced with fresh medium, and the tissue was incubated for an additional hour at 13°C. At the end of this 'baseline' period, 250 μl of medium were removed, flash frozen, and stored at -20°C for later analysis of cortisol concentrations. The medium was replaced with fresh medium (control), or fresh medium containing 0.01 or 0.5 IU ml^{-1} porcine $ACTH_{(1-39)}$ (Sigma), and the tissue was incubated with gentle shaking for 3 h at 13°C. The medium was then removed, flash frozen, and stored at -20°C for later analysis of cortisol concentrations. Incubation times, ACTH concentrations, and tissue weights were chosen based on preliminary experiments. Rates of cortisol production were calculated per mg wet tissue weight,

and rates of ACTH-stimulated cortisol production were then expressed as the percent increase over the control (no ACTH) value.

3.3.2.3 Series 3: Expression of HPI axis genes

Trout used in this experiment (mass = 86.3 ± 1.8 g, fork length = 20.7 ± 0.1 cm, *N* = 91 fish) were either paired with a conspecific for 5 d (fork-length difference = 1.4 ± 0.2%, behaviour score difference = 1.97 ± 0.10, *N* = 33 pairs) or were handled identically to the pairs but held individually ('sham' treatment group, *N* = 25 fish). Fish were euthanized by terminal anaesthesia at the end of the 5 d interaction period ('control'), or were subjected to a 1 h netting stressor (as in Series 1) and euthanized immediately following the stressor ('0 h'), or 1 h, or 4 h post-stress. Two blood samples were withdrawn by caudal puncture, using syringes rinsed with heparin (2500 IU ml^{-1} heparin sodium salt, Sigma) or 0.5 M Na_2-EDTA as anti-coagulants for the collection of samples used for cortisol and ACTH measurements, respectively. Blood samples were centrifuged (10,000 *g* for 2 min) to yield plasma that was flash frozen and stored at -80°C for later analysis. Head kidney and brain tissue (specifically POA) also were extracted, flash frozen in liquid nitrogen, and stored at -80°C for later analysis of mRNA abundance of stress axis genes.

Total RNA was extracted from 10-100 mg of tissue using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Tissues were homogenized by forcing the solution of TRIzol and tissue through 18 G and 23 G syringes until the solution passed easily through the syringe. Extracted RNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer. To generate cDNA, 1 or 2 μg of RNA

for POA or head kidney, respectively, were first treated with DNase (amplification grade, Invitrogen) according to the manufacturer's protocol. The RNA was then reverse transcribed using 100 U of RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) and 0.2 μg of random hexamer primer (IDT ReadyMade Primer) according to the manufacturer's protocol. For head kidney samples, the resultant cDNA was diluted with molecular grade water from the initial volume (20 μl) to a final volume of 40 μl. To improve the efficiency of gene-specific S-QPCR reactions, head kidney cDNA was purified using QIAquick PCR Purification kit (Qiagen) following the manufacturer's protocol to obtain an eluted volume of 40 μl.

Semi-quantitative real-time RT-PCR then was used to assess changes in mRNA relative abundances of target genes POA CRF and CRF-BP, and head kidney MC2R, StAR, and P450scc. Gene-specific primers (Table 3.1) were generated using GeneTool software (BioTools Inc.). The ribosomal 18S subunit (AF309412.1) was used as a reference gene. Two sets of primers were designed for this gene; "18S short" and "18S long" suited, respectively, the shorter products of CRF and CRF-BP primer sets, and the longer products of MC2R, StAR, and P450scc. The specificity of each primer pair was verified by sequencing the amplicons.

All S-QPCR reactions were carried out according to the protocols of Jeffrey et al. (2012) using SYBR green mastermix kit (Stratagene) and a Mx3000P real-time RT-PCR system (Stratagene). Briefly, the kit manufacturer's instructions were followed with the exception that the total reaction volume was scaled to 12.5 μl instead of 25 μl, and cDNA was diluted 25-fold for all target genes except StAR, and 1000-fold for the reference gene 18S. The annealing temperature of 60°C (60 s) and extension

temperature of 72°C (60 s) were used over 40 cycles. Standard curves were developed for each primer set (Table 3.1) and reaction compositions were adjusted to optimize the efficiency of the reaction; the efficiency for each primer set was between 0.98 and 1.04. For comparisons among treatment groups (sham, dominant, and subordinate) and across time points (control, 0, 1, and 4 h post-stress), the mRNA abundance of each gene was calculated relative to the sham control group using the modified delta-delta Ct method (Pfaffl, 2001) with 18S mRNA abundance as a normalizing gene.

3.3.3 Statistical analyses

Data are expressed as mean values ± 1 SEM. Series 1 (*In vivo* cortisol response) and 2 (Cortisol production *in vitro*) employed pairs of rainbow trout. Because the members of a pair are not independent of one another (see Briffa and Elwood, 2010), data from these series were analyzed using paired Student's t-tests or two-way repeated measures (RM) ANOVA, as appropriate. Series 3 (Expression of HPI axis genes) employed both sham-treated fish and pairs of trout, and in some cases data were not obtained from both members of the pair. Therefore, data from this series were analyzed by two-way ANOVA using social status (dominant, subordinate, or sham) and sample time (control, 0 h, 1 h, or 4 h) as factors. Because this approach did not take into account the links between members of a pair (where these occurred), it can be more difficult to reject the null hypothesis of no differences due to social status or sample time, i.e., this analysis is more prone to type 2 error (Briffa and Elwood, 2010). It is, however, a conservative approach to the analysis of the data. Where data did not meet assumptions of normality or equal variance, data were transformed or

equivalent non-parametric tests were employed. The fiducial limit of significance was 5%.

Table 3.1 Oligonucleotide primer pairs used for semi-quantitative real-time RT-PCR in rainbow trout (*Oncorhynchus mykiss*).

Gene	Primer pair	Accession number	Reference
18S "short"	F – cgg cgg cgt tat tcc cat gac R – ccc ccc gga acc caa aga ctt	AF309412.1	
CRF	F – aca acg act caa ctg aag atc tcg R – agg aaa ttg agc ttc atg tca gg	AF296672.1	Bernier et al. (2008)
CRF-BP	F – gga gga gac ttc atc aag gtg tt R – ctt ctc tcc ctt cat cac cca g	AY363677.1	Alderman et al. (2008)
18S "long"	F – ggc ggc gtt att ccc atg a R – tgc cct tcc gtc aat tcc ttt a	AF309412.1	
MC2R	F – gag aac ctg ttg gtg gtg gt R – gag gga gga gat ggt gtt ga	EU119870.1	Aluru and Vijayan (2008)
StAR	F – tgg gga agg tgt tta agc tg R – agg gtt cca gtc tcc cat ct	AB047032.2	Aluru and Vijayan (2008)
P450scc	F – gct tca tcc agt tgc agt ca R – cag gtc tgg gga aca cat ct	S57305.1	Aluru and Vijayan (2008)

Primer sequences are listed from 5' to 3'. CRF, corticotropin-releasing factor; CRF-BP, CRF-binding protein; MC2R, melanocortin 2 receptor; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 side chain cleavage enzyme.

3.4 Results

The objective of the initial series of experiments was to evaluate the cortisol response of dominant *versus* subordinate trout to an acute netting stressor. After 3 d of confinement in pairs, subordinate trout exhibited significantly higher baseline circulating cortisol concentrations than dominant trout (Wilcoxon signed rank test, $P = 0.002$), but did not differ from dominant trout in plasma glucose (paired Student's t-test, $P = 0.396$) or ACTH (paired Student's t-test, $P = 0.838$) concentrations (Fig. 3.1A-C). Following a 24 h recovery period, fish were subjected to the netting stressor. Dominant fish mounted significantly greater cortisol (one-tailed Wilcoxon signed rank test, $P = 0.033$) and glucose (one-tailed paired Student's t-test, $P = 0.015$) responses to the netting stressor than did subordinate fish (Fig. 3.1D,E), where the response to the stressor was calculated as the difference between post-stress and baseline values for an individual fish. The ACTH response to the netting stressor did not differ significantly between dominant and subordinate trout (one-tailed paired Student's t-test, $P = 0.183$; Fig. 3.1F). To control for possible effects of repeated sampling (i.e., to link the responses specifically to the netting stressor), a separate set of pairs was subjected to the same handling and sampling regime but without exposure to the netting stressor. Repeated sampling had no significant effect on baseline circulating cortisol concentrations in either dominant (8.2 ± 5.2 *vs.* 8.9 ± 3.2 ng ml^{-1}, $N = 8$; paired Student's t-test, $P = 0.902$) or subordinate trout (68.6 ± 35.8 *vs.* 34.6 ± 12.7 ng ml^{-1}, $N = 8$; Wilcoxon signed rank test, $P = 0.641$). Glucose concentrations increased slightly but significantly between sampling times in dominant trout (5.0 ± 0.3 *vs.* $6.6 \pm$

0.7 mmol L^{-1}, $N = 8$; paired Student's *t*-test, $P = 0.037$) but not in subordinate trout (7.9 ± 2.0 *vs.* 6.5 ± 1.3 mmol L^{-1}, $N = 8$; Wilcoxon signed rank test, $P = 0.313$).

To test the possibility that differences in stress responses between dominant and subordinate trout reflect differences in cortisol production by the interrenal cells, head kidney tissue from dominant and subordinate trout was incubated *in vitro* with porcine ACTH. As expected, circulating plasma cortisol concentrations of subordinate fish (93.3 ± 46 ng ml^{-1}, $N = 10$) were significantly greater than those of dominant fish (13.3 ± 4.6 ng ml^{-1}, $N = 10$; one-tailed Wilcoxon signed rank test, $P = 0.032$). Although baseline rates of *in vitro* cortisol production did not differ between dominant (0.85 ± 0.13 ng mg^{-1} tissue h^{-1}, $N = 10$) and subordinate fish (0.79 ± 0.17 ng mg^{-1} tissue h^{-1}, $N = 10$; paired Student's *t*-test, $P = 0.807$), rates of ACTH-stimulated cortisol production were significantly higher for preparations from dominant than subordinate trout (Fig. 3.2; two-way RM ANOVA on ranks, $P = 0.032$ for the effect of social status, $P = 0.302$ for the effect of [ACTH], $P = 1.000$ for social status x [ACTH]).

The mRNA abundance of key genes in the HPI axis was evaluated in the third series of experiments, which also provided an opportunity to examine cortisol and ACTH responses to the netting stressor across time. Sham-treated fish were included in the experimental design to control for effects of handling associated with social interactions. Because separate groups of fish were used for each sampling time, stress responses could not be determined for individual fish. Plasma cortisol concentrations were significantly affected by both social status and sampling time (Fig. 3.3A; two-way ANOVA on log-transformed data, $P < 0.001$ for social status, $P < 0.001$ for sampling time, $P = 0.044$ for social status x sampling time). Baseline plasma cortisol

concentrations were significantly higher in subordinate fish than in dominant or sham trout. Exposure to the netting stressor caused a significant elevation of plasma cortisol concentrations in all groups, although the difference in plasma cortisol concentrations in subordinate fish (~36 ng ml^{-1}) was substantially less than that in dominant (~84 ng ml^{-1}) or sham (~60 ng ml^{-1}) trout. In addition, cortisol concentrations post-stress fell more slowly in subordinate fish than dominant or sham fish; values 1 h post-stress did not differ from the value immediately following the netting stressor. Plasma ACTH concentrations were significantly affected only by sampling time (Fig. 3.3B; two-way ANOVA, P = 0.481 for social status, P = 0.024 for sampling time, P = 0.664 for social status x sampling time), being significantly lower 4 h post stress than immediately following the netting stressor.

Activation of the HPI axis begins with POA CRF, which is regulated at least in part by CRF-BP. The relative mRNA abundance of CRF was significantly affected by both sampling time and social status (Fig. 3.4A; two-way ANOVA on log-transformed data, P = 0.001 for social status, P = 0.412 for sampling time, P = 0.036 for social status x sampling time). At the 1 h post-stress sampling time, subordinate fish exhibited CRF relative mRNA abundance that was significantly higher than the values for dominant or sham-treated fish, as well as the control value for subordinate fish. Although CRF-BP relative mRNA abundance was not significantly affected by social status, a significant effect of sampling time was detected, with values immediately post-stress being higher than the control values (Fig. 3.4B; two-way ANOVA on log-transformed data, P = 0.151 for social status, P = 0.045 for sampling time, P = 0.299 for social status x sampling time).

At the level of the interrenal cells, MC2R plays a key role in activating cortisol biosynthesis, while StAR and P450scc contribute to determining the rate of cortisol production. Patterns of change in relative mRNA abundance were similar for these three genes. In all three cases, significant but independent effects of social status and sampling time on relative mRNA abundance were detected. The relative mRNA abundance of MC2R was significantly higher at all sampling times post-stress than at the control sampling time, and was significantly lower in sham fish than in dominant or subordinate trout (Fig. 3.5; two-way ANOVA on square root-transformed data, $P = 0.001$ for social status, $P = 0.010$ for sampling time, $P = 0.083$ for social status x sampling time). A similar patterns was detected for StAR relative mRNA abundance, although the post-stress elevation was not significant until the 1 h sampling time (Fig. 3.6A; two-way ANOVA on square root-transformed data, $P = 0.01$ for social status, $P = 0.038$ for sampling time, $P = 0.123$ for social status x sampling time). For P450scc, the stress-induced elevation of relative mRNA abundance was transient, having returned to control values by 4 h post-stress. In addition, the relative mRNA abundance of P450scc of subordinate fish was intermediate between those of sham and dominant trout (Fig. 3.6B; two-way ANOVA on square root-transformed data, $P = 0.047$ for social status, $P = 0.047$ for sampling time, $P = 0.177$ for social status x sampling time).

Figure 3.1 Plasma (A) cortisol, (B) glucose, and (C) adrenocorticotropic hormone (ACTH) concentrations in dominant (*N* = 19) and subordinate (*N* = 19) rainbow trout (*Oncorhynchus mykiss*). The plasma (D) cortisol, (E) glucose, and (F) ACTH responses to a 1 h netting stressor were calculated as the difference between the post-stress value and the baseline value 24 h earlier (see text for details). Values are means + SEM. An asterisk indicates a significant difference between dominant and subordinate fish (paired Student's *t*-test or Wilcoxon signed rank test; see text for details).

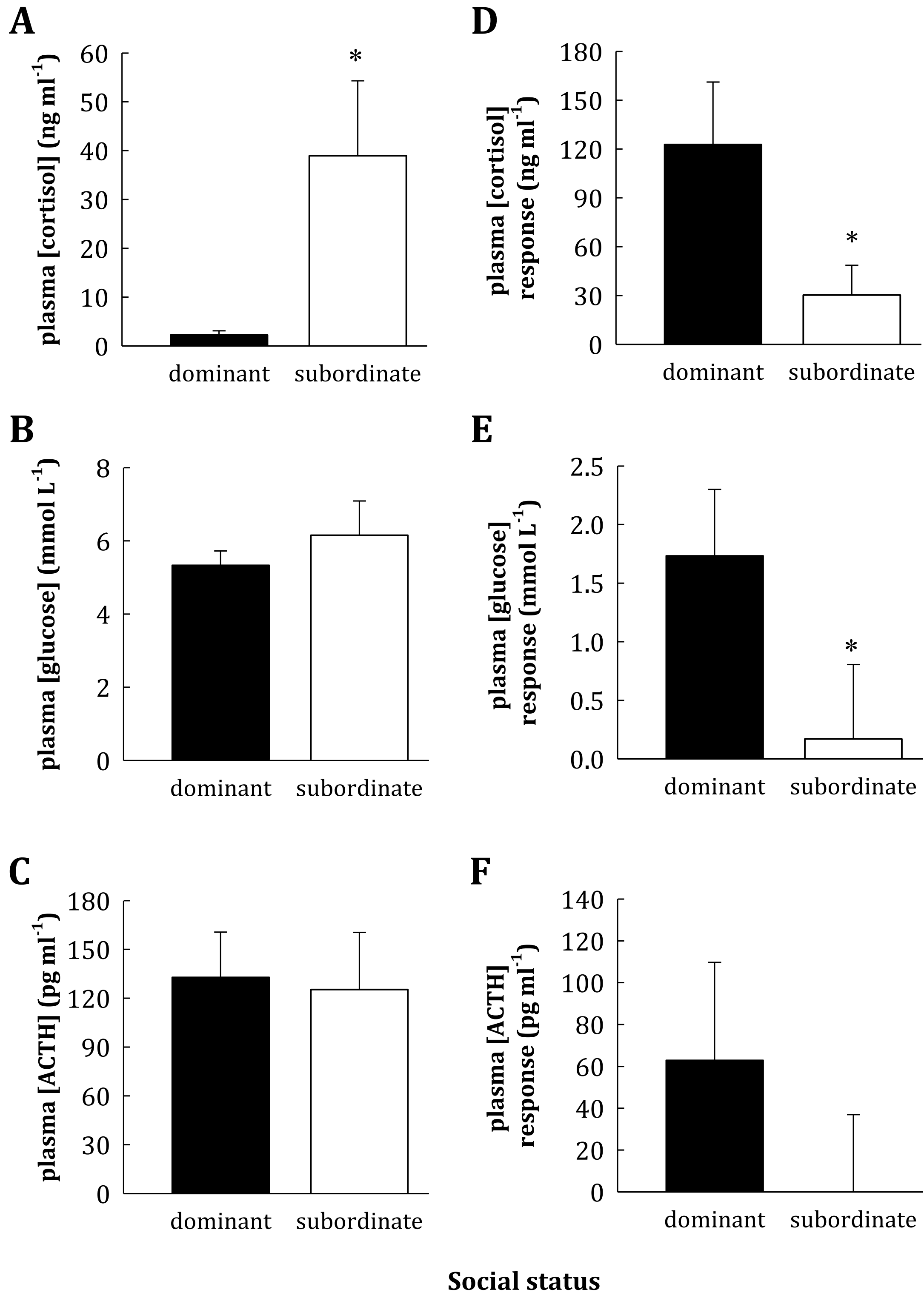

Figure 3.1

Figure 3.2 *In vitro* adrenocorticotropic hormone (ACTH)-stimulated cortisol production by head kidney preparations from dominant *versus* subordinate rainbow trout (*Oncorhynchus mykiss*). Values are means + SEM, *N* = 10 (for both dominant and subordinate fish). An asterisk indicates a significant difference between dominant and subordinate fish (two-way RM ANOVA; see text for details).

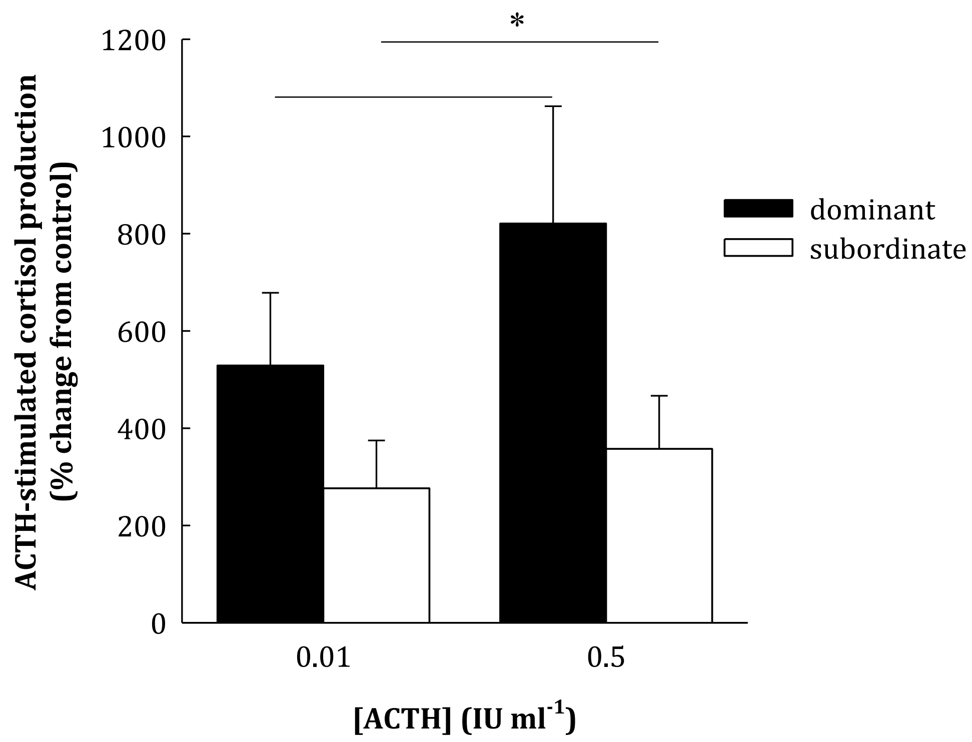

Figure 3.2

Figure 3.3 Plasma (A) cortisol and (B) adrenocorticotropic hormone (ACTH) concentrations of sham, dominant, and subordinate rainbow trout (*Oncorhynchus mykiss*) sampled under control conditions, or sampled directly (0 h), 1 h, or 4 h after exposure to a netting stressor. Values are means + SEM, with N = 8 in all cases. Data were analyzed by two-way ANOVA (see text for details). Sampling times that share a letter (within a social status category in panel A, or across all three categories in panel B) are not significantly different from one another. In panel A, significant effects of social status within a sampling time are indicated by an asterisk (significantly different from sham fish) and/or dagger (significantly different from dominant fish).

