

Regulation of cortisol production by serotonin and negative feedback in the head kidney of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Production of the glucocorticoid hormone cortisol in response to a stressor is initiated by activation of the hypothalamic-pituitary-interrenal (HPI) axis in fish. Serotonin (5-HT) and negative feedback regulate cortisol production at the whole-animal level; the objective of the present thesis was to investigate their roles in regulating cortisol production by interrenal cells of rainbow trout (*Oncorhynchus mykiss*). Messenger ribonucleic acid (mRNA) for the 5-HT₄ receptor was present in low abundance in interrenal cells. In addition, cortisol production was significantly increased for *in vitro* head kidney preparations incubated with 5-HT, and this elevated cortisol production was blocked by the 5-HT₄ receptor antagonist 5-fluoro-2-methoxy-[1-[2-[(methylsulphonyl) amino] ethyl]-4-piperidinyl]-1h-indole-3-methylcarboxylate sulphamate (GR125487). Thus, 5-HT acts at the head kidney level to regulate cortisol production, probably via the 5-HT₄ receptor. Chronic social stress did not appear to regulate the expression of key proteins involved in cortisol biosynthesis or corticosteroid receptors (CR). However, head kidney tissue incubated *in vitro* with cortisol for 2-8 h showed a reduction in adrenocorticotrophic hormone-stimulated cortisol production compared to controls, suggesting the existence of an ultra-short-loop negative feedback mechanism. Thus, the high circulating levels of cortisol in trout experiencing chronic social stress may activate this ultra-short-loop negative feedback mechanism to suppress cortisol production at the head kidney level.

Résumé

L'hormone glucocorticoïde cortisol est produite en réponse à un facteur de stress qui active l'axe hypothalamique-pituitaire-interrénal (HPI) chez les poissons. La sérotonine (5-HT) et la rétroaction négative régulent la production de cortisol au niveau de l'animal entier; l'objectif de cette thèse était d'étudier leurs rôles dans la régulation du cortisol au niveau des cellules interrénales chez les truites arc-en-ciel. L'acide ribonucléique messager (ARNm) pour le récepteur 5-HT₄ était présent en basse quantité au niveau des cellules interrénales. De plus, la production du cortisol a augmenté de manière significative lorsque les préparations *in vitro* du rein antérieur ont été incubées avec 5-HT et cette augmentation a été bloquée par l'antagoniste du récepteur 5-HT₄ 5-fluoro-2-methoxy-[1-[2-[(methylsulphonyl) amino] ethyl]-4-piperidiny]-1h-indole-3-methylcarboxylate sulphamate (GR125487). Donc, 5-HT agit au niveau du rein antérieur pour réguler la production du cortisol probablement via le récepteur 5-HT₄. Le stress social chronique n'a pas régulé l'expression des protéines clefs qui sont impliquées dans la synthèse du cortisol ou les récepteurs corticostéroïdes (RC). Par contre, le tissu du rein antérieur incubé *in vitro* avec du cortisol pour 2-8 h a démontré une réduction de la production de cortisol lorsque stimulé avec l'adrénocorticotrophine comparé aux contrôles, ce qui suggère l'existence d'un mécanisme de rétroaction négative ultra courte. Ainsi, le haut niveau de cortisol en circulation chez les truites qui subissent un stress social chronique peut activer ce mécanisme de rétroaction négative ultra courte pour supprimer la production du cortisol au niveau du rein antérieur.

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List of abbreviations

Abbreviation	Full name
11 β HSD2	11- β -hydroxysteroid dehydrogenase type 2
11 β -hyd	11beta-hydroxylase
3 β HSD	3 β -hydroxysteroid dehydrogenase/ Δ 5-4 isomerase
5-HIAA	5-hydroxyindole acetic acid
5-HT	serotonin
5-HT _R	serotonin receptor and the subtype is indicated by the subscripted number/letter
5-HTP	5-hydroxytryptophan
8-OH-DPAT	8-hydroxy-N,N-dipropyl-2-aminotetralin
AC	adenylate cyclase
ACTH	adrenocorticotrophic hormone
α -methylserotonin	3-(2-aminopropyl)-1H-indol-5-ol
ANOVA	analysis of variance
AT	acetylated tubulin
ATP	adenosine triphosphate
BBB	blood-brain barrier
cAMP	cyclic adenosine monophosphate
carbachol	2-[(Aminocarbonyl)oxy]-N,N,N-trimethylethanaminium chloride
CCAC	Canadian Council on Animal Care