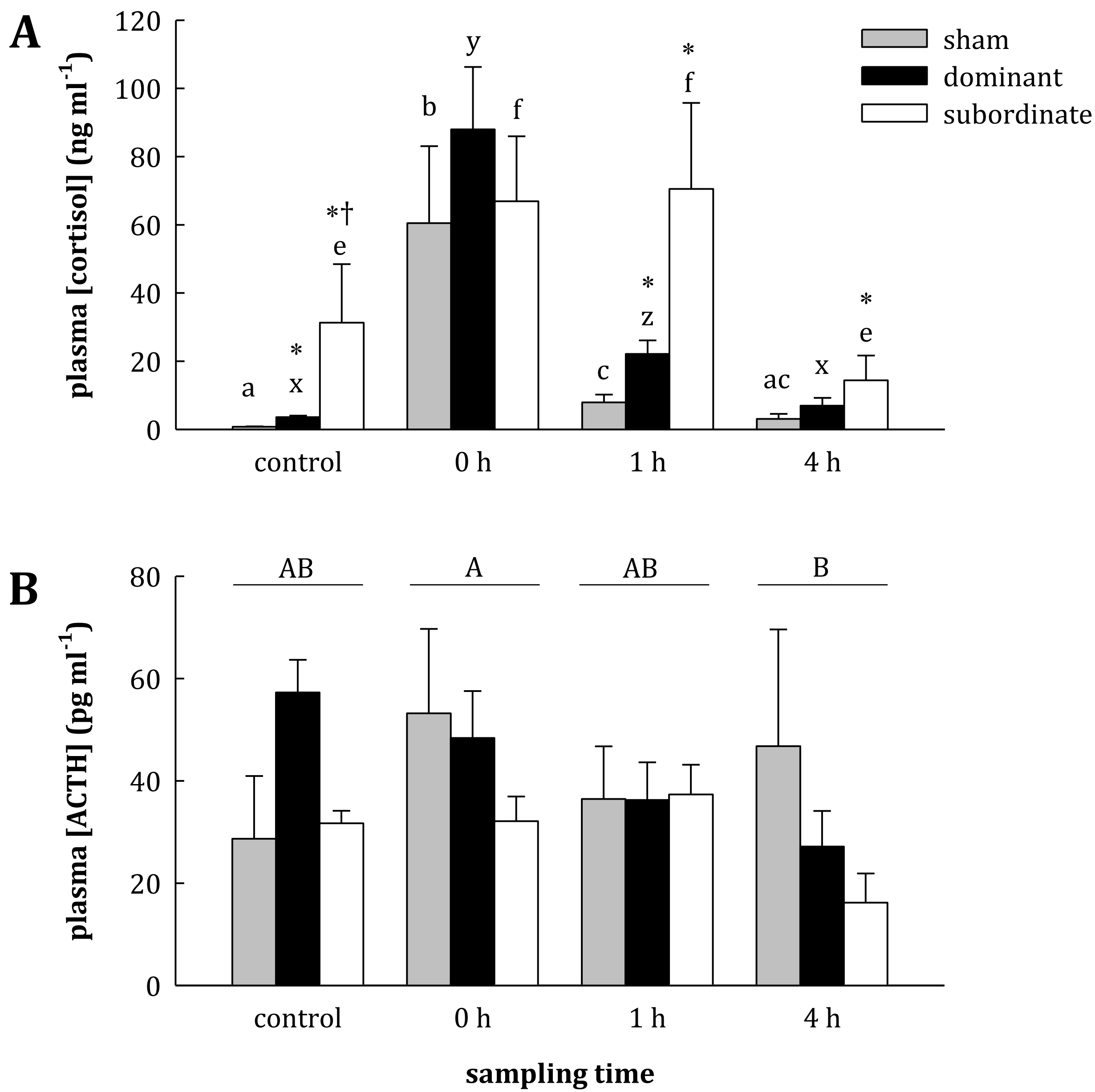

Figure 3.3

Figure 3.4 The relative mRNA abundance in the preoptic area of corticotropin-releasing factor (CRF; A) and CRF-binding protein (CRF-BP; B) for sham, dominant, and subordinate rainbow trout (*Oncorhynchus mykiss*) sampled under control conditions, or sampled directly (0 h), 1 h, or 4 h after exposure to a netting stressor. Values are means + SEM with N = 6 in all cases except dominant 0 h fish, where N = 4. All data were normalized against mRNA expression of 18S, and expressed relative to the sham control point. Data were analyzed by two-way ANOVA (see text for details). Sampling times that share a letter (within a social status category in panel A, or across all three categories in panel B) are not significantly different from one another. In panel A, significant effects of social status within a sampling time are indicated by an asterisk (significantly different from sham fish) and/or dagger (significantly different from dominant fish).

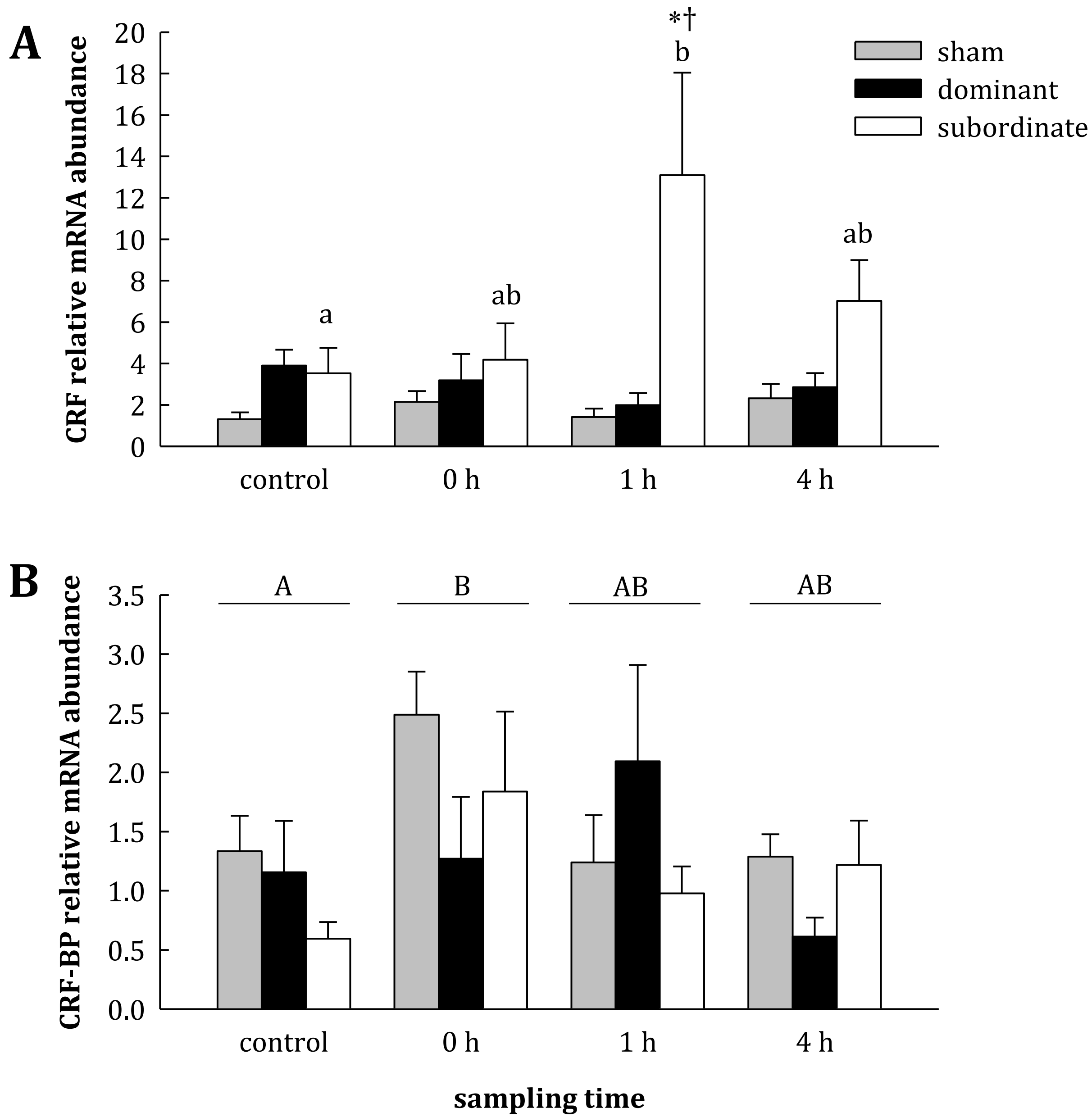

Figure 3.4

Figure 3.5 The relative mRNA abundance of head kidney melanocortin 2 receptor (MC2R) for sham, dominant, and subordinate rainbow trout (*Oncorhynchus mykiss*) sampled under control conditions, or sampled directly (0 h), 1 h, or 4 h after exposure to a netting stressor. Values are means + SEM with N = 6 in all cases except sham 0 and 1 h fish, and subordinate 4 h fish, where N = 5. All data were normalized against mRNA expression of 18S, and expressed relative to the sham control point. Data were analyzed by two-way ANOVA (see text for details). Sampling times that share a letter (across all three social status categories) are not significantly different from one another. Because no significant interaction between sampling time and social status was detected (see text for details), the data have been regrouped in the inset panel by social status category for presentation purposes only (not for statistical analysis); social status categories that share a letter are not significantly different from one another.

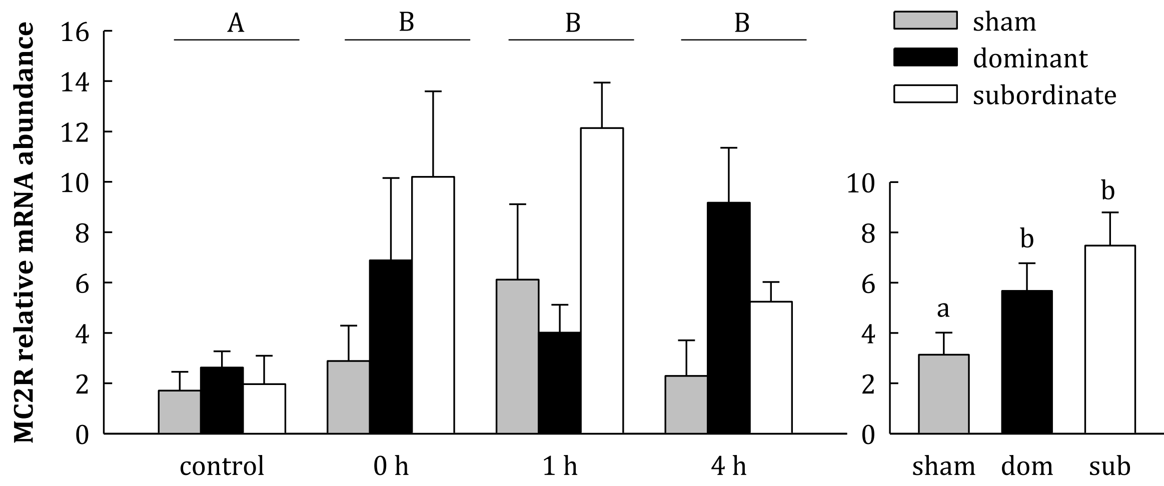

Figure 3.5

Figure 3.6 The relative mRNA abundance of head kidney (A) steroidogenic acute regulatory protein (StAR) and (B) cytochrome P450 side chain cleaveage enzyme (P450scc) for sham, dominant, and subordinate rainbow trout (*Oncorhynchus mykiss*) sampled under control conditions, or sampled directly (0 h), 1 h, or 4 h after exposure to a netting stressor. Values are means + SEM with N = 6 in all cases except sham 0 and 1 h fish, and subordinate 4 h fish, where N = 5. All data were normalized against mRNA expression of 18S, and expressed relative to the sham control point. Data were analyzed by two-way ANOVA (see text for details). Sampling times that share a letter (across all three social status categories) are not significantly different from one another. Because no significant interaction between sampling time and social status was detected in either case (see text for details), the data have been regrouped in the inset panels by social status category for presentation purposes only (not for statistical analysis); social status categories that share a letter are not significantly different from one another.

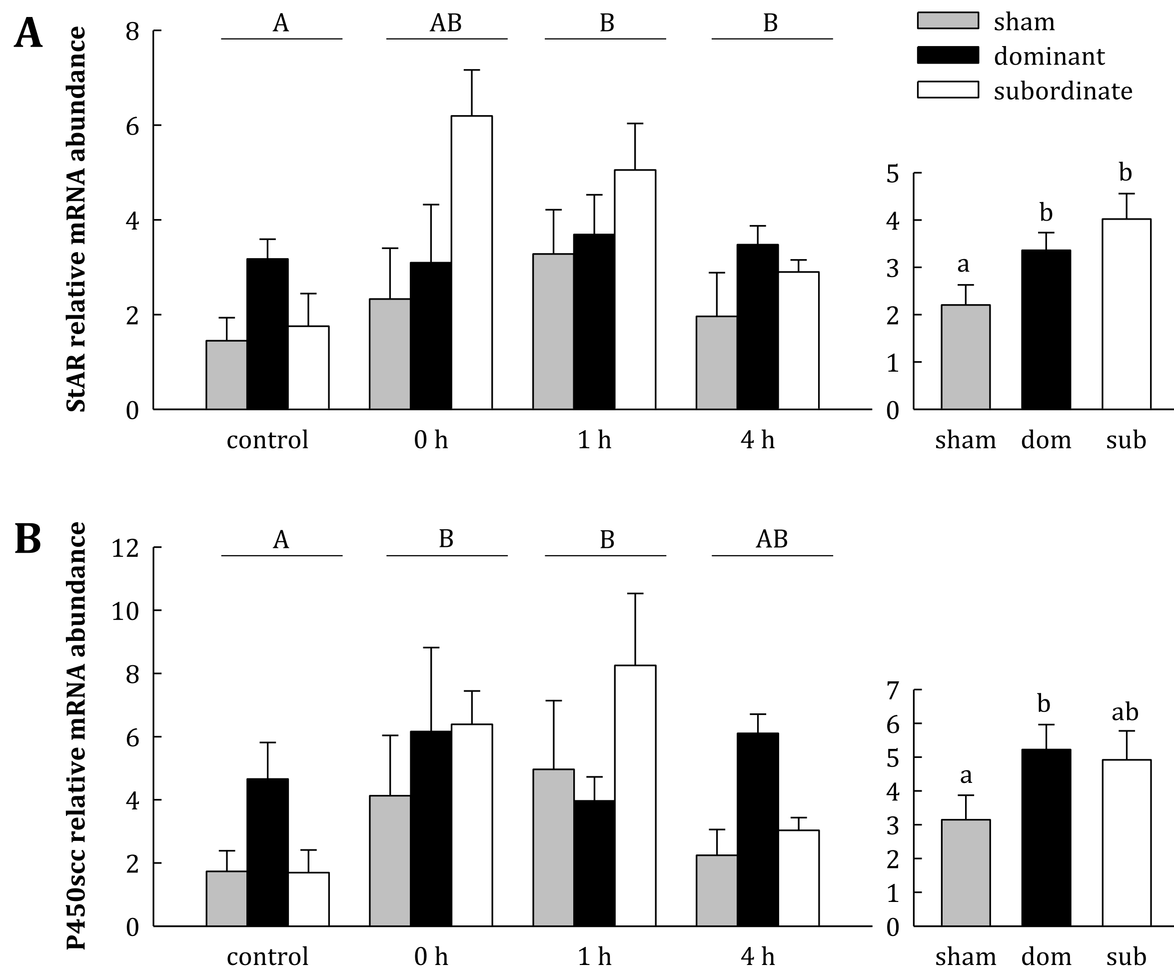

Figure 3.6

3.5 Discussion

The social stress experienced by subordinate rainbow trout causes chronic elevation of circulating cortisol concentrations and modulates HPI axis function (Bernier et al., 2008; Doyon et al., 2003; Jeffrey et al., 2012). The results of the present study demonstrated that modulation of HPI axis function in subordinate fish has functional consequences in the form of a compromised response to an acute stressor; the plasma cortisol and glucose responses to an acute netting stressor were significantly smaller in subordinate than dominant rainbow trout. Social status had little or no significant impact on POA CRF and CRF-BP relative mRNA abundance, or plasma ACTH concentration following exposure to the netting stressor, pointing to the interrenal cells themselves as the most likely source of differences between dominant and subordinate fish. Use of an *in vitro* preparation revealed that the attenuated cortisol response in subordinate fish primarily reflected decreased interrenal cell sensitivity to ACTH relative to dominant fish. However, the responses of MC2R, StAR and P450scc relative mRNA abundances to exposure to the netting stressor did not differ between dominant and subordinate fish, suggesting that transcriptional control of genes involved in cortisol biosynthesis did not contribute to differences in interrenal cell sensitivity to ACTH between dominant and subordinate fish. Collectively, the results of the present study support the hypothesis that chronic social stress modulates cortisol production at the level of the interrenal cells, resulting in an attenuated cortisol response to an acute stressor.

Chronic social stress in salmonid fish is associated with a suite of negative impacts, including lower growth rates and condition factor, immunosuppression, and

increased mortality (reviewed by Gilmour et al., 2005). On the basis of the present study, disruption of the acute stress response can be added to this list, in that subordinate trout mounted a significantly smaller cortisol response to an acute netting stressor than dominant trout. Similarly, Arctic charr deemed to be of dominant status on the basis of size, coloration, and bite marks exhibited greater increases in cortisol levels following a handling stressor than subordinate fish or fish of intermediate social status (Øverli et al., 1999b). Of note, however, was the tendency of both subordinate trout (present study) and Arctic charr (Øverli et al., 1999b) to exhibit a more sustained elevation of cortisol post-stress than dominant or sham fish. Subordinate trout also failed to elevate plasma glucose levels post-stress to the extent observed in dominant trout. The rapid plasma glucose response to an acute stressor reflects catecholamine-stimulated breakdown of liver glycogen stores, with cortisol contributing to this response through a permissive effect on liver ß$_2$-adrenoreceptors (Mommsen et al., 1999; Wendelaar Bonga, 1997). Subordinate trout were reported to exhibit lower liver glycogen levels, lower rates of glucose production by hepatocytes, and fewer liver β-adrenoreceptors than dominant trout (Gilmour et al., 2012), factors that could account for the attenuated plasma glucose response observed in the present study. By enhancing gluconeogenic potential, cortisol plays a key role in restoring liver glycogen stores during recovery from an acute stressor (Mommsen et al., 1999). The attenuated cortisol response to an acute netting stressor observed in the present study, in conjunction with the low liver GR protein levels reported previously (Jeffrey et al., 2012), suggest that this aspect of the metabolic response to acute stress also will be compromised in subordinate trout. In short, their reduced cortisol and glucose

responses imply that the capacity to deal with the metabolic burden of an acute stressor is diminished in subordinate trout.

A key objective of the present study was to identify mechanisms through which chronic social stress modulates the cortisol response to an acute stressor in subordinate rainbow trout. To identify possible mechanisms at the brain and pituitary levels of the HPI axis, the plasma ACTH response to the acute netting stressor was examined together with POA CRF and CRF-BP relative mRNA abundances. Plasma ACTH levels tended to increase in response to the stressor, peaking immediately post-stress and falling thereafter with the lowest levels observed 4 h post-stress. This response was not affected by social status and therefore differences in the ACTH response to a stressor probably do not account for the attenuated cortisol response of subordinate trout; it would, however, have been difficult to detect rapid, transient changes in ACTH responses between dominant and subordinate fish with the sampling protocol of the present study. Similarly, POA relative mRNA abundances of CRF and CRF-BP were largely unaffected or unaffected by social status, respectively. In subordinate trout, CRF relative mRNA abundance 1 h post-stress was significantly higher than values in dominant or sham fish, as well as control values for subordinate trout. Although the reasons for this spike in CRF relative mRNA abundance are not clear, it corresponded to a time at which plasma cortisol values in subordinate fish remained elevated, even though plasma cortisol values in dominant and sham fish had fallen significantly from the post-stress peak. Apart from this one point, CRF relative mRNA abundance did not change in response to the acute netting stressor. Other studies using relatively mild acute stressors, i.e., stressors eliciting peak cortisol

concentrations of less than ~100 ng ml^{-1}, also have failed to detect any impact on CRF mRNA abundance (Alderman et al., 2012; Backström et al., 2011; Bernier et al., 2008; Doyon et al., 2005; Huising et al., 2004). Longer (e.g., 24 h), more severe (e.g., plasma [cortisol] > 200 ng ml^{-1}) stressors appear to be necessary to elicit changes in CRF mRNA abundance (Bernier et al., 2008; Doyon et al., 2005; Huising et al., 2004), perhaps because it is only these types of stressors that deplete CRF protein stores sufficiently to require a transcriptional response (Huising et al., 2004). Interestingly, POA CRF-BP mRNA abundance responded to the netting stressor even though CRF mRNA abundance did not. Clear trends in the relationships among CRF-BP mRNA abundance, CRF mRNA abundance and circulating cortisol levels have not yet emerged (Alderman et al., 2012; Alderman et al., 2008; Doyon et al., 2005; Huising et al., 2004), underscoring a need to improve our understanding both of the role played by CRF-BP in regulating CRF signalling, and of the regulation of CRF-BP gene activation (Alderman et al., 2008). In summary, then, the results of the present study were not consistent with differences in activation of the stress axis at the hypothalamic or pituitary levels being the proximate cause of differences in the acute stress response between dominant and subordinate fish.

Dominant and subordinate fish did, however, differ in rates of ACTH-stimulated cortisol production by head kidney preparations *in vitro*. Similar results were reported by Sloman et al. (2002) using an *in situ* saline-perfused posterior cardinal vein preparation. In both studies, preparations from dominant and subordinate fish did not differ in baseline or unstimulated rates of cortisol production, suggesting that chronic social stress specifically modulates the activation of cortisol synthesis by ACTH. To

investigate this possibility, mRNA abundances of the ACTH receptor MC2R, StAR protein that transports cholesterol between the outer and inner mitochondrial membranes, and the enzyme P450scc that converts cholesterol to pregnenolone, were measured during exposure to and recovery from the acute netting stressor. The mRNA abundances of all three genes increased in response to the stressor, as expected based on previous work that reported increases in MC2R (Aluru and Vijayan, 2008), as well as StAR and P450scc (Aluru and Vijayan, 2006; Geslin and Auperin, 2004; Kusakabe et al., 2002) transcript levels in rainbow trout exposed to comparable stressors. At least for StAR, transcriptional responses seem to occur primarily with stressors causing substantial elevation of circulating cortisol levels (Alderman et al., 2012; Aluru and Vijayan, 2006; Geslin and Auperin, 2004; Kusakabe et al., 2002), or *in vitro* with high doses of ACTH (Aluru et al., 2005; Hagen et al., 2006). Despite the transcriptional responses observed for MC2R, StAR, and P450scc with exposure of trout to an acute netting stressor, no significant differences were detected between dominant and subordinate trout, although the relative mRNA abundances of all three genes in dominant and subordinate trout were generally significantly higher than those in sham fish. The latter observation could reflect a potential need to replenish proteins depleted as a result of HPI axis activation in both dominant and subordinate fish during hierarchy formation. Translational and post-translational regulation of StAR also appear to be important in ACTH-mediated activation of cortisol steroidogenesis (Geslin and Auperin, 2004; Hagen et al., 2006). In mammals, PKA activates StAR protein by phosphorylation of two specific serine residues, increasing steroid production (Arakane, 1997; Kallen et al., 1998). Protein kinase A has been implicated in

ACTH-stimulated cortisol synthesis in rainbow trout (Lacroix and Hontela, 2001), and Hagen et al. (2006) pointed out that the serine residues phosphorylated by PKA in mammals also are present in trout StAR protein, suggesting that this regulatory pathway may be conserved between mammals and fish. Thus, the decreased interrenal cell sensitivity to ACTH of subordinate relative to dominant trout probably reflected differences in translational and post-translational mechanisms of regulating cortisol biosynthesis rather than differences in transcriptional control.

In summary, chronic social stress decreased the capacity of subordinate rainbow trout to mount a cortisol response to an acute stressor. Based on data collected using an *in vitro* head kidney preparation, this attenuation of the *in vivo* cortisol response likely was the result of reduced rates of ACTH-stimulated cortisol biosynthesis in the interrenal cells in subordinate relative to dominant trout. The steroidogenic response to ACTH is thought to involve both transcriptional control of genes such as MC2R, StAR, and P450scc, as well as activation of existing StAR protein. Although dominant and subordinate fish did not differ in the transcriptional responses of MC2R, StAR, and P450scc to the acute netting stressor, differences in translational and post-translational regulation of cortisol biosynthesis warrant further investigation. It is clear, however, from the diminished cortisol and glucose responses of subordinate trout to an acute netting stressor, that the capacity of these chronically-stressed fish to respond to an acute stressor is impaired. Social stress therefore provides a useful experimental paradigm for investigating interactions between chronic and acute stress responses.

3.6 Acknowledgments

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CHAPTER 4

Effects of maternal social status on development of the hypothalamic-pituitary-interrenal axis and stress response in zebrafish (*Danio rerio*)

Notes on Chapter 4

The present chapter is formatted as a manuscript to be submitted to the journal *Proceedings of the Royal Society B: Biological Sciences* as:

Jeffrey, J.D., Gilmour, K.M., Effects of maternal social status on development of the hypothalamic-pituitary-interrenal axis and stress response in zebrafish (*Danio rerio*).

4.1 Abstract

The objective of the present study was to examine the effects of maternal social status on development of the HPI axis and stress response in zebrafish. Social stress results in chronically elevated levels of cortisol in subordinate fish. Maternal cortisol transferred to the egg has been suggested as a key mediator of programming offspring phenotypes. Female zebrafish were confined in pairs for 48 h to establish dominant/subordinate hierarchies; their embryos (fertilized *in vitro*) were reared to 144 hours post-fertilization (hpf) and sampled at five time points over development. Social status did not affect maternal contributions of either cortisol or mRNA of key genes of the HPI axis to offspring, nor did maternal treatment affect embryo volume (mm^3), survival, or hatching at 48 hpf. However, at 48 hpf (around the time of hatch and when embryos begin to synthesize cortisol *de novo*), larvae of dominant females exhibited significantly lower baseline cortisol levels than offspring of subordinate females. The lower cortisol levels in these fish may have resulted from reduced HPI axis activity, as CRF and P450scc mRNA levels also were lower in larvae from dominant than subordinate females at 48 hpf. Although maternal treatment did not affect baseline cortisol levels beyond 48 hpf, differences in mRNA abundance of HPI axis-related genes with maternal social status occurred at 96 and 144 hpf. Furthermore, at 144 hpf, stress-induced levels of cortisol in offspring from subordinate females were lower than those of offspring from sham-treated females (females handled in the same way as paired fish but not paired with a conspecific). This result might reflect an adaptive response to maternal experience (i.e., exposure to a stressor) in offspring of subordinate females. Our data suggest that maternal social status impacts development

of the HPI axis and stress response in early development; thus, providing an example of potential intergenerational effects of maternal social status in a teleost fish.

4.2 Introduction

Maternal contributions to offspring help to shape their development and phenotype. In addition to genetic information, these maternal contributions include proteins, mRNA, and steroid hormones. Perturbations of the female's environment and/or her condition can affect these contributions, resulting in changes to offspring physiology. For example, maternal stress that results in increased levels of glucocorticoid stress hormones (e.g., cortisol or corticosterone) can result in increased transfer of glucocorticoids to offspring (e.g., Almasi et al., 2012; Schreck et al., 2001; Talge et al., 2007). Although glucocorticoids play a key role in development (e.g., in embryogenesis), elevation of these hormones can affect offspring viability and growth rate as well as stress responsiveness and anxiety-related behaviours (reviewed by Cottrell and Seckl, 2009).

Social hierarchies are one situation in which female fish can experience elevated levels of cortisol, the main glucocorticoid in fish. Dominance hierarchies form in groups of fish where individuals compete aggressively for limited resources in an environment (reviewed by Johnsson et al., 2006). These social hierarchies have been studied extensively in juvenile salmonids and there is evidence of both sustained elevation of cortisol levels (e.g., Jeffrey et al., 2012; Sloman et al., 2001) in fish of low social status and an impaired ability to mount a cortisol response to subsequent stressors (Jeffrey et al., 2014; Sloman et al., 2002). In addition, function of the HPI axis, which is responsible for the release of cortisol (see below), is modulated in a social status-specific manner (Jeffrey et al., 2012; 2014). Similar social hierarchies have been observed in other species of fish. In particular, adult zebrafish held in small groups form social

hierarchies, with one fish becoming dominant through aggressive interactions (e.g., Dahlbom et al., 2011; Filby et al., 2010; Larson et al., 2006; Paull et al., 2010; Pavlidis et al., 2011), and these hierarchies influence reproductive success and mate choice (Paull et al., 2010). Whether maternal social status also has consequences for zebrafish offspring, specifically development of the stress response in offspring, remains to be determined. Zebrafish provide a useful model for such studies because they spawn easily in a lab setting and produce a large number of embryos with rapid external development, hatching by 2-3 days post-fertilization (dpf) and begin feeding between 4-6 dpf. In addition, owing to the sequencing of their genome and the large number of studies using zebrafish, many tools are available to study their physiology (Ekker and Akimenko, 2010; Xu and Zon, 2010). Although the impact of maternal stress on development of the stress axis in zebrafish remains poorly understood, ontogeny of the HPI axis itself has been well studied in zebrafish (e.g., Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Wilson et al., 2013).

The HPI axis begins with neurons that release CRF; the cell bodies of these neurons originate in the POA of the brain. These CRF-releasing neurons project to corticotropes in the pituitary causing the release of ACTH (reviewed by Bernier et al., 2009; Flik et al., 2006; Lederis et al., 1994). In most teleost fish, CRF signals through either CRF-R1 or CRF-R2, and the CRF-BP is thought to regulate bioavailability of CRF for its receptors (Alderman and Bernier, 2007; Alderman et al., 2008; Flik et al., 2006; Huising et al., 2004). Circulating ACTH binds to MC2R of the interrenal cells of the head kidney, which leads to the shuttling of cholesterol to the inner mitochondrial membrane as the first and rate-limiting step of cortisol synthesis, facilitated by StAR.

Cholesterol is cleaved to pregnenolone by P450scc (Aluru and Vijayan, 2006; Hagen et al., 2006; Mommsen et al., 1999). The final step of cortisol synthesis occurs with the conversion of 11-deoxycortisol to cortisol catalyzed by 11β-hyd (Aluru and Vijayan, 2006). Cortisol levels can be further regulated by its inactivation to cortisone catalyzed by 11β-HSD2 (Alderman and Vijayan, 2012). Finally, cortisol signalling at target tissues is mediated by GR and MR, which act as ligand-activated transcription factors (reviewed by Alsop and Vijayan, 2009a; Bury and Sturm, 2007; Prunet et al., 2006).

Certain maternal mRNAs associated with the HPI axis are transferred to eggs and are thought to play a key role in embryogenesis before embryos begin to synthesize mRNAs (i.e., become transcriptionally active). Transcripts for CRF, its binding protein and its receptors are present in eggs; however, their role in early development remains unclear (Alderman and Bernier, 2009). Maternal GR transcripts play an important role in mediating cortisol-dependent actions during early development (reviewed by Nesan and Vijayan, 2013). In addition, P450scc-synthesized pregnenolone is involved in early embryogenesis and epiboly (Hsu et al., 2002; 2006; 2009). As embryo development continues and embryos become transcriptionally active, maternal mRNA abundance decreases (Schier, 2007), but mRNA abundance of all of the above mentioned HPI-axis related genes increases between 24-72 hpf in zebrafish (reviewed by Alsop and Vijayan, 2009b). At this point, zebrafish larvae possess all of the machinery necessary to synthesize cortisol *de-novo*, and by 72 hpf they begin to increase cortisol levels in response to stress (Alderman and Bernier, 2009; Wilson et al., 2013).

The objectives of the present study were to examine the effect of maternal social status on maternal cortisol and HPI axis-related mRNA contributions to offspring (measured at 1 hpf), offspring cortisol levels and mRNA abundance of genes associated with the HPI axis over development, and the larval stress response. Zebrafish embryos from females of different social status were reared to 6 dpf and sampled at five time points over development. Baseline cortisol levels were measured throughout development, and the stress response was assessed at 96 and 144 hpf. In addition, mRNA abundance of key genes associated with the HPI axis was measured throughout development.

4.3 Materials and methods

4.3.1 Experimental animals

Adult zebrafish purchased from AQUAlity Tropical Fish Wholesale in Mississauga, Ontario were housed in 3 and 10 L flow-through polycarbonate tanks under a 14L:10D photoperiod supplied with aerated, dechloraminated City of Ottawa tap water at 28°C. Fish were fed once or twice daily to satiation with No.1 crumble-Zeigler (Aquatic Habitats, Apopka, FL, USA) as well as brine shrimp. Fish were allowed to acclimate to these holding conditions for at least 3 weeks prior to experimentation. Embryos were reared in 50 ml Petri dishes containing embryo media (0.01% methylene blue; NaCl 0.275 g L^{-1}, KCl 0.012 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.078 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 0.046 g L^{-1}) and kept in a 28°C incubator. All experiments were conducted in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) and after approval of the University of Ottawa Animal Care Committee (protocol BL-280).

4.3.2 Maternal social status

Adult zebrafish were maintained in four 3-L tanks in groups of 16 prior to social status experiments. Females (N = 183; 0.588 ± 0.015 g, mean ± SEM) that had previously spawned successfully were tagged with a unique mark using alcian blue (Sigma-Aldrich). Fish were lightly anaesthetized in a buffered solution of MS-222 [0.24 mg ml^{-1} 3-aminobenzoic acid ethyl ester; 21 mM Tris, pH 7; Sigma-Aldrich, (Westerfield, 2000)], to the point of losing equilibrium, blotted dry, weighed, measured for fork length, and marked. Tagged fish were returned to their original tank and allowed to recover for 2 d after which fork-length matched females were placed in pairs (N = 74; difference in fork length 1.25 ± 0.15 %) or on their own as shams (N = 35) in behaviour tanks (4.5 L). Fish were placed individually on either side of an opaque perforated divider (day 1) and on the morning of day 2, the divider was removed, allowing fish to interact. Each fish within a pair was observed twice daily for 3 min (6 min total per pair) on days 2 and 3. Fish were assessed for position within the water column, feeding (first fish to feed), acts of aggression, and retreats/freezing behaviour. Dominant behaviours (acts of aggression and monopolizing of food and territory) received higher scores and subordinate behaviours (retreats and freezing) received lower scores. The fish with the higher overall score was assigned dominant status. Previous studies have used similar scoring systems to assess dominance status in zebrafish (e.g., Filby et al., 2010; Paull et al., 2010) as well as other teleosts (e.g., Jeffrey et al., 2012; 2014). To improve the likelihood of collecting viable eggs, pairs of females

were moved to individual breeding tanks (2 L) at the end of day 3 and held overnight with a male on the other side of a transparent divider.

4.3.3 In vitro *fertilization and embryo rearing*

Females were terminally anaesthetized in a buffered solution of MS-222 (as above but using 0.72 mg ml^{-1}) on the morning of day 4, weighed, and stripped of their eggs before being flash frozen in liquid nitrogen. Each female's clutch was collected into a 50 ml Petri dish and fertilized with sperm, pooled from 11-20 males, collected in Hanks' solution (Sigma-Aldrich) (see Westerfield, 2000 for similar methods). After the addition of 100 µl of Hanks' solution containing sperm, system water (2 ml) was added over a period of 2 min to activate the sperm, after which embryo media was added and Petri dishes were placed into a 28°C incubator. Sham females were more likely to produce viable clutches (i.e., clutches in which the majority of eggs was successfully fertilized) than both dominant and subordinate females (90.3% of sham females *versus* 56.9% of dominant and subordinate females), accounting for the larger number of paired (N = 74) compared to sham (N = 35) females. For those females that produced a viable clutch, all embryos were counted to determine clutch size (which did not differ significantly among groups; one-way ANOVA on ranks; P = 0.181), and clutches were reared to 1, 24, 48, 96, or 144 hpf. At each time point, groups of 25 embryos were collected for cortisol analysis, and groups of 100 (1 hpf) to 15 (144 hpf) embryos were collected for analysis of mRNA abundance. Collected embryos were flash frozen in liquid nitrogen and stored at -80°C. A female's clutch constituted a single N for a time

point, and in most but not all cases embryos were collected for both mRNA and cortisol analysis from a single clutch.

4.3.4 Embryo physiological factors

After fertilization, embryo volume was determined from pictures using ImageJ. The equation for an ellipsoid (elps):

$$elps = \frac{3}{4}\pi \left(\frac{major\ axis\ length}{2}\right)\left(\frac{minor\ axis\ length}{2}\right)^2$$

was used to estimate embryo volume. Survival to 24 hpf (a critical time point in development, as was evident from the present study), hatching success (at 48 hpf), and survival over the developmental period also were assessed. Because embryo clutches were sampled throughout the rearing period, the number of clutches available for these measurements decreased at later points in development. All clutches assessed at 24 hpf and 48 hpf were used to determine survival at 24 hpf and hatching at 48 hpf, respectively. Only clutches that were reared to 144 hpf were used to assess survival over the full developmental period.

4.3.5 Whole-body cortisol levels

4.2.5.1 Embryo/larvae baseline and stress-induced cortisol levels

Baseline, whole-body cortisol levels of zebrafish embryos/larvae reared from dominant, subordinate, and sham females were assessed at all time points. Stress-induced whole-body cortisol levels in response to a 'swirling' stressor were determined at 96 and 144 hpf, when the stress response becomes active in developing zebrafish (Alsop and Vijayan, 2008; Wilson et al., 2013). Groups of 25 zebrafish larvae in 5 ml of

embryo media were placed in a 20 ml glass scintillation vial, swirled vigorously for 30 s, and collected after 5 min in a 28°C incubator, according to Alsop and Vijayan (2008).

Cortisol was extracted from pooled embryos/larvae using a protocol modified from Sopinka et al. (2014). In brief, samples were partially thawed on ice, and homogenized in 200 µl of extraction buffer (from Neogen cortisol EIA kit) in a 1.5 ml microtube using a battery operated pestle grinder (Kimble Chase Kontes). Samples were extracted three times with 1 ml of diethyl ether (Fisher Scientific) each time. After each addition of diethyl ether, samples were vortexed thoroughly, incubated at room temperature for 30 min (15 min for the second and third extractions), centrifuged at 3,000 *g* for 5 min, and flash frozen at -80°C for 30 min. The liquid phases of each extraction were combined, transferred to a clean 1.5 ml microtube, and evaporated under forced air. The extract was reconstituted in 250 µl of extraction buffer (Neogen), vortexed, heated at 65°C for 5 min twice, and stored at -80°C until analysis by enzyme-linked immunoassay (Neogen Cortisol EIA). Samples were assayed in duplicate over four plates where inter-assay variability was 3.1% and intra-assay variability was 7.8%. Since solid phase extraction was not used, a subset of samples were diluted by a factor of 2 to verify for the influence of interfering substances, which was determined to be negligible. Extraction efficiency was determined by spiking homogenates with a known amount of ^{3}H-hydrocortisone and was 85% for embryos at 1 hpf and 79% for larvae at 144 hpf.

4.3.5.2 Maternal cortisol levels

Cortisol was extracted from adult female zebrafish using a protocol adapted from Fuzzen et al. (2011b; 2010). In brief, zebrafish were powdered in liquid nitrogen with a mortar and pestle prior to homogenization with a handheld homogenizer (PowerGen 125, Fisher Scientific) on ice in 400 µl of homogenization buffer (80 mM Na_2HPO_4; 20 mM NaH_2PO_4; 100 mM NaCl; 1 mM EDTA) in a 2 ml microtube. Homogenates were extracted three times with 1 ml of methanol each time. After each addition of methanol, samples were vortexed thoroughly and incubated at 4°C in the dark for 60 min (for the first extraction) or 30 min (for the second and third extractions). Samples were centrifuged at 3000 *g* for 5 min at 4°C and flash frozen at -80°C for 10 min. After each extraction, the supernatant was transferred to a 2 ml microtube and evaporated under forced air. Combined extractions were reconstituted in 2 ml of acetate buffer (2.35 ml glacial acetic acid and 1.23 g sodium acetate trihydrate per 1 L).

Reconstituted samples were purified by passing them through a 500 g C_{18} solid phase extraction column (Fisher Scientific) primed with methanol and miliQ water. Prior to elution, ultra-pure water (Caymen Chemical) and hexane were passed through the column. Steroids were eluted slowly from the column four times with 1 ml of ethyl acetate (1% methanol) each time. The eluates were combined into a 2 ml microtube, dried under forced air and then reconstituted in 1 ml of extraction buffer (Neogen). Samples were heated at 60°C for 5 min, vortexed twice, and stored at -80°C until analysis by EIA. Samples were diluted 2-fold and assayed in duplicate on a single plate

where intra-assay variability was 5.6%. Extraction efficiency was determined by spiking homogenates with a known amount of ^{3}H-hydrocortisone and was 59%.

4.3.6 Embryo mRNA abundance

Total RNA was extracted from pools of 15-100 embryos/larvae using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Embryos/larvae were homogenized in 0.5 ml of TRIzol reagent using a battery operated pestle grinder (Kimble Chase Kontes). Extracted RNA was quantified using a NanoDrop® ND-2000c UV-Vis Spectrophotometer (Thermo Scientific). Next, cDNA was synthesized from 0.5 μg of RNA using QuantiTect Reverse Transcription Kit (Qiagen) and following the manufacturer's protocol with the exception that half reaction volumes, with a final volume of 10 μl, were used.

Semi-quantitative real-time RT-PCR was used to assess relative mRNA levels of genes of the HPI axis, specifically CRF, CRF-R1, CRF-R2, StAR, P450scc, 11β-hyd, 11β-HSD2, GR, and MR (Table 4.1). All genes were assessed over 1 to 144 hpf with the exception of 11β-HSD2, which was measured over 48 to 144 hpf because mRNA levels are not detectable in zebrafish until after 25 hpf (Alsop and Vijayan, 2008). Where primers were designed for the present study, Primer3 or Primer3Plus was used, and primer specificity was verified by sequencing (GenScript, USA). All S-QPCR reactions were performed in a similar manner using Rotor-Gene SYBR Green PCR Kit (Qiagen) and a Rotor-Gene Q real-time PCR system (Qiagen), following the manufacturer's protocol with the exception that reaction volumes were scaled to10 μl instead of 25 μl. Standard curves using serial dilutions of pooled cDNA were performed to optimize

reactions compositions; the efficiency for each primer set was between 0.92 and 1.10. Negative controls, including no template controls (where cDNA was replaced with water) and no reverse transcriptase controls (where RNA was treated in the same way as in other cDNA reactions with the exception that reverse transcriptase was replaced with water), were used to confirm that primers did not bind to genomic DNA.

The mRNA abundances of three reference genes (18S, β-actin, and ubiquitin) were measured over the developmental period and among treatments. However, levels of these housekeeping genes differed either by maternal treatment (18S) or with developmental time (β-actin and ubiquitin). Because more than 5 genes were assessed in the present study, the NORMA-gene approach of Heckmann et al. (2011) was used to normalize Ct values. Normalized Ct values were then expressed relative to either the sham 1 hpf mean (for CRF, CRF-R1, CRF-R2, P450scc, and GR), the sham 24 hpf mean (for StAR, 11β-hyd, and MR) or the sham 48 hpf mean (for 11β-HSD2) using the −ΔCt method (Livak and Schmittgen, 2001; Medeiros et al., 2010). Genes were expressed relative to a group other than the sham 1 hpf when expression levels for this gene were undetectable at 1 hpf (48 hpf for 11β-HSD2).

4.3.7 Statistical analysis

Cortisol and HPI axis-related gene mRNA have been well characterized throughout development in zebrafish (see Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Wilson et al., 2013). In the present study, cortisol and mRNA abundance of genes associated with the HPI axis followed similar patterns to those of previous studies. Because the goal of the present study was to evaluate the effects of maternal

social status on cortisol or mRNA levels of HPI axis-related genes, only maternal effects within a developmental time point were evaluated statistically; differences among time points were not analyzed statistically owing to previous characterization in the literature (Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Wilson et al., 2013).

All data are expressed as mean values ± 1 SEM. Maternal treatment effects on embryo size at 1 hpf, survival at 24 hpf, hatching at 48 hpf, baseline cortisol levels within each time point, stress-induced cortisol levels at 96 hpf and 144 hpf, and mRNA abundance within each time point were analyzed by one-way ANOVA. Paired Student's *t*-tests were used to analyze differences between baseline and stress-induced cortisol levels at 96 and 144 hpf. Survival over the developmental period was analyzed by two-way RM ANOVA. Where data were not normally distributed, equivalent non-parametric tests were used. The level of significance (α) was 0.05. All analyses were carried out using JMP V.11.

Table 4.1 Oligonucleotide primer sets for semi-quantitative real-time RT-PCR in zebrafish (*Danio rerio*).