cDNA	complementary deoxyribonucleic acid
CR	corticosteroid receptors
CRF	corticotropin releasing factor
CRF-BP	corticotropin releasing factor binding-protein
CRFR	corticotropin releasing factor receptor and the type is indicated by the number
DAPI	4',6-diamidino-2-phenylindole
db-cAMP	dibutyryl cyclic adenosine monophosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DOI	1-(2,5-dimethoxy-4-iodophenyl) 2-aminopropane
DRN	dorsal raphe nucleus
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
G _{q/11}	G protein subunit that activates phospholipase C
GR	glucocorticoid receptor and the type is indicated by the number
GR125487	5-fluoro-2-methoxy-[1-[2-[(methylsulphonyl) amino] ethyl]-4-piperidinyl]-1h-indole-3-methylcarboxylate sulphamate
GRE	glucocorticoid response elements
G α_i	G protein subunit that inhibits adenylate cyclase
HK	head kidney
HPA	hypothalamic-pituitary-adrenal
HPI	hypothalamic-pituitary-interrenal

HRP	horseradish peroxidase
HSP90	heat shock protein 90
IU	international unit
MAO-A	monoamine oxidase-A
MCR	melanocortin receptor and the type is indicated by the number
MEM	minimum essential medium
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
MSH	melanocyte-stimulating hormone
<i>N</i>	sample size
P450 _{scc}	cytochrome P450 side chain cleavage enzyme
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline-Tween
PCA	principal component analysis
PCR	polymerase chain reactions
PCV	posterior cardinal vein
PFA	paraformaldehyde
PKA	protein kinase A
PLC	phospholipase C
POA	preoptic area
POMC	proopiomelanocortin
PVN	paraventricular nucleus
qPCR	real-time reverse transcription polymerase chain reaction

RIA	radioimmunoassay
RIPA	radioimmunoprecipitation assay
RM	repeated measures
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SER	smooth endoplasmic reticulum
SERT	serotonin transporters
StAR	steroidogenic acute regulatory protein
SV2	synaptic vesicle protein 2
TBST	tris-buffered saline-tween
TH	tyrosine hydroxylase
TPH	tryptophan hydroxylase and the type is indicated by the number
zacopride	4-amino-N-(1-azabicyclo[2.2.2]oct-3-yl)-5-chloro-2-methoxybenzamide
ZN-12	zebrafish neuron-specific surface antigen

CHAPTER 1

GENERAL INTRODUCTION

Exposure to stressors encountered in the natural environment and/or in aquaculture situations activates a stress response that can affect factors such as growth, resistance to disease, and reproduction (Wendelaar Bonga, 1997). Therefore, it is important to know how the stress axis is regulated. In addition, many species of fish form social hierarchies in which subordinate fish experience chronic stress (Gilmour et al., 2005). How the stress axis is regulated during chronic stress remains an open question. Thus, the overarching objective of the present thesis was to examine regulation of the stress axis using rainbow trout as the study species. The rainbow trout (*Oncorhynchus mykiss*) is part of the family Salmonidae and is of particular interest because of its importance in aquaculture. For instance, Canadian trout farmers produced over 5,000 tonnes of trout valued at over \$21 million in 2006 according to the Canadian Aquaculture Industry Alliance, and produced 6,700 tonnes of trout valued at \$39.1 million in 2013 according to Fisheries and Oceans Canada. This rapid expansion of the industry demonstrates the importance of aquaculture in Canada. Farmed fish experience numerous stressors such as handling, sorting, grading, intra- and interspecific interactions, poor water quality, overcrowding, and transport (Schreck, 1982; Barton and Iwama, 1991). For the purpose of this thesis, two potential regulators were studied with a focus on the head kidney, which is the tissue containing the cortisol-producing interrenal cells. Chapter 2 focused on serotonin (5-HT) as a potential regulator of cortisol production and Chapter 3 focused on negative feedback regulation of cortisol production during chronic social stress. This general introduction will review the key concepts and literature on which the experimental work was based, including the stress axis, 5-HT as a modulator of the stress axis, and negative feedback as a regulator of the stress axis.

1.1 The stress axis

1.1.1 Stressors and the hypothalamic-pituitary-interrenal (HPI) axis

Vertebrate animals may encounter extrinsic stimuli that are perceived as a threat and therefore disturb the animal's homeostasis; these stimuli may be described as stressors (Chrousos and Gold, 1992). Examples of such stressors include capture, transport, forced exercise, hypoxia, osmotic and temperature shocks, social interactions, or exposure to water pollutants (Chrousos and Gold, 1992; Wendelaar Bonga, 1997). Stressors can be of different duration or severity. For instance, an acute stressor is of short duration, minutes to hours, with examples being 3 h at high stocking density, a 1 h netting stressor, 2 h of confinement, or capture and transfer to low water for 2 min, whereas a chronic stressor is of longer duration, days to weeks, and examples include social subordination for 2-5 days, or high stocking density for 5 days (Gilchrist et al., 2000; Ramsay et al., 2006; Jeffrey et al., 2014). To cope with exposure to a stressor, vertebrate animals activate their stress response which results in the release of primary stress hormones, the catecholamines (adrenaline and noradrenaline) and the glucocorticoid hormones, with cortisol being the primary glucocorticoid in teleost fish (Wendelaar Bonga, 1997; Barton, 2002). The focus of the present study is cortisol, which is produced as the end-product of HPI axis activation.