Gene	Primer (5' – 3')	Accession number	Reference*
CRF	F – gcc gcg caa agt tca aaa R – gcgaggagaatctgtgcgtaa	BC085458	Alsop and Vijayan (2008)
CRF-R1	F – gaa atg cca cct ggt tcg tg R – agc ctg cac cag atc aca tt	XM691254	
CRF-R2	F – aat ggt gag gtt cgg tct gc R – tgt ggg aat gga cat cgc tc	ENSDART 00000055713	
StAR	F – ttg aac aag ctc tcc gga cc R – cac tgt atg tct cct cgg ca	NM131663	
P450scc	F – agg gcc atc acc cca ata g R – cca ggc ctt ccc ttc ttt tag	NM152953	Alderman and Bernier (2009)
11β-hyd	F – gct cat gca cat tct gag ga R – tgt gct gaa ggt gat tct cg	NM001080204	Ings and Van Der Kraak (2006)
11β-HSD2	F – cac gac tta ccc tcc tgc aa R – aca agc ccc cac aaa tct ct	NM212720	
GR	F – aca gct tct tcc agc ctc ag R – ccg gtg ttc tcc tgt ttg at	NM001020711	Alsop and Vijayan (2008)
MR	F – ccc att gag gac caa atc ac R – agt aga gca ttt ggg cgt tg	EF567113	Alsop and Vijayan (2008)
18S	F – ggc ggc gtt att ccc atg acc R – ggt ggt gcc ctt ccg tca att c	FJ915075	
β-actin	F – tgt ccc tgt atg cct ctg gt R – aag tcc aga cgg agg atg g	AF025305	Alsop and Vijayan (2008)
Ubiquitin	F – tgt ccc tgt atg cct ctg gt R – aag tcc aga cgg agg atg g	BC105746	Alsop and Vijayan (2008)

* Where no reference is provided, primers were designed specifically for this study. CRF, corticotropin-releasing factor; CRF-R, CRF receptor; StAR, steroidogenic acute regulatory protein; 11β-hyd, 11β-hydroxylase; 11β-HSD2, 11β-hydroxysteroid dehydrogenase 2; GR, glucocorticoid receptor; MR, mineralocorticoids receptor

4.4 Results

Maternal social status had no significant effect on offspring survival to 144 hpf; embryo survival decreased significantly over the initial 24 h but remained constant thereafter (Fig. 4.1; two-way RM ANOVA; $P = 0.475$ for maternal status, $P < 0.0001$ for time point, $P = 0.546$ for treatment x time). Similarly, none of embryo survival to 24 hpf (for all embryos reared to 24 hpf), embryo size at 1 hpf, or hatching success at 48 hpf differed significantly with maternal status (Table 4.2).

No significant effect of maternal treatment on offspring baseline whole-body cortisol levels was detected, except at 48 hpf, where cortisol levels were significantly lower in offspring of dominant females compared to those of subordinate females (Fig. 4.2). Similarly, maternal whole-body cortisol levels measured at the end of the experiment did not differ with social status (sham, 7.74 ± 1.31 ng g^{-1} body weight (bw); dominant, 8.03 ± 1.40 ng g^{-1} bw; subordinate, 6.17 ± 1.29 ng g^{-1} bw; one-way ANOVA, $P = 0.574$). Cortisol levels also were measured 5 min after exposure to a 30 s swirling stressor (stressed) for groups of 25 larvae at 96 and 144 hpf (Fig. 4.3). At 96 hpf, only larvae of subordinate females significantly elevated cortisol levels (Fig. 4.3A; paired Student's *t*-test; $P = 0.009$); in addition, larvae of sham females tended to increase cortisol levels in response to a stressor (Fig. 4.3A; paired Student's *t*-test; $P = 0.061$). Overall however, stressed cortisol levels did not differ significantly with maternal treatment at 96 hpf (Fig. 4.3A; one-way ANOVA; $P = 0.426$). At 144 hpf, regardless of maternal treatment, all larvae significantly elevated cortisol levels in response to the swirling stressor (Fig. 4.3B; paired Student's *t*-tests; $P = 0.003$ for offspring of sham, $P = 0.006$ for offspring of dominant, and $P = 0.031$ for offspring of subordinate females).

Stress-induced cortisol levels were significantly lower in larvae of subordinate females compared to offspring of sham females, with offspring of dominant females having intermediate values (Fig. 4.3B; one-way ANOVA; $P = 0.030$).

Whole-embryo or whole-larva levels of mRNA for genes of the stress axis were measured at 1 to 144 hpf. For CRF, offspring of dominant females exhibited significantly lower CRF mRNA abundance at 48 hpf compared to offspring of subordinate females and these levels also tended to be lower at 96 hpf (Fig. 4.4A). There were no other significant effects of maternal treatment on CRF levels, although 144 hpf larvae from dominant mothers tended to have higher levels of CRF mRNA (Fig. 4.4A). For CRF-R1, there was no significant effect of maternal treatment except for a subtle effect at 144 hpf, where offspring of dominant females had significantly higher levels of CRF-R1 mRNA compared to offspring of both sham and subordinate females (Fig. 4.4B). For CRF-R2, significantly higher CRF-R2 mRNA abundance was detected in offspring of subordinate females at 96 hpf compared to offspring of both sham and dominant females (Fig. 4.4C). There were no other significant effects of maternal treatment on CRF-R2 mRNA abundance (Fig. 4.4C).

Genes involved in cortisol synthesis (StAR, P450scc, and 11β-hyd) exhibited some effects of maternal social status. For StAR, mRNA levels were significantly lower in offspring of dominant females at 96 hpf, but there were no other significant effects of maternal treatment (Fig. 4.5A). Similarly, for P450scc, again maternal dominance resulted in significantly lower levels of P450scc mRNA although this time at 48 hpf, and maternal treatment had no other significant effect (Fig. 4.5B). No significant effect of maternal treatment on 11β-hyd was detected (Fig. 4.5C).

To evaluate possible differences of maternal social status on the effects of cortisol, relative mRNA levels of the receptors for cortisol (GR and MR) as well as an enzyme responsible for the breakdown of cortisol (11β-HSD2) were measured. Although mRNA levels of GR (Fig. 4.6A) and MR (Fig. 4.6B) did not generally differ with maternal treatment, MR mRNA levels were significantly lower in offspring of dominant females compared to offspring of both sham and subordinate females at 96 hpf. Only a subtle effect of maternal treatment was detected for the level of 11β-HSD2 mRNA; offspring of dominant females had significantly higher levels of 11β-HSD2 mRNA compared to offspring of subordinate females at 144 hpf (Fig. 4.6C).

Figure 4.1 Percent survival of offspring of sham (*N* = 8), dominant (*N* = 12), and subordinate (*N* = 13) female zebrafish (*Danio rerio*) over 1 to 144 hours post-fertilization (hpf). Values are presented as means ± SEM. No significant effect of maternal treatment was found. Time points that do not share a letter are significantly different from one another (two-way RM ANOVA; see text for details).

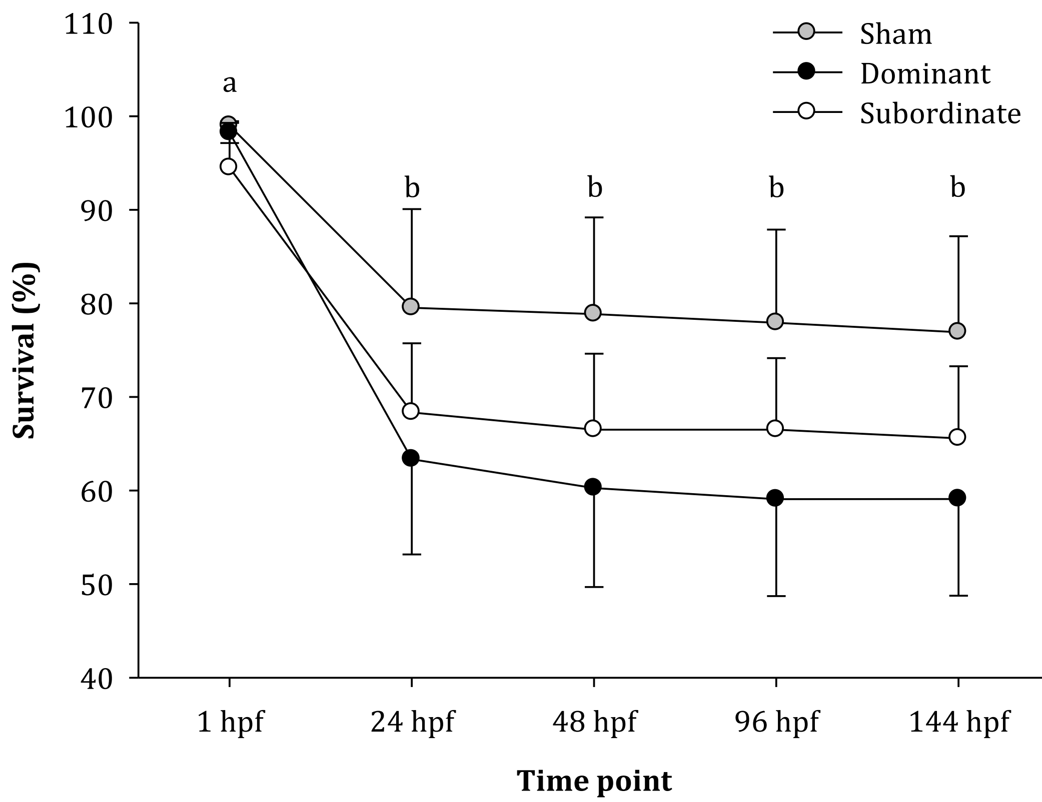

Figure 4.1

Figure 4.2 Baseline whole-body cortisol levels of offspring of sham (*N* = 5-6), dominant (*N* = 6-8), and subordinate (*N* = 6-8) female zebrafish (*Danio rerio*) over 1 to 144 hours post-fertilization (hpf). Values are presented as means + SEM. Maternal status effects were analyzed by one-way ANOVA (on ranks indicated by italics, and significant values are in bold font). Within a time point, groups that do not share a letter are significantly different from one another.

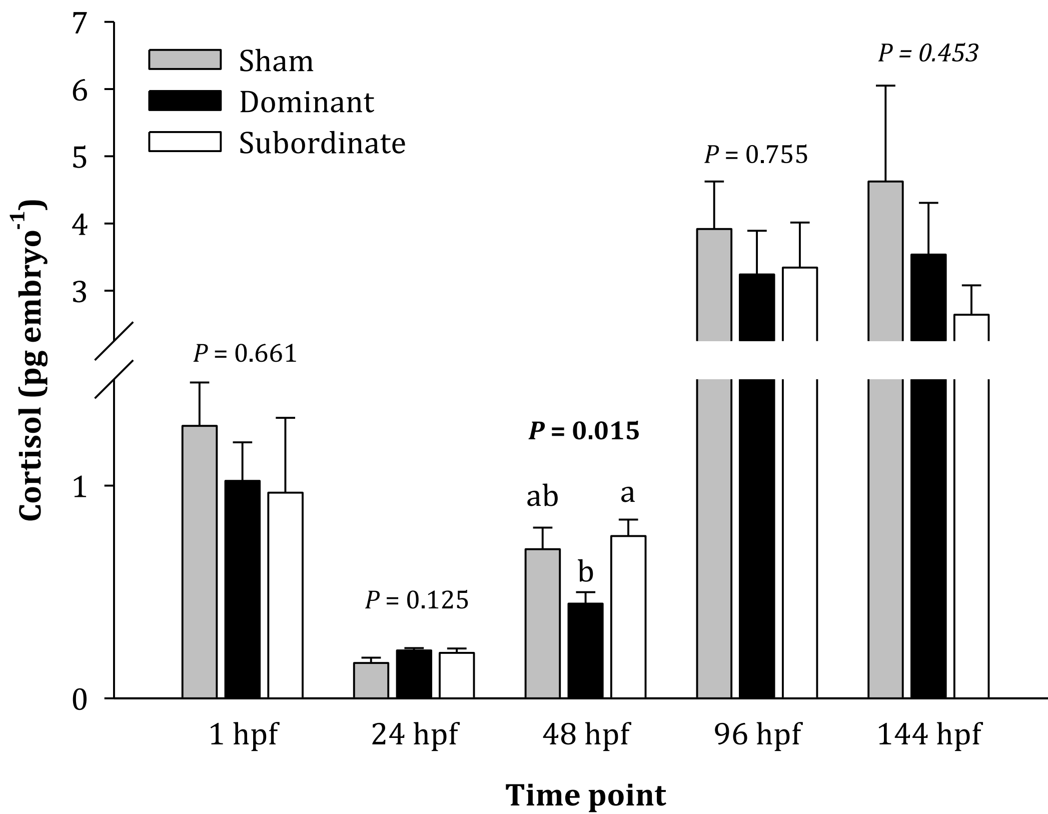

Figure 4.2

Figure 4.3 Whole-body cortisol levels of offspring of sham (*N* = 6), dominant (*N* = 7-8), and subordinate (*N* = 7-8) female zebrafish (*Danio rerio*) at 96 (A) and 144 (B) hours post-fertilization (hpf) following exposure to a swirling stressor. Groups of 25 larvae were sampled before (baseline; solid bars) or 5 min after exposure to a 30 s swirling stressor (stressed; hatched bars). Values are presented as means + SEM. Baseline values are repeated from Fig. 2 (see Fig. 2 for statistical analysis). An asterisk represents a significant difference between baseline and stressed values within a maternal status group (paired Student's *t*-test; see text for details). Stressed groups that do not share a letter are significantly different from one another (one-way ANOVA; see text for details).

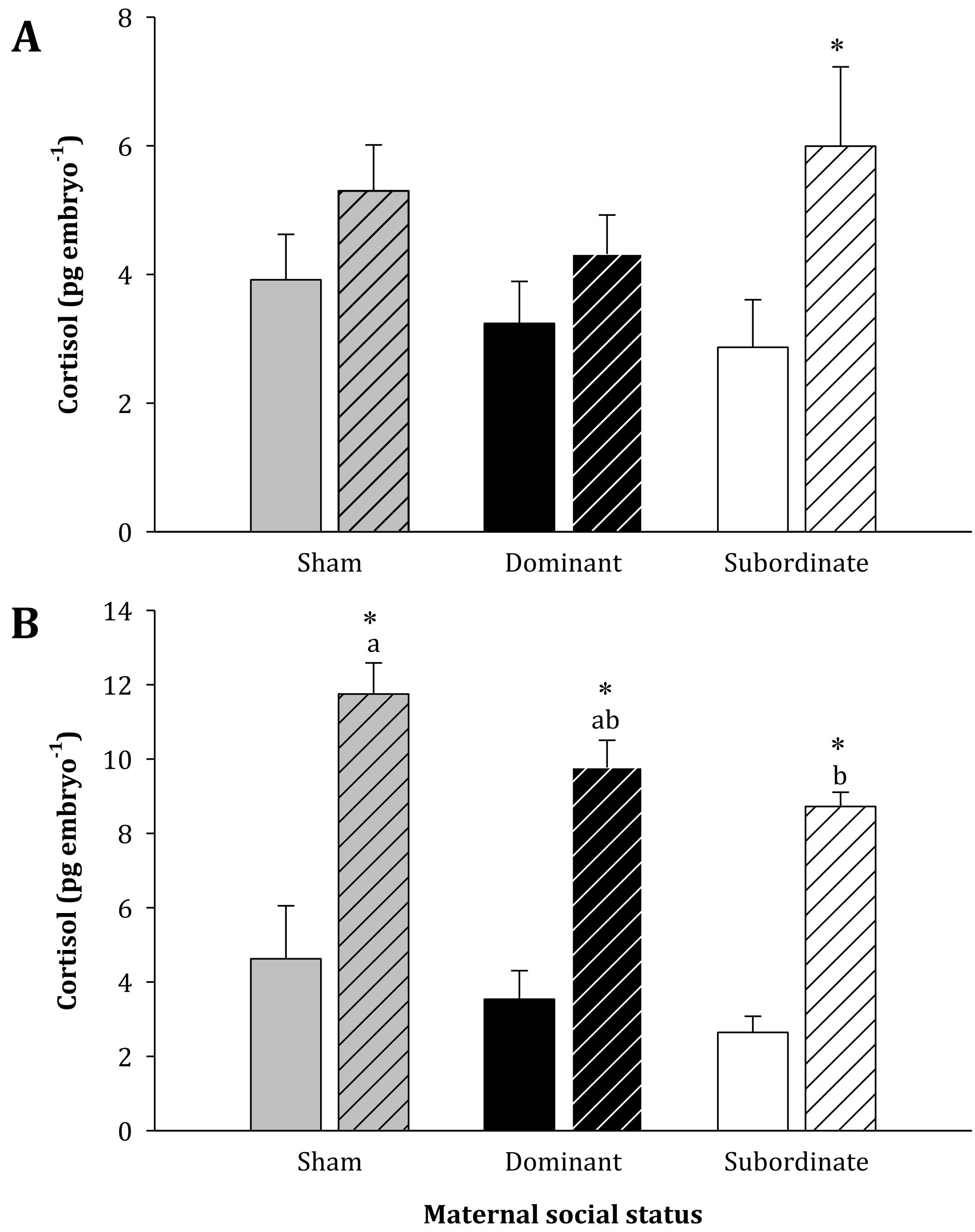

Figure 4.3

Figure 4.4 Relative mRNA abundance of corticotropin-releasing factor (CRF; A), CRF-receptor 1 (CRF-R1; B), and CRF-R2 (C) in offspring of sham (*N* = 5-6), dominant (*N* = 6), and subordinate (*N* = 4-6) female zebrafish (*Danio rerio*) over 1 to 144 hours post-fertilization (hpf). Values are presented as means + SEM. All data were expressed relative to the 1 hpf group from sham females. Maternal status effects were analyzed by one-way ANOVA (on ranks indicated by italics, and significant values are in bold font). Within a time point, groups that do not share a letter are significantly different from one another.

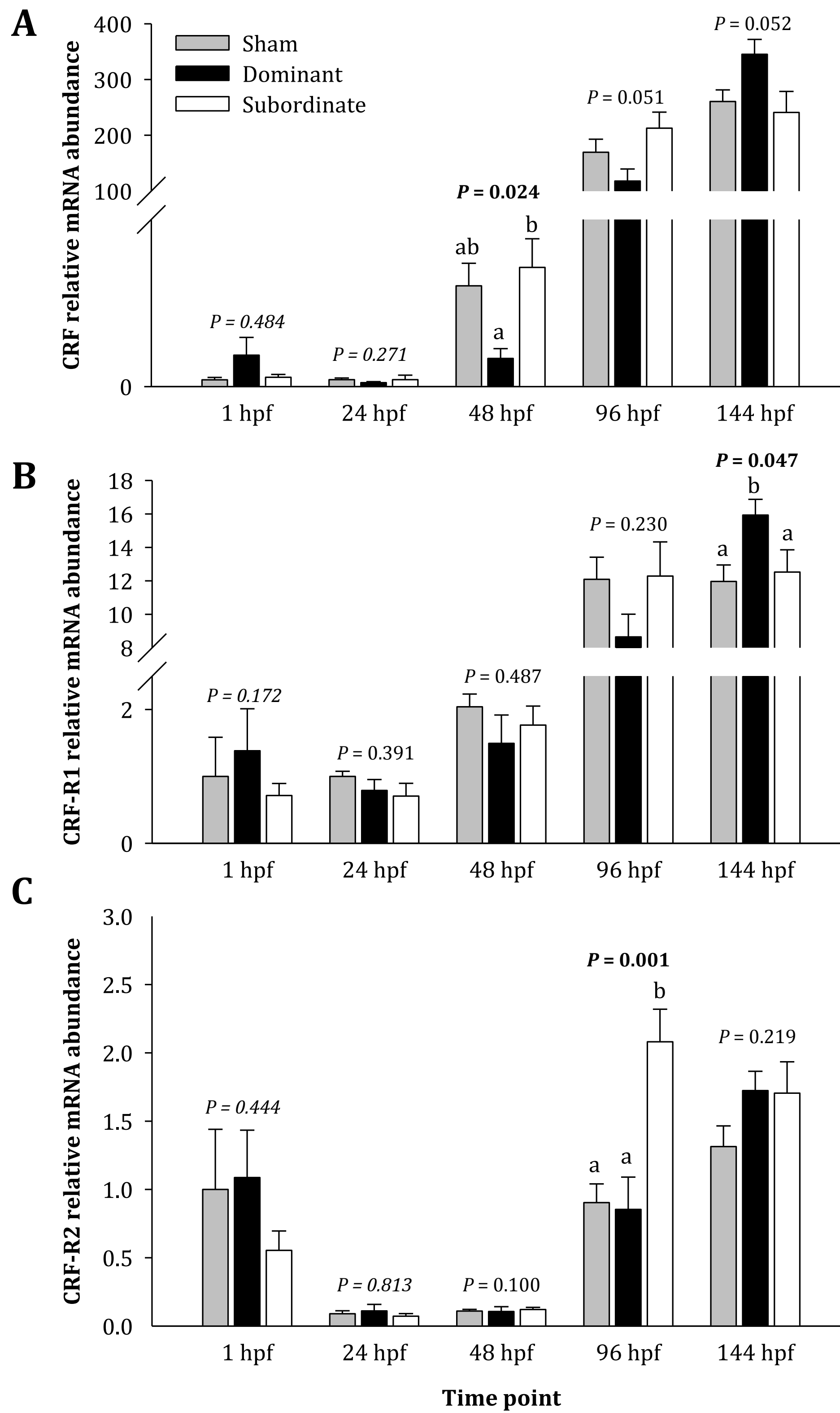

Figure 4.4

Figure 4.5 Relative mRNA abundance of steroidogenic acute regulatory protein (StAR; A), cytochrome P450 side chain cleavage enzyme (P450scc; B), and 11β-hydroxylase (11β-hyd; C) in offspring of sham, dominant, and subordinate female zebrafish (*Danio rerio*) over 1 to 144 hours post-fertilization (hpf). Values are presented as means + SEM (*N* = 5-6 for all groups). Data were expressed relative to the 24 hpf group from sham females for StAR and 11β-hyd, and relative to the 144 hpf group from sham females for P450scc. Maternal status effects were analyzed by one-way ANOVA (on ranks indicated by italics, and significant values are in bold font). Within a time point, groups that do not share a letter are significantly different from one another.