Cortisol is produced on demand by interrenal cells (Chester et al, 1980; Wendelaar Bonga, 1997; Mommsen et al., 1999). However, the time course of the cortisol response differs according to whether an acute or chronic stressor is experienced. In response to an acute stressor, cortisol levels begin to increase after approximately 5 min, reach a maximum response, and then decrease to baseline levels within hours (Pickering and Pottinger, 1989; Wendelaar Bonga, 1997;

Gilmour et al., 2005). However, in response to a chronic stressor, cortisol levels begin to increase after ~5 min but may remain elevated for days and even weeks (Pickering and Pottinger, 1989; Wendelaar Bonga, 1997; Gilmour et al., 2005). Activation of the HPI axis triggers the release of corticotropin releasing factor (CRF), a 41-amino acid peptide of the CRF family, which includes CRF, sauvagine, urotensins, and urocortins (Barton, 2002; Huising et al., 2004; Flik et al., 2006). Levels of CRF messenger ribonucleic acid (mRNA) are high in the preoptic area (POA) of the telencephalon in fish (Doyon et al., 2003; Craig et al., 2005), and axons of CRF-expressing cells in the POA project directly to the corticotropes of the pituitary gland (Matz and Hofeldt, 1999; Zupanc et al., 1999; Flik et al., 2006). At the corticotropes, CRF binds to the CRF receptor (CRFR), a seven-helix G protein-coupled receptor (Flik et al., 2006). Two CRFRs exist (CRFR1 and CRFR2, although note that CRFR3 has been found in a single catfish species, *Ameiurus nebulosus*), but CRFR1 has been found to be the important receptor in the stress axis (Flik et al., 2006). The activity of CRF can be modulated by CRF binding-protein (CRF-BP), which is found in the POA as well as the pituitary gland in fish (Seasholtz et al., 2002; Huising et al., 2004; Alderman et al., 2008). Although no colocalization of CRF and CRF-BP has been detected in common carp (*Cyprinus carpio*) POA, both are found in nerve fibres projecting onto adrenocorticotrophic hormone (ACTH)-positive cells of the pituitary, suggesting that CRF-BP may regulate the stress response in teleost fish, at least in part, by binding to CRF (Huising et al., 2004).

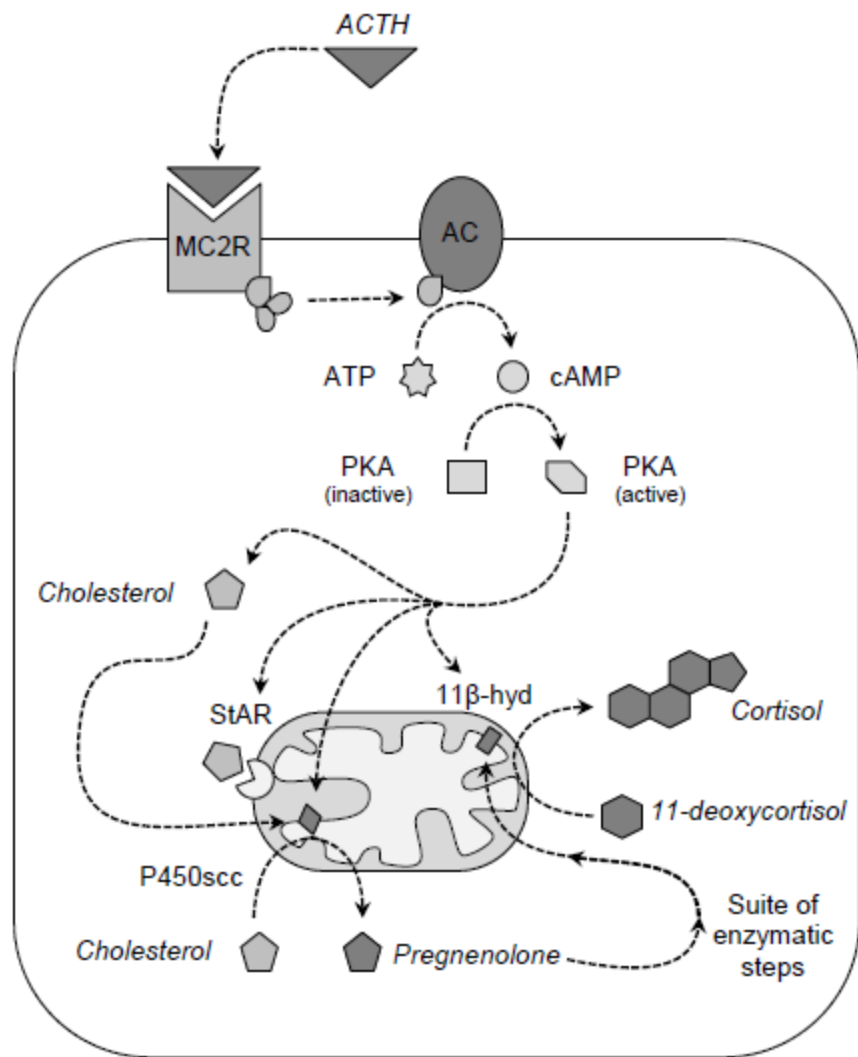
The activation of CRFR stimulates the release of ACTH from the corticotropes of the anterior pituitary (Barton, 2002). Adrenocorticotrophic hormone is derived from the precursor proopiomelanocortin (POMC), a member of the opioid/orphanin gene family that encodes α - melanocyte-stimulating hormone (α -MSH)/ACTH, β -MSH, γ -MSH, and β -endorphin, as well