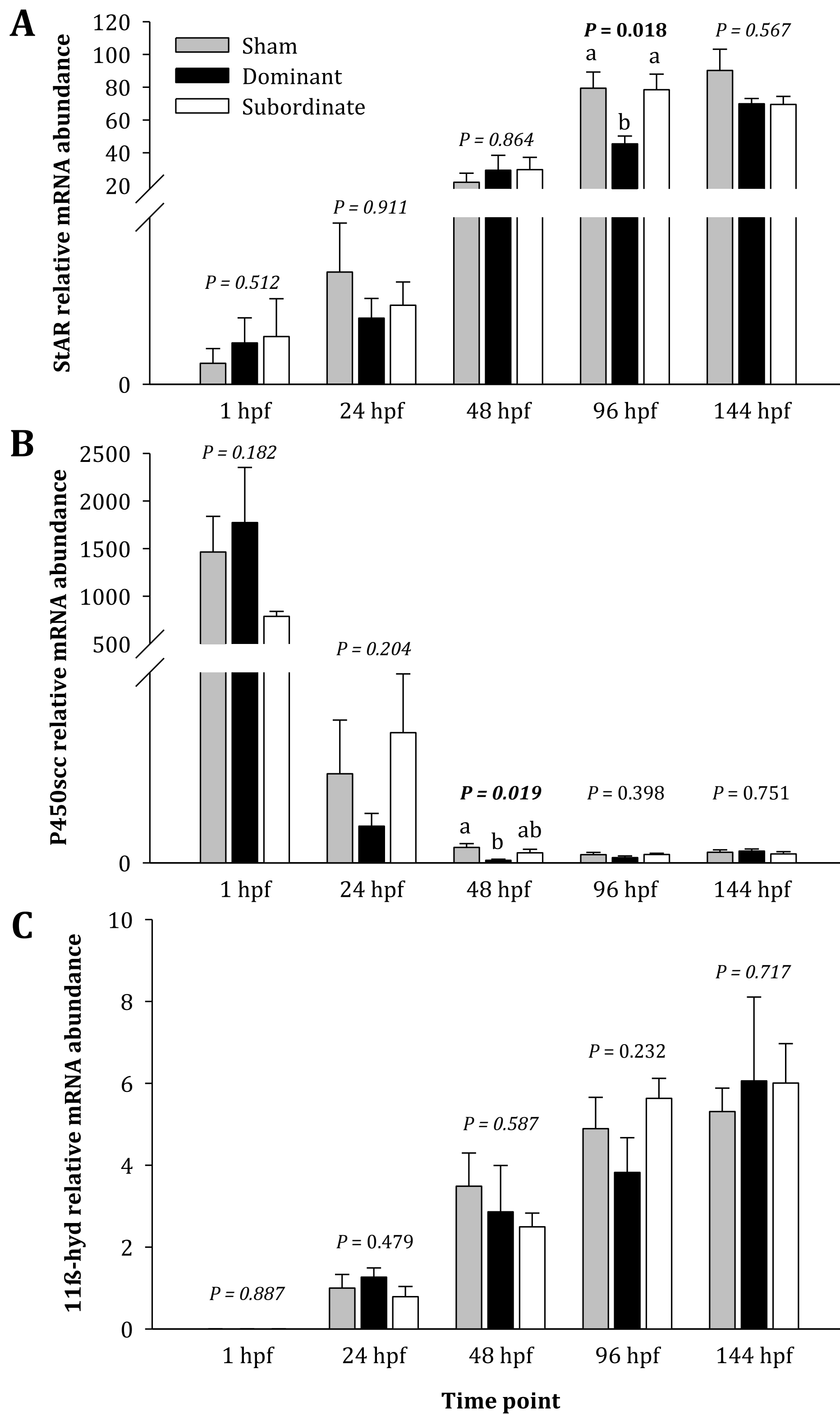

Figure 4.5

Figure 4.6 Relative mRNA abundance of glucocorticoid receptor (GR; A), mineralocorticoid receptor (MR; B), and 11β-bydroxysteroid dehydrogenase type 2 (11β-HSD2; C) in offspring of sham (*N* = 5-6), dominant (*N* = 5-6), and subordinate (*N* = 6) female zebrafish (*Danio rerio*) over 1 to 144 hours post-fertilization (hpf) for GR and MR, and over 48 to 144 hpf for 11β-HSD2 (see Materials and Methods section). Values are presented as means + SEM. Data were expressed relative to the 1 hpf offspring group from sham females for GR, relative to the 24 hpf offspring group from sham females for MR, and relative to the 48 hpf offspring group from sham females for 11β-HSD2. Maternal status effects were analyzed by one-way ANOVA (on ranks indicated by italics, and significant values are in bold font). Within a time point, groups that do not share a letter are significantly different from one another.

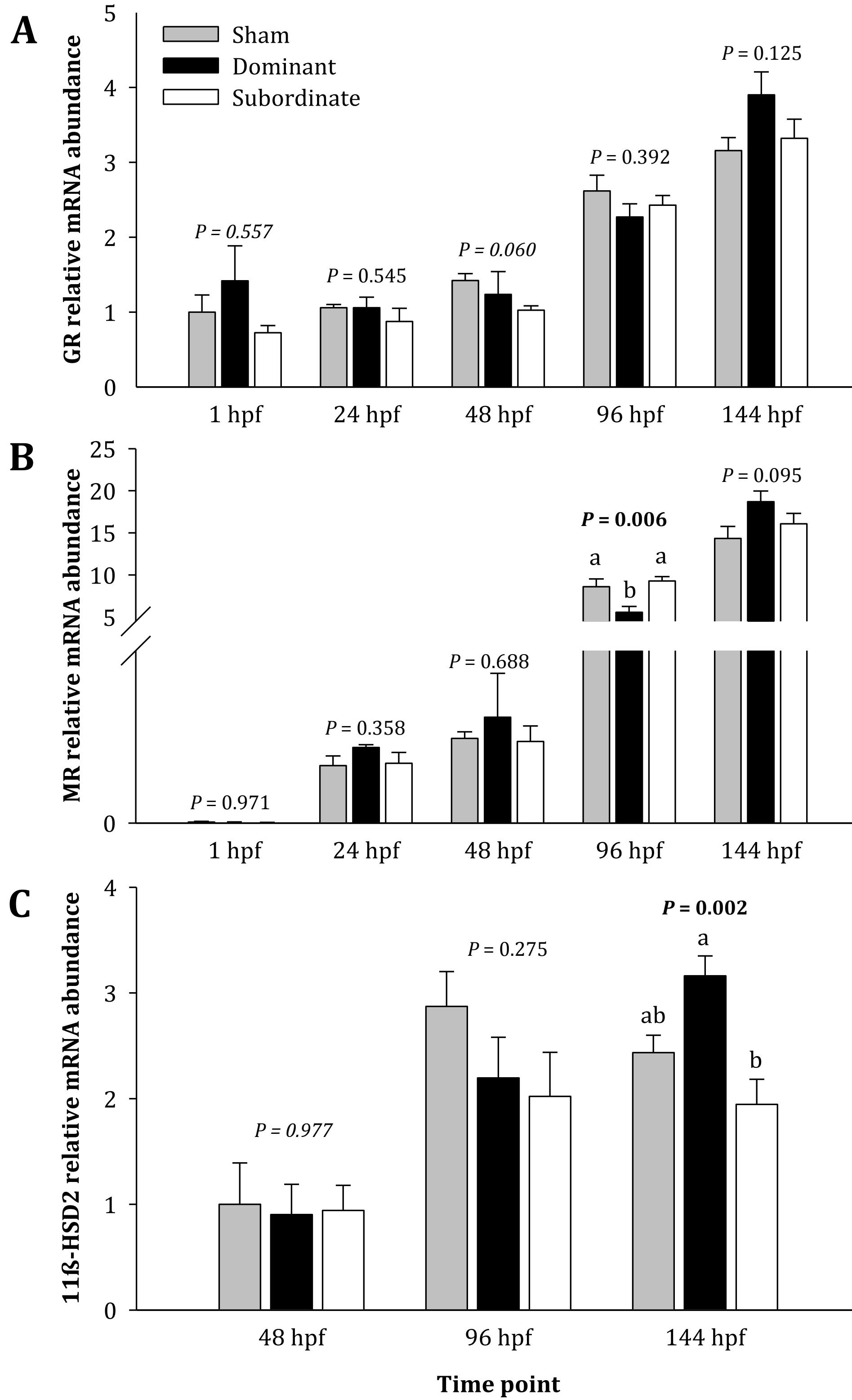

Figure 4.6

Table 4.2 Embryo volume at 1 hpf, survival at 24 hpf, and hatching at 48 hpf for offspring of sham, dominant, and subordinate female zebrafish (*Danio rerio*).

	Maternal social status			
	Sham	**Dominant**	**Subordinate**	***P*-value**
Embryo volume at 1 hpf (mm^3)	0.0697 ± 0.0023 (18)	0.0723 ± 0.0025 (17)	0.0751 ± 0.0028 (22)	0.331
Survival to 24 hpf (%)	81.7 ± 3.6 (26)	70.1 ± 4.7 (36)	63.8 ± 5.0 (38)	*0.098*
Hatching at 48 hpf (%)	52.3 ± 6.8 (20)	41.9 ± 6.4 (28)	37.1 ± 6.6 (27)	*0.349*

Data are presented as means ± SEM (*N*). Effects of maternal status were analyzed by one-way ANOVA (on ranks indicated by italics).

4.5 Discussion

The present study reports that maternal social status affected development of the HPI axis and the larval cortisol response to a stressor, effects that occurred even in the absence of a difference in maternal cortisol contribution to eggs. Most strikingly, maternal dominance resulted in lower baseline cortisol levels in larvae at 48 hpf, and this difference was accompanied by lower mRNA levels of key HPI axis genes. Although effects of maternal social status on baseline cortisol levels did not persist at 96 and 144 hpf, maternal dominance did play a role in regulating HPI axis function at these stages of development. Maternal subordinance also resulted in higher mRNA levels of CRF-R2 in larvae at 96 hpf. At 144 hpf, stressor-induced levels of cortisol were attenuated in offspring of subordinate females, suggesting the possibility of an adaptive role for social status-induced modulation of stress axis development. These results will be discussed within the context of possible implications of maternal social status on offspring HPI axis development.

4.5.1 Effects of maternal social status on egg production and early embryo development

Competition for dominance requires fish to invest energy in agonistic contests (reviewed by Johnsson et al., 2006), and thus energy may have been reallocated from reproduction to social contests, resulting in dominant and subordinate females producing viable clutches less often than sham females. Of the viable clutches produced, maternal social status had no effect on offspring viability during early development (i.e., embryo size, survivability, or hatching at 48 hpf), but this does not exclude the possibility that maternal effects might be observed in older offspring. In

contrast to the results of the present study, both repeated bouts of acute stress and chronic stress in rainbow and/or brown trout (*S. trutta*) reduced egg size and survivability; however, both male and female trout were stressed in these studies (Campbell et al., 1992, 1994). In the present study, *in vitro* fertilization using the sperm pooled from several males (not exposed to dyadic interactions) was used to eliminate potential male effects and focus solely on the effects of female social status. Although additional studies have also found that maternal stress affected egg or larval size in other teleost species [rainbow trout (Contreras-Sánchez et al., 1998), damselfish (*Pomacentrus amboinensis*) (McCormick, 2006, 2009), *N. pulcher* (Mileva et al., 2011), and three-spined stickleback (Giesing et al., 2010)], similar to the findings of the present study, maternal stress had no effect on gamete quality in coho salmon (Stratholt et al., 1997), nor on egg size or early offspring survival in sockeye salmon (Sopinka et al., 2014). Despite the lack of obvious differences in early embryo quality in the present study, it is possible that other physiological factors may have been influenced by maternal social status, such as yolk protein, lipid content, yolk utilization rate, metabolic rate, and growth over development (e.g., Eriksen et al., 2006; Giesing et al., 2010; Sloman, 2010); these factors warrant further investigation.

4.5.2 Maternal social status affects cortisol and HPI axis function over development

Maternal condition and experience are thought to play a key role in programming of offspring, at least in part through their effects on contributions of cortisol and maternal mRNA to eggs. Maternal cortisol is widely accepted as a key player in offspring programing (Schreck et al., 2001), and is involved in a number of

developmental aspects of normal gametogenesis and embryogenesis in teleost fish (see Li et al., 2012b). In zebrafish, maternal cortisol likely elicits the majority of its effects on early development through its action on GRs, which are also maternally contributed to the oocyte as mRNA (Alsop and Vijayan, 2008; Nesan et al., 2012; Nesan and Vijayan, 2012; Pikulkaew et al., 2011). Owing to the key role of cortisol in early teleost fish development, its level is tightly regulated, and *de novo* synthesis of cortisol and activation of the stress response likely are delayed in teleost fish until after hatch to prevent elevation in cortisol during early, critical time points in development (e.g., Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Barry et al., 1995). Although increases in embryo cortisol by exogenous elevation (i.e., egg/embryo exposure or microinjection) have revealed negative impacts during early development (e.g., Auperin and Geslin, 2008; Li et al., 2010; Nesan and Vijayan, 2012; Sloman, 2010), results with maternal stress have been mixed. Previous studies have found that maternal stress can increase transfer of cortisol to embryos (Giesing et al., 2010; Stratholt et al., 1997); however, similar to the results of the present study, Mileva et al. (2011) and Sopinka et al. (2014) were unable to detect an effect of maternal stress on egg cortisol levels. Even in the absence of an increase in maternal cortisol transfer to embryos, Sopinka et al. (2014) reported that maternal stress caused differences in offspring swim performance, suggesting that maternal cortisol is not the only mediator of offspring programming by maternal stress. In the present study, no difference in maternal cortisol levels with social status was detected, which may account for the lack of a difference in embryo cortisol levels. However, previous studies suggest that cortisol levels are elevated in subordinate zebrafish during social interaction (Filby et al.,

2010); the lack of a difference in the present study may reflect the measurement of cortisol levels in females at the end of the experiment, after fish were moved and handled. As with cortisol, maternal mRNA contributions of HPI axis-related genes were not influenced by social status in the present study. However, even in the absence of differences in maternal contributions of cortisol or mRNA levels, the present study found evidence for effects of maternal social status on development of the HPI axis in zebrafish. Effects of maternal social status were first observed at 48 hpf, which is around the time of hatching and the point in development at which zebrafish begin to synthesize cortisol *de novo* owing to the presence of HPI axis machinery.

At 48 hpf, offspring of dominant females exhibited lower baseline levels of cortisol than offspring of subordinate females. Correspondingly, mRNA levels of both CRF, the first hormone released in the HPI axis (reviewed by Bernier et al., 2009; Flik et al., 2006; Lederis et al., 1994), and P450scc, the first enzyme in cortisol synthesis (Aluru and Vijayan, 2006; Mommsen et al., 1999), were lower in offspring of dominant females. Lower levels of cortisol, perhaps owing to lower mRNA levels of these key HPI axis-related genes, might reflect a delay in the *de novo* synthesis of cortisol in offspring of dominant females. Cortisol, through its actions on GRs, affects hatching rate in zebrafish and blocking cortisol synthesis either by morpholino knockdown of cyp11b1 (the gene coding for 11β-hyd) or with metyrapone (an 11β-hyd blocker) decreased the proportion of hatched embryos at 72 hpf (Wilson et al., 2013). Thus, the lower baseline cortisol levels in embryos of dominant females might have been predicted to delay hatching, but no effect of maternal treatment on hatching rate was detected in the present study. However, hatching was only measured at a single time point (48 hpf) in

the present study, which may have hindered the detection of differences in hatching rate. For example, Wilson et al. (2013) detected significant effects of metyrapone and morpholino knockdown of cyp11b1 on hatching rate at 72 but not 48 hpf.

Effects of maternal social status on HPI axis-related genes were also observed at 96 and 144 hpf, although baseline cortisol levels did not differ significantly with maternal treatment at these time points. Interestingly, the level of CRF-R2 mRNA in the offspring of subordinate females was elevated at 96 hpf. Recent evidence in common carp suggests that CRF receptor subtypes may have distinct roles in fish based on differences in their responsiveness to CRF and CRF-like peptides (Manuel et al., 2014); however, the significance of an elevation in CRF-R2 mRNA at 96 hpf remains unknown.

4.5.3 Cortisol production in response to a stressor is affected by maternal social status

In the present study, 96 hpf larvae did not exhibit a robust cortisol response to a swirling stressor (less than a two-fold increase). Indeed, only larvae of subordinate females significantly elevated cortisol levels, making it difficult to detect effects of maternal social status at this stage. Although stress-induced elevation of cortisol has been observed as early as 72 hpf in zebrafish larvae (Alderman and Bernier, 2009; Wilson et al., 2013), these studies used different, perhaps more severe, stressors (osmotic challenge, Alderman and Bernier, 2009; prolonged kinetic stressor, Wilson et al., 2013) than the swirling stressor used in the present study and by Alsop and Vijayan (2008); notably, Alsop and Vijayan (2008) detected an acute cortisol response to the swirling stressor only at 97 hpf. At 144 hpf, a more robust elevation of cortisol in response to stressor exposure was observed across maternal treatments, and stressor-

induced cortisol levels were significantly lower in the offspring of subordinate females. This apparent plasticity of the HPI axis during early development also has emerged in other studies. For example, Auperin and Geslin (2008) found that either exposure of developing young to a stressor (air exposure/cold water) or cortisol treatment during early development attenuated the stress response of rainbow trout fingerlings at 5 months of age. In addition, using predator exposure as a maternal stressor, Mommer and Bell (2013) found that offspring of predator-exposed female three-spined stickleback exhibited a cortisol response that was different to that of offspring of unstressed females; the extent of this difference was unclear but the authors suggested that offspring of predator-exposed females mounted a stronger stress response, an effect opposite in direction to that of the current study. Although few studies have assessed HPI responsiveness in fish as a result of maternal stress, studies on avian and mammalian species are more abundant (reviewed by Love et al., 2013). For instance, exposure to elevated egg corticosterone attenuated the stress response in European starling fledglings, whereas decreasing the quality of the postnatal environment increased responsiveness of the HPA axis (Love and Williams, 2008). The level of maternal care delivered by rats during early development plays an important role in mediating responsiveness of the HPA axis through epigenetic modification of hippocampal GR (Weaver et al., 2004; reviewed by Weaver, 2007; Weaver, 2009). Collectively, these data suggest that maternal stress reduces HPI axis responsiveness and this effect may be considered adaptive, where maternal experience takes advantage of developmental plasticity to produce a phenotype that may be beneficial in a future life-history stage (i.e., 'predictive adaptive response', see Love and Williams,

2008). Put another way, predictive programming of the HPI axis may be beneficial for offspring that are likely to experience a stressful environment. Further investigation is required to determine whether the lower level of stress-induced cortisol in offspring of subordinate mothers is maintained in older fish, and if so, when might this response be advantageous in zebrafish.

4.5.4 Conclusion

Although early embryo viability and measured maternal contributions to offspring (i.e., cortisol) were unaffected by maternal social experience in the present study, development of the stress axis was affected once embryos began to express HPI axis-related genes (i.e., after 48 hpf). Furthermore, stress-induced elevation in cortisol was attenuated at 144 hpf in larvae of subordinate females, begging the question of whether maternal social experience shapes the stress response of their offspring in an adaptive fashion. Further investigation into the mechanisms mediating these differences (e.g., epigenetic modifications) and the potential effects of differences in HPI axis development on adult physiology and behaviour clearly is warranted.

4.6 Acknowledgements

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CHAPTER 5

Regulation of hypothalamic-pituitary-interrenal axis function in male smallmouth bass (*Micropterus dolomieu*) during parental care

Notes on Chapter 5

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S.J. Cooke was a collaborator at Carleton University who made possible the field work necessary for this study.

5.1 Abstract

Male smallmouth bass provide sole parental care until offspring reach independence, a period of several weeks. During the early parental care period when males are guarding fresh eggs (MG-FE), cortisol responsiveness is attenuated; the response is re-established when males reach the end of the parental care period and are guarding free-swimming fry (MG-FSF). It was hypothesized that attenuation of the cortisol response in male smallmouth bass during early parental care reflected modulation of HPI axis function. Male smallmouth bass were sampled at the beginning and end of the parental care period, before and/or 25 min after exposure to a standardized stressor consisting of 3 min of air exposure. Repeated sampling of stressed fish for analysis of plasma cortisol and ACTH levels was carried out. Males significantly elevated both plasma cortisol and ACTH levels when guarding free-swimming fry but not during early parental care. Control and stressed fish were terminally sampled for tissue mRNA abundance of POA and hypothalamic CRF as well as head kidney MC2R, StAR, and P450scc. No significant differences in either hypothalamus CRF or head kidney P450scc mRNA abundance were found across parental care stages or in response to a stressor. However, POA CRF mRNA abundance and interrenal cell MC2R and StAR mRNA abundances failed to increase in response to a stressor in males guarding fresh eggs. Thus, the attenuated cortisol response in males guarding fresh eggs may be explained by hypoactive HPI axis function in response to a stressor. The present is one of few studies, and the first teleost fish study, to address the mechanisms underlying resistance to stress during the reproductive/parental care period.

5.2 Introduction

All centrarchid fish provide sole male parental (i.e., paternal) care although the level of parental investment varies markedly among species (Warren, 2009). Male smallmouth bass invest extensively in parental care because males provide care to offspring over a period of 4-6 weeks in the late spring and early summer. During this period, males exert considerable energy providing nest defense, either directly by aerating the eggs and defending the nest from predators, or indirectly by limiting their foraging opportunities (Cooke et al., 2006; Cooke et al., 2002; Ridgway, 1988; Ridgway et al., 1991). Indeed, although they rarely move more than 3 m away from the nest, telemetry revealed that bass swim the equivalent of over 40 km day^{-1} (Cooke et al., 2002). The energy exerted during this period can negatively impact adult growth as well as survival probability over the following winter (Ridgway et al., 1991). Owing to these energy limitations, a life-history trade-off exists between investing in defense of a current brood (current reproductive outcome) and investing in growth and survival (future reproductive outcome) (Williams, 1966). Indeed, reproductive holidays (i.e., when fish skip reproduction in one or more years despite being sexually mature) are common for smallmouth bass, which is presumably a reflection of this trade-off (Barthel et al., 2008; Gravel et al., 2010).

A rise in glucocorticoid levels in response to a stressor initiates a sequence of physiological effects that are important to the survival of an organism (Wendelaar Bonga, 1997). Cortisol is the main glucocorticoid in teleost fish, and acts to mobilize energy resources to cope with increased energy demand during stress and to restore homeostasis (Barton, 2002; Mommsen et al., 1999; Wendelaar Bonga, 1997).

Stress-induced levels of cortisol are adaptive, but when stressors are severe or prolonged, cortisol can negatively impact reproductive function (reviewed by Fuzzen et al., 2011a; Schreck, 2010; Schreck et al., 2001). For example, nest abandonment in male smallmouth bass increased when cortisol levels were raised exogenously (Dey et al., 2010; O'Connor et al., 2009). Owing to the negative impacts of high cortisol levels on current reproductive outcome, 'resistance to stress' (reviewed by Wingfield and Sapolsky, 2003) may allow for successful reproduction in fish invested in defending a current brood. O'Connor et al. (2011b) found that male smallmouth bass attenuated their cortisol response to a standardized stressor at the early stages of parental care, with the stress response being re-established toward the end of the parental care period. The aim of the present study was to investigate the mechanisms underlying attenuation of the cortisol response during early parental care in smallmouth bass.

Cortisol elevation reflects activation of the HPI axis in fish (HPA in other vertebrates). When the HPI axis is activated, CRF is released from the POA of the brain to the pituitary corticotropes (reviewed by Bernier et al., 2009; Flik et al., 2006; Lederis et al., 1994). Stimulation of corticotropes by CRF causes the release of ACTH, the main secretagogue of cortisol (reviewed by Lederis et al., 1994; Wendelaar Bonga, 1997). Circulating ACTH binds to MC2R, G protein-coupled receptors on interrenal cells in the head kidney (Aluru and Vijayan, 2008), a structure homologous to the mammalian adrenal gland. Binding of ACTH to MC2R activates a cAMP-signaling cascade that facilitates the movement of cholesterol to the inner mitochondrial membrane, via StAR, where it is cleaved to pregnenolone by P450scc in the first and rate-limiting step of cortisol synthesis (Alsop and Vijayan, 2008; Hagen et al., 2006; Mommsen et al., 1999).

Because key mediators of cortisol synthesis are expected to increase in response to a stressor (eg., Alsop and Vijayan, 2008; Aluru and Vijayan, 2006; Jeffrey et al., 2014) it was hypothesized that attenuation of the cortisol response in male smallmouth bass during early parental care was a result of modulation of HPI axis activity.

To test the above hypothesis, male smallmouth bass guarding nests were sampled during early and late parental care, when the cortisol response is, respectively, attenuated and re-established (O'Connor et al., 2011b). Circulating cortisol and ACTH levels were assessed together with mRNA abundance of POA and hypothalamus CRF and head kidney MC2R, StAR, and P450scc in males pre- and post-exposure to a stressor.

5.3 Materials and methods

5.3.1 Experimental animals

In the spring of 2012, male smallmouth bass [N = 27, total length = 36.8 ± 0.9 cm (mean ± SEM); size range = 29.5-46.0 cm] guarding nests were identified by snorkeling on Charleston Lake in eastern Ontario. Males were sampled on two occasions representing the beginning (May 17, 2012; water temperature 16°C) and end (May 29, 2012; water temperature 20°C) of the parental care period when males were guarding fresh eggs (MG-FE; N = 13) and free-swimming fry (MG-FSF; N = 14), respectively. Only males with an egg or fry score of 3-4 (where the maximum score of 5 represents a nest of > 4000 eggs/fry and the minimum score of 1 represents a nest of < 500 egg/fry) were sampled (O'Connor et al., 2011b).

Male smallmouth bass were either sampled as unstressed controls (N = 14) or were exposed to a standardized stressor (N = 13). Males were angled using a standard rod-and-reel with barbless hooks and a rubber mesh landing net and immediately were placed in a foam-lined trough filled with fresh lake water (O'Connor et al., 2011b). Once a fish was hooked, it was landed and placed in the trough within 20 s and total length was measured. A baseline blood sample (1.5 ml) was collected immediately from all fish by caudal puncture using EDTA-coated 3 ml vacutainers (21 G needle; BD Vacutainer). Subsequently, fish were either euthanized as controls or subjected to 3 min of air exposure in a damp, foam-lined plastic tub. Stressed fish were allowed to recover in individual tubs filled with fresh lake water until a second blood sample (O'Connor et al., 2011b) was collected 25 min post-stress, and again until 2 h post-stress, when fish were euthanized. O'Connor et al. (2011b) validated this approach and reported that the peak cortisol responsiveness occurred 25 min post-stress. Fish were euthanized via cerebral percussion in accordance with approved standard operating procedures approved by institutional animal care committees (protocol # B10-09, Carleton University) and in accordance with the guidelines of the Canadian Council on Animal Care for the use of animals in research and teaching. Hypothalamus, POA, and head kidney tissue were collected immediately after phlebotomy (after blood withdrawal for control fish and 2 h post-stress for stressed fish). Blood samples were centrifuged at 10,000 g for 5 min in the field. Plasma and tissue samples were flash frozen in liquid nitrogen, and stored at -80°C for later analysis of plasma cortisol and ACTH levels or mRNA abundance of HPI axis-related genes.

5.3.2 Hormone analysis

Circulating levels of cortisol and ACTH were determined by RIA using commercially available kits (MP Biomedicals). All samples were analyzed together in a single assay where intra-assay variability (% CV) was 6.79% for ACTH. In our hands, the intra-assay variability for cortisol is typically 7.3% (Jeffrey et al., 2014). These kits have been validated for use on teleost samples (Doyon et al., 2006; Gamperl et al., 1994; Lim et al., 2013; O'Connor et al., 2011b).

5.3.3 RNA and first strand cDNA synthesis

RNA extraction and cDNA synthesis were performed as in (Jeffrey et al., 2012). Briefly, total RNA was extracted from tissues (10-100 mg) using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and quantified using a NanoDrop® ND-1000 UV–Vis Spectrophotometer. Tissues were homogenized by repeatedly passing the solution of TRIzol and tissue though syringes with 18 G and 23 G needles. Prior to cDNA synthesis, 1 μg of POA and hypothalamus, and 2 μg head kidney RNA were treated with DNase I (amplification grade; Invitrogen) according to the manufacturer's protocol. The cDNA was synthesized by reverse transcription using 100 U of RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) and 0.2 μg of random hexamer primer (IDT ReadyMade Primer) according to the manufacturer's protocol.

5.3.4 Gene sequences

Partial nucleotide sequences for CRF, MC2R, StAR, and P450scc were generated from cDNA synthesized from POA (for CRF) and head kidney (for MC2R, StAR, and P450scc) using PCR with gene-specific primers (Table 5.1) that were designed using Primer3 (SimGene.com) based on conserved sequences for several teleost fish species. For StAR and P450scc, a second set of primers was used (forward 2 and reverse 2) to extend the sequence, where forward 2 was a nested primer within the sequence produced from forward 1 and reverse 1 primer set. Primers for CRF were based on conserved regions in *C. auratus* (AF098629), *C. carpio* (AJ317955), *D. rerio* (NM001007379), *D. labrax* (JF274994*), G. aculeatus* (ENSGACT00000003899), *Haplochromis burtoni* (EF363131), *O. mykiss* (AF296672), *O. latipes* (NM001128518), *Platichthys flesus* (AJ555623), *Salmo salar* (NM001141590), *Takifugu rubripes* (ENSTRUT00000022109), and *Tetraodon nigroviridis* (ENSTNIT00000000684). Primers for MC2R were based on conserved regions in *C. carpio* (AJ605725), *D. rerio* (AY161848), *G. aculeatus* (ENSGACT00000004920), *Gadus morhua* (ENSGMOT00000000108), *O. latipes* (ENSORLT00000005412), *O. mykiss* (NM001124680), *T. rubripes* (AY227793), and *T. nigroviridis* (AY332239). Steroidogenic acute regulatory protein primers were based on the *M. salmoides* sequence for StAR (DQ166820). Primers for P450scc were based on conserved regions in *D. rerio* (BC154309), *G. aculeatus* (ENSGACT00000006237), *G. morhua* (ENSGMOT00000009165), *Ictalurus punctatus* (AF063836), *Odontesthes bonariensis* (GQ381266), *O. latipes* (ENSORLT00000009022), *O. mykiss* (S57305),

Tautogolabrus adspersus (GU596480), *T. nigroviridis* (ENSTNIT00000011435), and *T. rubripes* (ENSTRUT00000035401).

In each case, 25 µl reaction compositions were as follows; 2 µl cDNA, 0.25 µl Choice Taq DNA polymerase (Denville), 0.2 mM dNTPs (Invitrogen), and 0.2 µM primer. All PCR reactions were performed using a Bio-Rad S1000 Thermal Cycler (Bio-Rad) with the following cycling conditions; annealing temperature 55°C (30 s) and elongation temperature 72°C (30 s) for 38 cycles. Amplicons were run on 1.5% agarose (Life Technologies) gels with ethidium bromide (Fisher Scientific), extracted using QIAquick gel extraction kit (QIAGEN), and cloned using QIAGEN PCR cloning kit and Subcloning Efficiency DH5a Competent Cells (Invitrogen) following the manufacturers' protocols. Plasmids were extracted using QIAprep Spin Miniprep Kit (QIAGEN) and were sequenced by GenScript USA Inc. Gene cloning resulted in partial sequences; 386 bp for CRF, 397 bp for MC2R, 403 bp for StAR, and 861 bp for P450scc. These sequences were sufficient to generate gene-specific primers for real-time RT-PCR.

5.3.5 Semi-quantitative real-time RT-PCR

Semi-quantitative real-time RT-PCR was used to assess the mRNA abundance of target genes. Primers for CRF, MC2R, StAR, and P450scc (Table 5.2) were generated using Primer3 (SimGene.com). The ribosomal 18S subunit was used as a reference gene, and primers were retrieved from Robertson et al. (2009). Primer specificity was verified by sequencing of amplicons for each gene-specific primer set. In addition, standard curves were generated for each primer set to optimize the reaction composition based on the efficiency of the reaction; the efficiency for each primer set

was between 0.97 and 1.04. Real-time RT-PCR reactions were carried out using SYBR green III mastermix kit (Stratagene) and Bio-Rad CFX96 Real-Time PCR System (Bio-Rad). For all reactions, the manufacturer's instructions were followed with the exception that reaction volumes were scaled to 10 μl rather than 20 μl. Primer concentrations were 0.07 μM for MC2R, 0.1 μM for CRF and P450scc, and 0.12 μM for 18S and StAR; in addition, cDNA was diluted 25-fold for CRF, 10-fold for MC2R, StAR, and P450scc, and 1000-fold for 18S. In each case, the cycling conditions were 95°C (10 s) and 60°C (6 s) for 39 cycles. Dissociation curves were generated for each product to confirm primer specificity and no template controls (cDNA replaced with water for S-QPCR) as well as no reverse transcriptase controls (cDNA synthesis reaction carried out without reverse transcriptase) were included to eliminate the possibility of genomic DNA contamination. The mRNA abundance of each gene was calculated relative to the control MG-FE using the modified delta-delta Ct method (Pfaffl, 2001) with 18S as the normalizing gene, as in Jeffrey et al. (2012).

5.3.6 Statistical analyses

All data are expressed as mean values ± 1 SEM. For plasma ACTH and cortisol concentrations, only repeated samples from stressed fish were used, and these data were analyzed by two-way RM ANOVA. For mRNA abundance, data were analyzed by two-way ANOVA. Data were transformed using a square root transformation where they did not pass the normality test (for StAR). Where trends were present in the data but no overall (i.e., by parental care stage or stress treatment for the entire ANOVA model) statistical differences were present, within-stage differences were further

analyzed by one-tailed Student's *t*-test (plasma ACTH, POA CRF, and head kidney StAR) for the purpose of discussion. Our overall statistical power was low given conservation concerns with sampling wild fish engaged in reproduction so we felt that this approach would not lead to spurious type I errors given the overall conservatism associated with low sample size. All analyses were carried out using Sigma Plot V.11. The level of significance (α) was 0.05.

Table 5.1 Oligonucleotide primer sets used for gene cloning in smallmouth bass (*Micropterus dolomieu*).

Gene	Primer direction	Primers (5' to 3')
CRF	Forward	cgc tat gaa tgt agg gct att g
	Reverse	tct gtt gct ttg cgc ttg ctg
MC2R	Forward	cac tcg ccc atg tac tgc tt
	Reverse	cgt gcc agc agg aac atg ta
StAR	Forward 1	tgg agc aaa tgg ggg agt gg
	Reverse 1	gat tgt ctt tgg gat cca gc
	Forward 2	atg tcc act cag cac ccg aa
	Reverse 2	tca gca ggc gtg agc cat ct
P450scc	Forward 1	gag att cct gga ctg tgg aag
	Reverse 1	gga ggt ggt ctt gaa cat gag
	Forward 2	atc ctg ttc aaa gcg gag gg
	Reverse 2	tcc tgg agg ttg gga tgt ct

CRF, corticotropin-releasing factor; MC2R, melanocortin 2 receptor;
StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 side chain cleavage enzyme.

Table 5.2 Oligonucleotide primer sets used for semi-quantitative real-time RT-PCR in smallmouth bass (*Micropterus dolomieu*).

Gene	Primers (5' to 3')	Accession number
18S	F – gca aag ctg aaa ctt aaa gga att g R – tcc cgt gtt gag tca aat taa gc	EU502753
CRF	F – cat aag cag ctt cgg aga cc R – gac atc tcc atc atc tcc cg	KJ534544
MC2R	F – tca cca aaa cct ggg aga ac R – agc agg gag tcc atc aca tc	KJ534545
StAR	F – tgt tgt cag agc gga gaa tg R – aaa gtc cac ctg cgt ctg ag	KJ534546
P450scc	F – atc ttc aac caa gcg gac cg R – ttg cca gga ctc ctg ggt at	KJ534547

CRF, corticotropin-releasing factor; MC2R, melanocortin 2 receptor;
StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 side chain cleavage enzyme

5.4 Results

Plasma cortisol levels were significantly elevated by a standardized stressor in MG-FSF but not in MG-FE, confirming that the cortisol response during the early period of parental care was attenuated relative to that later in the parental care period (Fig. 5.1A; two-way RM ANOVA $P = 0.067$ for parental care stage, $P < 0.001$ for sampling time, and $P = 0.024$ for stage x time). The post-stress cortisol levels of MG-FSF were significantly higher than those of MG-FE. Plasma ACTH levels appeared to be elevated in response to the stressor but this trend was not significant with analysis by two-way RM ANOVA (Fig. 5.1B; $P = 0.747$ for stage, $P = 0.066$ for sampling time, and $P = 0.444$ for stage x time). However, when males at different parental stages were analyzed separately, ACTH levels were significantly elevated in MG-FSF (one-tailed Student's *t*-test, $P = 0.0175$) but not in MG-FE (one-tailed Student's *t*-test, $P = 0.250$).

Corticotropin-releasing factor mRNA abundance was assessed in the POA and hypothalamus of control and stressed males at each parental-care stage. No significant differences in hypothalamic CRF mRNA levels were found (data not shown). Preoptic area CRF mRNA levels were significantly higher in MG-FSF compared to MG-FE (Fig. 5.2; two-way ANOVA, $P = 0.050$ for parental care stage, $P = 0.418$ for treatment group, and $P = 0.111$ for stage x treatment). When analyzed individually, CRF mRNA abundance was significantly higher in stress-exposed males in MG-FSF (one-tailed Student's *t*-test, $P = 0.037$) but not in MG-FE (one-tailed Student's *t*-test, $P = 0.302$).

Exposure to a standardized stressor resulted in significantly higher MC2R mRNA abundance in MG-FSF but not MG-FE, and levels of MC2R mRNA in males of the

stress-exposed group were significantly higher in MG-FSF compared to MG-FE (Fig. 5.3; two-way ANOVA, $P = 0.097$ for parental care stage, $P = 0.20$ for treatment group, and $P = 0.014$ for stage x treatment). Although no significant differences with parental stage or exposure to a stressor were detected in P450scc mRNA levels (two-way ANOVA, $P = 0.106$ for stage, $P = 0.883$ for treatment group, and $P = 0.664$ for stage x treatment), StAR mRNA abundance appeared to be higher in MG-FSF compared to MG-FE (two-way ANOVA, $P = 0.068$ for stage, $P = 0.107$ for treatment group, and $P = 0.179$ for stage x treatment). Analysis of these data by parental care stage suggested a trend for StAR mRNA abundance to be higher in males exposed to a stressor than in control males in MG-FSF (one-tailed Student's t-test, $P = 0.0585$) but not MG-FE ($P = 0.314$) (see Fig. 5.4).

Figure 5.1 Plasma (A) cortisol and (B) adrenocorticotripic hormone (ACTH) levels in male smallmouth bass (*Micropterus dolomieu*) guarding fresh eggs (MG-FE; N = 6) or free-swimming fry (MG-FSF; N = 6-7). Males were subjected to 3 min of air exposure and blood samples were collected before (baseline) and 25 min after the stressor. Values are means + SEM. An asterisk indicates a significant difference in 25 min post-stress cortisol levels between MG-FE and MG-FSF, and a dagger indicates a significant difference between baseline and 25 min post-stress cortisol levels within a parental care stage (two-way RM ANOVA; see text for details).

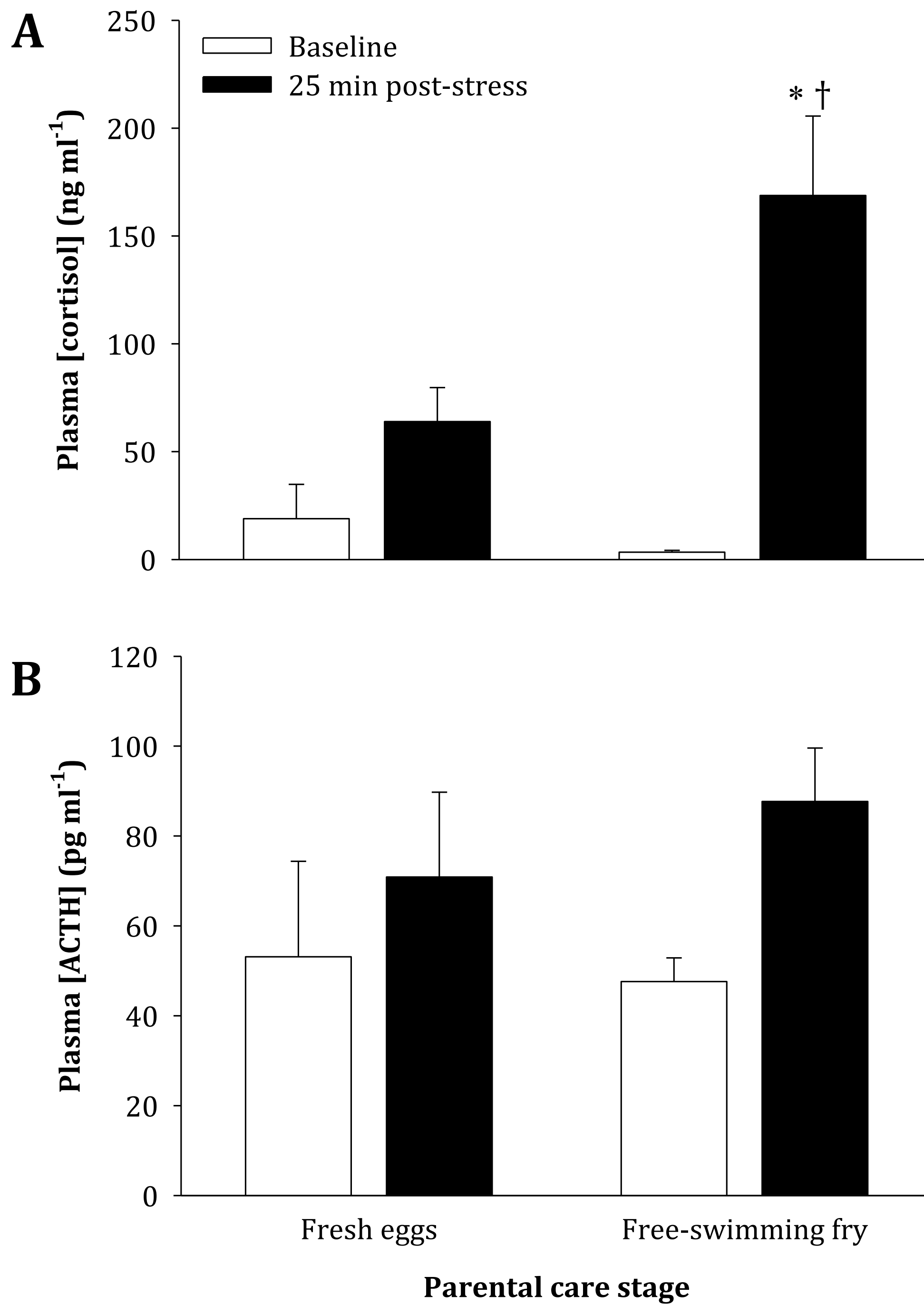

Figure 5.1

Figure 5.2 Relative mRNA abundance of preoptic area (POA) corticotropin-releasing factor (CRF) in male smallmouth bass (*Micropterus dolomieu*) guarding fresh eggs (MG-FE) or free-swimming fry (MG-FSF). Males (N = 6) were euthanized immediately post-capture (control), or 2 h after 3 min of air exposure (stressed). Values are means + SEM. All data were normalized to the mRNA abundance of 18S and expressed relative to the MG-FE control group. An asterisk indicates a significant difference between MG-FE and MG-FSF, with no significant effect of exposure to the stressor (two-way ANOVA; see text for details).

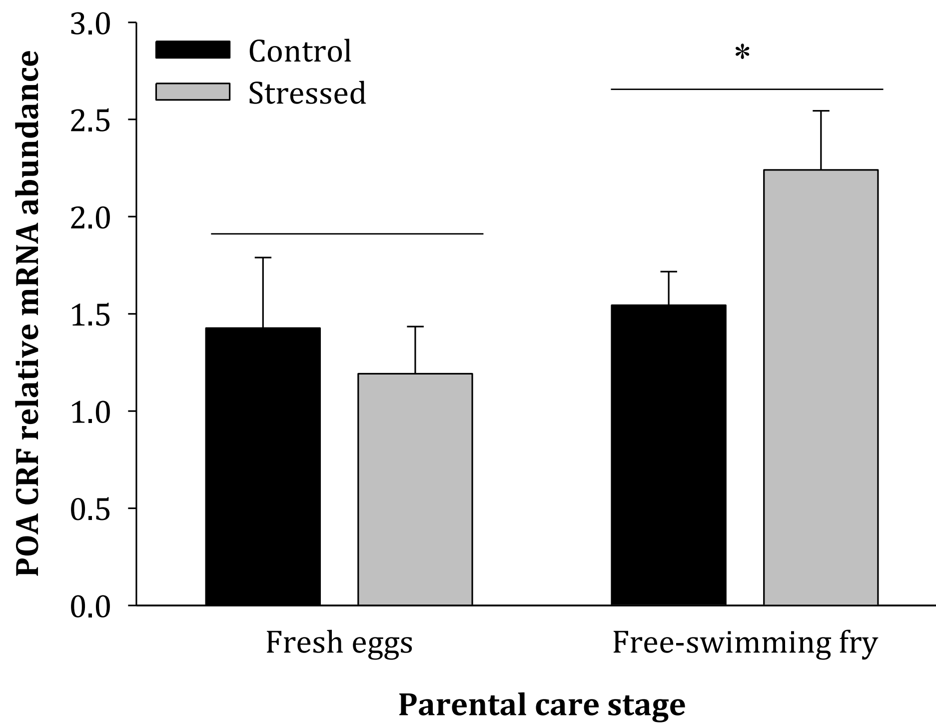

Figure 5.2

Figure 5.3 Relative mRNA abundance of head kidney melanocortin 2 receptor (MC2R) in male smallmouth bass (*Micropterus dolomieu*) guarding fresh eggs (MG-FE; N = 6-7) and free-swimming fry (MG-FSF; N = 6). Males were either euthanized immediately post-capture (control), or 2 h after 3 min of air exposure (stressed). Values are means + SEM. All data were normalized to the mRNA abundance of 18S and expressed relative to the MG-FE control group. An asterisk indicates a significant difference between stressed MG-FE and MG-FSF males, and a dagger indicates a significant difference between control and stressed groups within MG-FSF (two-way ANOVA; see text for details).

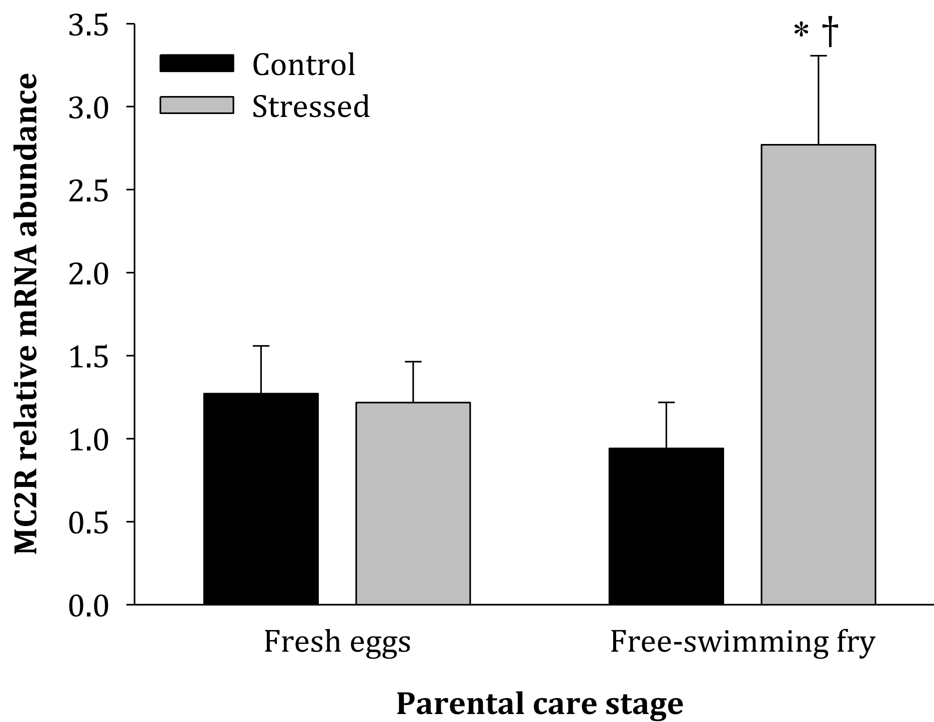

Figure 5.3

Figure 5.4 Relative mRNA abundance of head kidney steroidogenic acute regulatory protein (StAR; A) and cytochrome P450 side change cleavage enzyme (P450scc; B) in male smallmouth bass (*Micropterus dolomieu*) guarding fresh eggs (MG-FE; N = 6-7) or free-swimming fry (MG-FSF; N = 6). Males were either euthanized immediately post-capture (control), or 2 h after 3 min of air exposure (stressed). Values are means + SEM. All data were normalized to the mRNA abundance of 18S and expressed relative to the MG-FE control group. Data were analyzed by two-way ANOVA (see text for details).

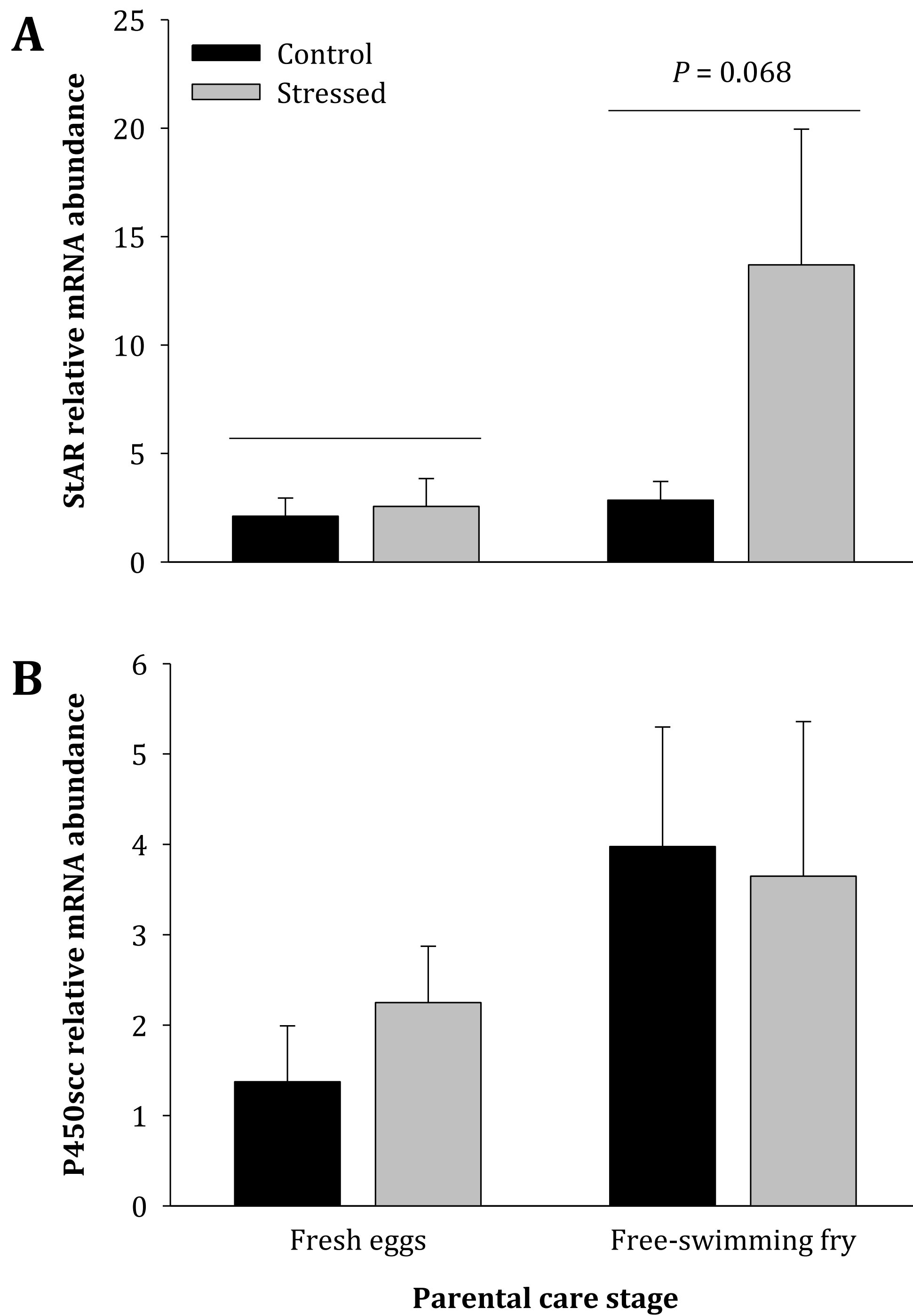

Figure 5.4

5.5 Discussion

Male smallmouth bass attenuated their cortisol response to a stressor during early parental care, a response that was recovered by the end of the parental care period (see also O'Connor et al., 2011b). The results of the present study supported the hypothesis that this attenuated cortisol response reflected modulation of HPI axis activity. Activity of the HPI axis was modulated at multiple levels, and in directions that would be expected to decrease cortisol synthesis in response to a stressor. With the exception of hypothalamic CRF and head kidney P450scc mRNA abundance, which did not vary significantly with parental care stage, all other variables tested, including POA CRF mRNA abundance, circulating ACTH concentration, and MC2R and StAR mRNA levels (the latter as a trend), increased in response to a stressor in MG-FSF but not in MG-FE.

We acknowledge that it was not possible to control for water temperature given that it rose steadily during the parental care period for bass (i.e., water temperatures were 16°C at the FE sampling period and 20°C at the FSF period), a common problem with studies of parental care in the wild (Wilson et al., 2012). To partially address this issue, in the present study, two additional groups of males were angled, euthanized and sampled outside of the reproductive period (in the fall) at water temperatures (16 and 20°C) equivalent to those experienced by males engaged in parental care. Only control (non-stressed) fish were used, to decrease the number of fish that had to be euthanized, particularly because distinguishing between males and females is challenging in non-reproductive bass. With the exception of POA CRF mRNA, key mediators of HPI axis function did not vary significantly between males sampled at low or high

temperatures, representing water temperatures for MG-FE and MG-FSF, respectively. Preoptic area CRF levels were significantly higher in males sampled at high water temperature compared to those sampled at low water temperature. Although this difference could be related to water temperature, it is also possible that seasonal differences may have been a contributing factor because no significant difference in POA CRF was found between control MG-FE and MG-FSF. Although a potential impact of water temperature cannot be ruled out, the data for non-reproductive fish provide some confidence that temperature effects were of little importance, and that parental care stage effects dominated the data set collected.

We also acknowledge that the relatively low sample size in this study made statistical analysis difficult. The most appropriate way to search for differences between parental care stages and treatments was a two-way ANOVA; however, our ability to detect statistically significant differences in the data was constrained by the relatively low *N* numbers. To this end, Student's *t*-tests were used to assess differences between treatments within a parental care period where differences were suspected but were not found to be significant by two-way ANOVA. Although increasing the sample size would be preferred, sampling additional males during the parental care period becomes problematic from a conservation perspective and would not be permitted by regulators.

5.5.1 Modulation of HPI axis function at multiple levels during early parental care

Corticosteroid axis reactivity and reproductive status are tightly linked in that reproductive status can dictate stress responsiveness, and corticosteroid levels can

affect reproductive success. During the reproductive period and subsequent parental care, robust activation of the stress axis can negatively impact reproduction (e.g., Schreck et al., 2001; Silverin, 1986; Wingfield and Silverin, 1986) as well as parental care behaviours (e.g., Almasi et al., 2008; Kitaysky et al., 2001; Silverin, 1986). The effect of high glucocorticoid levels on reproduction and parental care has been assessed in a number of avian species. Experimental increases in glucocorticoid levels decreased reproductive success in pied flycatchers (*Ficedula hypoleuca*) (Silverin, 1986), decreased territorial behaviour in male song sparrows (*Melospiza melodia*) (Wingfield and Silverin, 1986), reduced nestling provisioning in male barn owls (*Tyto alba*) (Almasi et al., 2008), and increased the time spent away from the nest in black-legged kittiwakes (*Rissa tridactyla*) (Kitaysky et al., 2001). Similarly, nest abandonment increased in male smallmouth bass with exogenously elevated cortisol levels (Dey et al., 2010; O'Connor et al., 2009). Owing to the possible negative impacts of high glucocorticoid levels on reproductive success, it has been hypothesized that 'resistance to stress' (e.g., an attenuated glucocorticoid response to a stressor) is an adaptive response to increase current reproductive success (Wingfield and Sapolsky, 2003). Resistance to stress has been observed in a number of vertebrate species and is a strategy that can vary across species; blockade at the level of the central nervous system, HPA/HPI axis, or hypothalamic-pituitary-gonadal (HPG) axis is possible (reviewed by Wingfield and Sapolsky, 2003). In the current study and that of O'Connor et al. (2011b), an attenuated cortisol response to an acute stressor was found in parental care-providing male smallmouth bass, with the most severe attenuation of the stress response occurring early in the parental care period when males were guarding

fresh eggs; the response was re-established by the end of the parental care period when fry were free-swimming. To our knowledge, few studies have assessed stress responsiveness in teleosts providing parental care. However, several studies on avian species reported similar attenuation of the acute stress response during parental care (e.g., Done et al., 2011; Meddle et al., 2003; O'Reilly and Wingfield, 2003; Silverin and Wingfield, 1998; Wingfield et al., 1992). Although the dynamics of reproduction and parental care are different between birds and teleosts (e.g., smallmouth bass), they share similarities in that parental investment can fluctuate over the period of parental care (Sargent and Gross, 1986), which in turn is reflected in the extent of their response to acute stressors. In the Arctic-breeding polygynandrous songbird Smith's longspur (*Calcarius pictus*), for example, males arriving on the breeding ground retained a sensitive stress response, but this response was suppressed once paternity was established during the nestling stage and parental investment was highest (Meddle et al., 2003). In smallmouth bass, parental investment arguably may be higher in the early stages of parental care when the stress response exhibits the greatest degree of attenuation, compared to the late stages of parental care when offspring near independence (Cooke et al., 2002). Although extensive work has documented (presumably) adaptive changes in the stress response during breeding and parental care, few studies have attempted to elucidate the mechanisms through which HPA/HPI axis function is modulated (Meddle et al., 2003; Romero et al., 1998). In the current study, HPI axis function was altered at multiple levels during early but not late parental care, which was in agreement with cortisol responsiveness during these periods.

Activation of the HPI axis and subsequent cortisol production begins with the release of CRF to the pituitary from neurons whose cell bodies originate in the POA (reviewed by Bernier et al., 2009; Flik et al., 2006; Lederis et al., 1994). Although CRF is produced in other areas of the brain, POA CRF is thought to play the largest role in activating pituitary corticotropes to produce ACTH in teleosts (Bernier et al., 2009). In mammals, CRF is depleted from CRF-containing neurosecretory neurons of the HPI axis in response to stress and elicits an increase in CRF mRNA expression in the PVN, the mammalian equivalent of the POA (e.g., Chen et al., 2004; Imaki et al., 1995). Although this process is less well understood in fish, there is evidence for a similar increase in POA CRF mRNA in response to stress (confinement, Ando et al., 1999; hypoxia, Bernier and Craig, 2005; subordinance, Doyon et al., 2003; repeated chasing, isolation, and confinement, Doyon et al., 2005; vortex stress, Fuzzen et al., 2010). In accordance with these findings, POA CRF mRNA levels were elevated in response to a stressor in MG-FSF but not MG-FE in the present study. Suppression of stress-induced increases in POA CRF mRNA abundance in MG-FE may explain, at least in part, the attenuated cortisol response observed in these fish. Factors such as CRF-receptors and CRF-BP mediate the downstream actions of CRF (Alderman and Bernier, 2007; Pohl et al., 2001; Seasholtz et al., 2002) and as such, future studies should investigate how these factors may be regulated by parental care stage.

Pituitary corticotropes release ACTH, which then binds to MC2R of interrenal cells in the head kidney. Previous studies in teleost fish have shown that ACTH levels increase in response to an acute stressor (Aluru and Vijayan, 2008; Balm and Pottinger, 1995; Pickering et al., 1986; Sumpter et al., 1986), and that this increase in circulating

ACTH levels causes an increase in MC2R mRNA abundance (Aluru and Vijayan, 2008). As expected, plasma ACTH and MC2R mRNA levels increased in response to stress in MG-FSF. Again, however, circulating ACTH and MC2R mRNA levels failed to increase in response to stress in MG-FE. Autoregulation of MC2R mRNA by ACTH is thought to play an important role in maintaining interrenal responsiveness to ACTH (Aluru and Vijayan, 2008). A failure to increase circulating ACTH levels and interrenal MC2R mRNA abundance in response to a stressor suggests a desensitization of cortisol responsiveness at the level of the interrenal cells which could help to explain the attenuated cortisol response in MG-FE. Stimulation of the interrenal cells with ACTH in males at different stages of parental care could shed further light on whether interrenal insensitivity plays a role in modulation of the cortisol response in these fish. For example, Jeffrey et al. (2014) demonstrated that interrenal cell insensitivity to ACTH was responsible for attenuation of the cortisol response in subordinate rainbow trout.

Cortisol synthesis also is mediated by other factors including StAR and P450scc. Cholesterol is moved to the inner mitochondrial membrane by StAR and cleaved to pregnenolone by P450scc as the first and rate-limiting step in cortisol synthesis (Aluru and Vijayan, 2006, 2008; Hagen et al., 2006). The current study presents some evidence to support an increase in StAR but not P450scc mRNA abundance in response to stress in MG-FSF, a response that was not observed in MG-FE. Like MC2R, both StAR and P450scc mRNA levels have been shown to increase in response to a stressor in teleosts (Aluru and Vijayan, 2006; Geslin and Auperin, 2004; Jeffrey et al., 2014), and during ACTH-stimulation *in vitro* (Aluru and Vijayan, 2008; Hagen et al., 2006). However, transcriptional regulation of StAR and P450scc remains poorly understood in

teleosts since Alderman et al. (2012) and Geslin and Auperin (2004) failed to observe an increase in mRNA levels of these steroidogenic genes following ACTH-stimulation *in vitro* (Alderman et al., 2012; Geslin and Auperin, 2004) or chasing (Geslin and Auperin, 2004). Geslin and Auperin (2004) suggested that the severity of the stressor and the sampling time may influence transcriptional regulation of and observation of an mRNA increase in both StAR and P450scc. The lack of an increase in StAR in response to stress in males at the early parental care stage provides further evidence for mediation of the cortisol response at the level of the interrenal cells in these smallmouth bass; however, transcriptional regulation of steroidogenic genes in teleost fish warrants further investigation.

5.5.2 Conclusion

The trade-off between current and future reproduction in relation to the glucocorticoid stress response has been documented in avian species as well as other taxa, and evidence for similar trade-offs in teleosts is increasing. However, most of these studies focused on the ecological relevance of the trade-off and did not probe more deeply into the physiological mechanisms underlying changes in the glucocorticoid response during the period of parental care. The present study is one of the first that has attempted to fill this gap. The current study provides evidence to support a multi-level modulation of HPI axis activity during the early parental care period, with key mediators in the brain (POA CRF), pituitary (circulating levels of ACTH), and interrenal cells (MC2R and StAR) failing to increase in response to stress, accounting for the attenuated cortisol response in male smallmouth bass during early

parental care. Further studies into the causes of a hypoactive HPI axis during early parental care are warranted. Of particular interest would be the role of androgens in mediating HPI axis function.

5.6 Acknowledgments

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CHAPTER 6

General Discussion

6.1 Overview

This thesis has provided a detailed examination of several mechanisms by which HPI axis function is regulated in teleost fish. An analysis of modulation of HPI axis function by social status in juvenile rainbow trout revealed that both dominant and subordinate status resulted in changes in the expression of key genes and hormones of the HPI axis. Examination of the impact of subordinate social status on a fish's response to additional stressors pointed to the head kidney as a key target for attenuation of the cortisol response in subordinate fish. Intergenerational impacts of social status on zebrafish offspring were evident in that both maternal dominance and maternal subordinance affected aspects of HPI axis function over early development. Interestingly, maternal subordinance resulted in larval offspring with attenuated stress-induced cortisol levels, suggesting a possible adaptive response to maternal social stress in these fish. Finally, the mechanisms underlying attenuation of the stress response during early parental care in male smallmouth bass were studied. Compared to the parental care period later in the reproductive season, during early parental care, the stress response is dampened (O'Connor et al., 2011b) and fish also displayed hypoactive functioning of the HPI axis. The present chapter will focus on major themes that arise from these three studies: multi-level control of HPI axis function; plasticity of the HPI axis; and epigenetics as a mechanism of gene regulation. In addition, the broader implications of the thesis for the practice of aquaculture will be considered.

6.2 Multi-level control of HPI axis function

A common theme that emerges from each of the chapters of this thesis is that the stress axis is regulated at multiple levels. Social status, maternal social status and parental care affected circulating hormone levels and/or expression of key genes at the level of the hypothalamus [i.e., POA CRF system], the pituitary (i.e., CRF-receptors and plasma ACTH), and the interrenal cells of the head kidney (i.e., cortisol steroidogenesis). Regulation of HPI axis activity in response to factors such as acute or chronic stressors, contaminants, and reproduction have also been studied in fish. To a large extent these studies have focused on single level effects in response to factors that regulate HPI axis activity [hypothalamus/pituitary: e.g., Doyon et al. (2003), Filby et al. (2010), Wunderink et al. (2011); or interrenal cell: e.g., Aluru and Vijayan (2006), Sandhu and Vijayan (2011), Young et al. (1996)], which is useful when teasing out specific targets (see below) but it also limits the ability to capture potential multi-level effects of these regulators. Recently, Fuzzen and colleagues assessed the impact of reproductive hormones (17β-estradiol and 11-ketotestosterone) (Fuzzen et al., 2011b), and exposure to an acute 1 h vortex stressor (Fuzzen et al., 2010) on multiple HPI axis genes (e.g., POA CRF, pituitary POMC and PCs, head kidney steroidogenic genes, and/or cortisol production using an *in vitro* head kidney preparation). From a mechanistic standpoint, the role of serotonin (5-HT) on HPI axis activity was assessed at the level of the POA, pituitary, and interrenal cells by Lim et al. (2013) in goldfish. Earlier studies also found multi-level effects of chronic contaminant exposure on HPI axis function (pituitary corticotrope and interrenal cell size, and circulating cortisol levels in response to acute stress) in yellow perch and northern pike (*Esox lucius*) (Hontela et al.,

1997; Hontela et al., 1992). Together, these studies highlight the importance of going beyond single-level assessments of HPI axis function to fully understand the mechanisms that underlie responses to various factors. The findings from this thesis add to the current understanding of how regulators can act at multiple levels of the HPI axis to adjust its functioning. With an understanding that factors can regulate function at multiple levels, it begs the question of how to tease apart such multi-level effects.

In an integrated system like the stress axis it is difficult to distinguish between effects specific to a particular level (e.g., POA, pituitary, or interrenal cells) and knock-on effects of changes higher up in the axis. To overcome this issue, head kidney preparations were used in rainbow trout, an approach that helped to clarify the role played by the head kidney in the reduced production of cortisol by subordinate fish in response to a netting stressor *in vivo* (Fig. 3.1D, Fig. 3.2, and Fig. 3.3A). As indicated above, similar approaches have been used in previous studies to determine the role of interrenal cells in mediating changes in stress responsiveness (e.g., Aluru et al., 2005; Gravel and Vijayan, 2006; Miller and Hontela, 2011).

Use of wild fish in the smallmouth bass work made this type of approach difficult (but not impossible, see Brodeur et al., 1997); however, others working on wild animals have provided an alternative approach. Instead of *in vitro* preparations to identify tissue specific contributions to cortisol production, injections of either CRF or ACTH and measurement of the resulting increases in circulating ACTH and/or corticosteroids have been used in the bird literature (e.g., Meddle et al., 2003; Romero et al., 1998) as well as in teleost fish (e.g., Girard et al., 1998). Similar methods could be used in smallmouth bass to assess whether the level of ACTH production by the pituitary (via

CRF injection) and/or interrenal cell sensitivity to ACTH (via ACTH injection) are limited during early parental care. The next logical step to move this work forward would be to determine what factors (e.g., hormonal) are responsible for altered stress axis activity (i.e., what triggers the onset of reduced HPI axis function). Although evidence for sex steroid effects on stress responsiveness in fish is equivocal (see review by Fuzzen et al., 2011a), two early studies suggested that stress responsiveness is attenuated in fish with elevated androgen levels (Pottinger et al., 1995; Pottinger et al., 1996). In smallmouth bass, androgens are elevated at the early stages of parental care when the HPI axis is attenuated (O'Connor et al., 2011b), and may thus be a candidate regulator of HPI axis function. Measuring the impact of elevated androgens (through 11-ketotestosterone or testosterone implants) on stress responsiveness in non-reproductive bass, or bass near the end of the reproductive period, as well as blocking the effects of androgens (through cyproterone acetate implants, an androgen antagonist) in bass during early parental care, could elucidate the role of androgens in suppressing HPI axis activity. Additionally, assessing androgen receptor expression at the multiple levels of the HPI axis would help in targeting possible level-specific effects of androgens.

The use of zebrafish embryos to assess maternal effects added the challenge of reduced tissue size. Pools of whole embryos/larvae were necessary to generate usable levels of RNA for S-QPCR analysis of HPI axis gene expression. Although these methods allowed for comparison with previous studies that used similar techniques to assess mRNA abundance of HPI axis genes over early development (e.g., Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Wilson et al., 2013), tissue-specific effects were

not measurable. Using *in situ* hybridization (e.g., Alderman and Bernier, 2009; Chandrasekar et al., 2007; Herzog et al., 2003; Hsu et al., 2002; To et al., 2007) or immunohistochemical (where zebrafish antibodies are available) approaches in developing zebrafish would provide information on tissue-specific, and HPI axis-specific (i.e., some HPI axis genes are additionally expressed in non-HPI axis-related tissues) effects of maternal social status. The findings of this thesis add to those of previous studies to emphasize the value of complementing whole-animal studies with reductionist approaches.

6.3 Plasticity of the HPI axis

Phenotypic plasticity is broadly defined as the expression of multiple phenotypes from a single genotype in response to environmental variation (Miner et al., 2005; Sultan and Stearns, 2005). The broad definition of phenotypic plasticity applies to changes in behaviour, physiology, morphology, life history, and growth, and can occur within the lifetime of an individual or across generations (Miner et al., 2005; Sultan and Stearns, 2005). Because more permanent effects of developmental plasticity differ from reversible short-term changes (e.g., metabolic or physiological responses), making distinctions between these types of phenotypic plasticity becomes important (see reviews by Dewitt and Scheiner, 2004; Piersma and Drent, 2003; Sultan and Stearns, 2005). Piersma and Drent (2003) proposed separating phenotypic plasticity into four categories: developmental plasticity (irreversible variation in traits due to processes in early development), polyphenism (a subcategory of developmental plasticity that applies to arthopods and other invertebrates; irreversible changes to the

phenotype of a series of generations within the same season), phenotypic flexibility (reversible phenotypic variation within a single individual), and life-cycle staging (a subcategory of phenotypic flexibility; reversible cyclic phenotypic changes in response to seasonal environmental variation in long-lived species). The findings of this thesis provide an opportunity to consider plastic changes in HPI axis function, keeping in mind the distinctions between developmental plasticity and phenotypic flexibility.

Developmental plasticity produces irreversible changes to an organism's phenotype due to processes that occur in early development as a consequence of environmental variation. Classically, developmental plasticity has been considered the most accepted form of phenotypic plasticity and often reflects parental effects (Miner et al., 2005; Piersma and Drent, 2003; Sultan and Stearns, 2005). For instance, exposure to elevated glucocorticoids in early development, either through elevated maternal or yolk glucocorticoid levels, reprograms HPA axis activity in birds (e.g., Hayward et al., 2006; Hayward and Wingfield, 2004; Love and Williams, 2008; Marasco et al., 2012). Similarly, maternal social status invoked plasticity of the HPI axis in developing zebrafish (Chapter 4). Specifically, offspring from dominant mothers may experience delayed activation of *de novo* cortisol synthesis, as evidenced by the lower baseline cortisol levels reported for these fish at 48 hpf (Fig. 4.2). Expression of CRF and P450scc (transcript levels) were accordingly lower in fish from dominant mothers (Fig. 4.4A and Fig. 4.5B), which may point to plastic changes at these levels of the HPI axis. Maternal subordinance also affected offspring, where stress induced levels of cortisol were attenuated at 6 dpf (Fig. 4.3B). In both cases, early experience (i.e., maternal social status) induced phenotypic changes in developing zebrafish

offspring. Determining whether these phenotypic changes are maintained into adulthood and have consequences for adult offspring will help to determine whether these phenotypic variations truly fall under the category of 'developmental plasticity' (i.e., irreversible changes) or whether this is a case of phenotypic flexibility during early development (i.e., a transient change).

Another interesting avenue to pursue would be other long-term effects of maternal social status on offspring behaviour (e.g., learning and anxiety-related behaviours) and social status. For instance, maternal exposure to a predator elicited a number of effects in three-spined stickleback offspring including altered anti-predator defence in juveniles (Giesing et al., 2010) and adults (McGhee et al., 2012), as well as adult learning (Roche et al., 2012). Exogenous cortisol treatment of eggs as a proxy for maternal stress altered learning, aggressiveness, and/or the chance of becoming subordinate in brown trout (Burton et al., 2011; Sloman, 2010). Thus, there is precedence for phenotypic changes due to maternal stress on factors beyond HPI axis function that can be further explored in offspring of dominant and subordinate mothers.

Reversible and transient phenotypic changes, referred to as phenotypic flexibility, can occur across different life stages. These variations in the phenotype enable organisms to adjust to rapid changes in their environment. For instance, a recent review by Angelier and Wingfield (2013) highlighted the importance of an animal's ability to alter the glucocorticoid response in response to a range of situations through integrative mechanisms including phenotypic flexibility. Phenotypic flexibility of HPI axis function was observed in juvenile rainbow trout of different social status

(Chapter 2 and 3). Notably, subordinate fish exhibited a chronically activated stress axis, resulting in decreased circulating levels of ACTH (likely due to negative feedback control of cortisol; Fig. 2.1B), desensitization of the liver to cortisol (decreased GR expression; Fig. 2.4), and reduced cortisol steroidogenic capacity at the level of the interrenal cells (Fig. 3.2). Subordinate salmonid fish also exhibit decreased growth relative to dominant fish (e.g., Abbott and Dill, 1989; DiBattista et al., 2006; Metcalfe, 1986), in part due to monopolization of food resources by dominant fish (e.g., Adams and Huntingford, 1996; Adams et al., 1998; Øverli et al., 1998), but also due to appetite suppression and impaired digestive function (DiBattista et al., 2006). These physiological adjustments in juvenile subordinate trout may be reversible, at least in part, if the stressor (i.e., dominant fish) were removed.

Interestingly, dominant fish also experienced changes in expression of interrenal cell steroidogenic genes (Fig. 2.3). Dominant fish are often considered to be 'control' fish in studies involving examination of social hierarchies. The phenotypic consequences of dominant status revealed in this work suggest that dominant fish should not be equated to 'control' fish. This conclusion is further supported by the observation that dominant fish also exhibit differences in hepatic metabolism (i.e., higher hepatic glycogen content likely owing to reduced liver glycogen phosphorylase activity and increased energy intake from food) compared to control fish (Gilmour et al., 2012).

Phenotypic flexibility can also apply to predictable changes in the environment (i.e., seasonal changes) that trigger physiological responses in long-lived species, referred to as life-cycle staging. For instance, many bird species suppress the

glucocorticoid response to stress during the reproductive and/or parental care period (e.g., Done et al., 2011; Meddle et al., 2003; O'Reilly and Wingfield, 2003; Silverin and Wingfield, 1998; Wingfield et al., 1992), likely owing to potential negative impacts of high glucocorticoids on reproduction (e.g., Silverin, 1986; Wingfield and Silverin, 1986) and parental care behaviours (e.g., Almasi et al., 2008; Kitaysky et al., 2001; Silverin, 1986). During the parental care period, male smallmouth bass also show transient regulation of the stress axis (O'Connor et al., 2011b; Fig. 5.1A), demonstrating phenotypic flexibility. The relatively fast change in stress axis responsiveness (within a period of 4-6 weeks) spawns several questions. For instance, is there variation among individual bass in the regulation of HPI axis function and hence the repression of stress axis activity, and does this have consequences for the level of care provided to their offspring? Two previous studies investigated the effects of exogenously-elevated cortisol levels on parental care in smallmouth (Dey et al., 2010) and largemouth (O'Connor et al., 2009) bass, and found increased incidence of nest abandonment with cortisol treatment. Bass are likely to experience stress (e.g., predation, fisheries-capture, and release) over the parental care period and while their stress response is repressed during early parental care, whether the bass stress response is equally repressed across individuals is an important point for consideration. It is widely accepted that variation in responses to stressors exists among individuals of a species (e.g., Hori et al., 2012; Pottinger and Carrick, 1999; Tort et al., 2001; Wang et al., 2004). An interesting avenue of study would be to assess whether the extent to which a male's stress response is attenuated correlates with nest success or the quality of parental care. Following from this idea, if the quality of care reflects HPI axis activity in the

paternal male, how does this impact offspring plasticity (i.e., is offspring HPI axis activity affected by the level of paternal care)? For instance, maternal factors (i.e., social status) affected stress axis activity in zebrafish, and the level of maternal care in rats has long-term effects on HPA axis activity (Weaver et al., 2004; Weaver et al., 2005; Weaver et al., 2007).

The adaptive potential of maternal stress, juvenile social status, and parental investment provide interesting areas for further study. Questions that arise from the findings of this thesis include those around heritability in or adaptive value to the variation in phenotypes observed in this thesis. For instance, in the case of developmental changes in zebrafish offspring that resulted from maternal social status, are changes to HPI axis function maintained in mature offspring (see above) and if so, can these phenotypes be transferred to their offspring (i.e., trans-generational effects)? Growing zebrafish to adults and assessing trans-generational effects of maternal social status will help to address this question. The attenuated stress response observed in offspring from subordinate mothers and male smallmouth bass during early parental care begs the question of whether these phenotypic changes in HPI axis activity are adaptive? The adaptive value of transient changes in HPI axis function in both dominant and subordinate juvenile rainbow trout also requires further examination. For instance, in an African cichlid, *Simochromis pleurospilus*, females raised in a poor quality environment (i.e., food deprived) as juveniles produced larger young due to increased investment in the eggs and faster larval growth (Taborsky, 2006). Could a similar situation apply to mature female rainbow trout that were subordinate as juveniles? Determining the fitness benefits of HPI axis plasticity would provide a

means for establishing whether these changes are in fact adaptive. For instance, are offspring of subordinate zebrafish better equipped to deal with stressors (e.g., social stress) as adults?

6.4 Epigenetics as a mechanism for gene regulation

The field of epigenetics has gained substantial momentum over the past decade, with more than 90 000 articles written on the topic in just the last 4 years (according to Google Scholar with the search query 'epigenetics'). Epigenetics is defined as the "study of mitotically (and potentially meiotically) heritable alterations in gene expression not caused by changes in DNA sequence" (Waterland, 2006). Waterland (2006) elegantly described epigenetic gene regulation as synergistic mechanisms that modify chromatin conformation and transcriptional activity. These mechanisms include methylation of cytosines within cytosine-phosphodiester-guanosine (CpG) dinucleotides in DNA, modification of histone proteins, and modification of numerous autoregulatory DNA-binding proteins (Jaenisch and Bird, 2003). Epigenetic mechanisms modulate gene expression in a tissue-specific faction and epigenetic gene regulation can be altered by experience. Epigenetic changes most often have been studied from a developmental point of view (e.g., Caldji et al., 2011; Champagne, 2012; Darnaudery and Maccari, 2008), but recently, epigenetic regulation of adult gene expression (e.g., transient regulation of physiological process in response to environmental changes; see below) also has been suggested (e.g., Elliott et al., 2010). Thus, epigenetic regulation provides a prime candidate for explaining phenotypic plasticity within the HPI axis.

Previous studies strongly suggest a role for epigenetic gene regulation in modulating effects of early environment on developmental outcomes (e.g., Mueller and Bale, 2008; Weaver et al., 2004; Weaver et al., 2007). For instance, prenatal stress resulted in epigenetic modification to CRF and GR that correlated with altered gene expression in mice (Mueller and Bale, 2008). Similarly, the effects observed in zebrafish offspring from mothers of different social status could potentially be achieved through epigenetic regulation. Potential targets to explore in this respect are methylation levels of the corticosteroid receptor (GR and MR) promoters in specific brain areas that would affect their expression (mRNA and protein). In mammals, the role of hippocampal GR and MR in negative feedback control of stress axis activity has been well characterized (de Kloet, 1998), and in rats, the level of maternal care regulates the methylation of hippocampal GR promoters, altering access of the promoter to NGFI-A, a transcription factor (Weaver et al., 2004; Weaver et al., 2005; Weaver et al., 2007). Although the picture remains less clear in fish, there is evidence for regulation of stress axis activity by both GR and MR at the level of the brain (e.g., POA) (Alderman et al., 2012; Alderman and Vijayan, 2012). Few differences in GR or MR transcript levels were observed in zebrafish in the current thesis (Fig. 4.6A and B), but only whole-body mRNA levels were measured. Likely it will be necessary to measure tissue-specific levels of both GR and MR transcripts and proteins to obtain specific information on the capacity for GR and MR regulation of the stress axis. Thus, GR and MR promoter regions in zebrafish would provide interesting targets to analyze for differences in methylation status arising from effects of maternal subordinance in zebrafish.

Recent studies have looked to epigenetic gene regulation as a possible mechanism for adjusting physiological responses in mature animals as well. Elliott et al. (2010) found evidence for epigenetic regulation of the CRF gene by chronic social stress. In this study, a subset of mice that showed increased social avoidance because of chronic social stress exhibited decreased demethylation of the promoter region for CRF, resulting in increased CRF mRNA expression (Elliott et al., 2010). Interestingly, antidepressant administration reversed this effect. Together, these results provide evidence that chronic stress affects DNA methylation in the adult mouse, and the authors suggested that regulation of DNA methylation could be a primary mechanism for behavioural changes in response to chronic stress (Elliott et al., 2010). Determining whether similar mechanisms regulate HPI axis function in dominant or subordinate juvenile rainbow trout provides exciting directions to explore. For instance, our study did not show changes in the level of CRF mRNA in subordinate individuals after 5 d of interaction (Fig. 2.2A), but Doyon et al. (2003) did find elevated CRF mRNA in subordinate fish after 3 d of interaction. Additionally, CRF mRNA expression was only elevated in subordinate trout (and not in sham-treated or dominant fish) 1 h after exposure to a netting stressor (Fig. 3.4A), which may indicate increased CRF turnover in subordinate fish. Methylation status of the CRF promoter in juvenile subordinate trout after 3-5 d of interaction may provide an interesting avenue to explore to better understand regulation of HPI axis function in these fish.

6.5 Conclusion

Collectively, the findings of this thesis present a thorough analysis of multi-level control of HPI axis function, as well as demonstrating situations of both developmental plasticity and phenotypic flexibility. Functioning of the HPI axis was mediated by social status, and for the first time, maternal social status effects on development of the HPI axis in teleost fish offspring were examined. The effects of parental investment in smallmouth bass also presented an example of multi-level control of HPI axis function and together with the social status studies, underscore the importance for further studies to consider multi-level approaches when assessing HPI axis function under different conditions. In addition developmental plasticity resulting in phenotypic changes in HPI axis function over early development by maternal social status in zebrafish, transient phenotypic changes were also observed in juvenile rainbow trout of different social status and in paternal smallmouth bass. These forms of phenotypic plasticity, in the general sense, spark questions such as whether epigenetic gene regulation might contribute to mechanisms underlying changes in the stress axis of these fish. Together, the findings from this thesis provide further insight into how behaviour and life-history modulate stress axis function.

APPENDIX

Early cortisol exposure effects on hypothalamic-pituitary-interrenal axis function in larval zebrafish

Notes on Appendix

This work was carried out at the University of Ottawa by J.D. Jeffrey and M. Brannen, in collaboration with P.L. McNeil and K.A. Sloman from the University of the West of Scotland.

i.i Objective and Methodology

Elevated cortisol levels during early embryogenesis can have a multitude of effects on teleost fish (see Chapter 1, section 1.4.3). To understand the possible implications of high cortisol levels on expression of HPI axis genes during early development, zebrafish embryos were treated exogenously with cortisol (2 h treatment; 500 ng ml^{-1}) immediately following fertilization. Zebrafish females were lightly anaesthetized, stripped of their eggs, and these eggs were fertilized *in vitro* using Hanks' solution containing the sperm from a group of males (see Chapter 4 section 4.4.3). Embryos were reared to 24, 48, and 96 hpf, after which embryos/larvae were sampled, flash frozen in liquid nitrogen, and stored at -80°C for later analysis by S-QPCR. Total RNA was extracted, cDNA was synthesized, and S-QPCR (for primers see Table 4.1) was performed as in Sections 5.3.3 and 5.3.5 of Chapter 5. To assess differences between cortisol-treated and control embryos/larvae, Student *t*-tests were used (non-parametric equivalents were used where data were not normally distributed), where the level of significance (α) was 0.05.

i.ii Results and Discussion

Cortisol treatment in the time following fertilization largely did not affect HPI axis gene transcript levels during early development in zebrafish (Table i.i). However, at 48 hpf, StAR mRNA was significantly higher in cortisol-treated compared to control embryos. This reflects a time in early development when embryos begin to synthesize cortisol *de novo* (Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Wilson et al., 2013). In addition, embryos start to hatch at 48 hpf, and cortisol plays role in

regulating hatching rate (Wilson et al., 2013). Although cortisol levels were not measured in these embryos, if higher StAR mRNA was also reflected by higher active protein levels, this may indicate higher cortisol production in cortisol-treated fish. Wilson et al. (2013) found that dexamethasone-treatment (a synthetic glucocorticoid) significantly increased hatching rate in developing zebrafish. Whether hatching rate was significantly affected in response to cortisol treatment in the current study remains unknown.

Together these results suggest, that at least for the genes assessed in the present study, cortisol treatment did not largely affect HPI axis genes during early development. However, further analysis of other HPI axis genes such as CRF and its receptors as well as other parameters such as hatching rate and survival should be measured. In addition, maternal social stress in zebrafish (Chapter 4) and cortisol treatment during early embryogenesis in rainbow trout (Auperin and Geslin, 2008) affected stress responsiveness in offspring. Similar studies using cortisol-treatment in zebrafish would shed further light on whether HPI axis function is modulated by early cortisol exposure in zebrafish.

Table i.i Relative mRNA abundance of hypothalamic-pituitary-interrenal axis genes in zebrafish (*Danio rerio*) embryos/larvae exposed to cortisol.

	Time point								
	24 hpf			**48 hpf**			**96 hpf**		
	Control	**Cortisol**	***P***	**Control**	**Cortisol**	***P***	**Control**	**Cortisol**	***P***
StAR	1.00 ± 0.28 (7)	1.17 ± 0.45 (5)	0.754	7.17 ± 1.12 (7)	22.15 ± 5.79 (6)	***0.038***	9.26 ± 1.54 (8)	7.33 ± 1.29 (5)	0.359
P450scc	182.09 ± 48.11 (7)	314.93 ± 162.72 (6)	*0.943*	3.709 ± 0.71 (7)	5.24 ± 1.52 (6)	0.391	1.00 ± 0.27 (8)	0.91 ± 0.25 (6)	0.804
GR	1.00 ± 0.07 (7)	1.12 ± 0.21 (6)	*0.721*	1.31 ± 0.21 (8)	2.47 ± 0.76 (6)	*0.272*	0.90 ± 0.10 (8)	0.94 ± 0.22 (6)	0.860
11β-HSD2	1.00 ± 0.4 (6)	1.08 ± 0.26 (5)	0.772	2.81 ± 0.58 (7)	4.47 ± 1.22 (6)	*0.432*	2.98 ± 0.21 (7)	3.27 ± 0.50 (6)	0.612

Values are means ± SEM (*N*); All mRNA data are expressed relative to mRNA expression of the normalizing 18S gene, and to the control 24 hpf group. Data were analyzed by Student *t*-tests (on ranks indicated by italics, and significant values are in bold font). 11β-HSD2, 11-β hydroxysteroid dehydrogenase type 2 ; GR, glucocorticoid receptor; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 side chain cleavage enzyme.

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