as δ -MSH which has been reported only in cartilaginous fish (Amemiya et al., 1999; Bernier et al., 2009; Does and Baron, 2011). The POMC gene is expressed in corticotropes of the anterior pituitary and melanotropes of the intermediate pituitary (Does and Baron, 2011). Once in the circulation, ACTH binds to the melanocortin 2 receptor (MC2R), a G protein-coupled receptor that is found on the plasma membrane of interrenal cells in the head kidney of teleost fish (Aluru and Vijayan, 2008; Bernier et al., 2009). Other MCRs exist (MC1R-MC5R), but MC2R has been found to be exclusively selective for ACTH (Haitina et al., 2005). From what is known in mammals, binding of ACTH to MC2R activates adenylate cyclase (AC), which in turn activates a cyclic adenosine monophosphate (cAMP)-signalling cascade (Payne and Hales, 2004). Through activation of protein kinase A (PKA) and/or C, one of the several roles of cAMP is to stimulate steroidogenic acute regulatory protein (StAR), which facilitates the transport of cholesterol from the outer to the inner mitochondrial membrane (Stocco, 2000; Sewer and Waterman, 2003) (Fig.1.1). Cholesterol, the precursor of cortisol as well as other steroid hormones, is converted to pregnenolone in a reaction catalyzed by cytochrome P450 side chain cleavage enzyme (P450_{scc}), which also is stimulated by the cAMP signalling cascade following on from MC2R activation (Stocco, 2000; Sewer and Waterman, 2003). The rate-limiting steps in cortisol synthesis involve cholesterol transport by StAR and the conversion of cholesterol to pregnenolone (Sewer and Waterman, 2003). Pregnenolone is then converted to cortisol through a series of reactions catalyzed by different enzymes in the smooth endoplasmic reticulum (SER) (Payne and Hales, 2004). The last step involves the conversion of 11-deoxycortisol to cortisol by 11 β -hydroxylase (11 β -hyd), an enzyme present in the inner mitochondrial membrane (Payne and Hales, 2004). Although most of our knowledge of cortisol biosynthesis is based on what is known in mammals, several elements of this pathway have been confirmed in fish. For instance,

studies have shown that MC2R is present in the head kidney of rainbow trout and incubation of head kidney tissue with ACTH or dibutyryl cAMP (db-cAMP), a cAMP analogue that mimics the action of endogenous cAMP, resulted in an increase in cortisol production (Hagen et al., 2006; Aluru and Vijayan, 2008). Furthermore, incubation of head kidney tissue with ACTH resulted in increases in MC2R, StAR, P450_{scc} and 11 β -hyd mRNA abundances which suggests a highly conserved ACTH signalling system in vertebrates (Aluru et al., 2005; Hagen et al., 2006; Aluru and Vijayan, 2008).

Once in the circulation, cortisol enters cells by passive diffusion and either binds to its receptor to have effects on target tissues (see next section) or is broken down (Mommsen et al., 1999). Cortisol has been found to be metabolized by the enzymes 4-ene-5 β -reductase, 3 α -hydroxysteroid dehydrogenase, 20 β -hydroxysteroid dehydrogenase, and 11 β -hydroxysteroid dehydrogenase in fish (Truscott, 1979; Mommsen et al., 1999). The main metabolites found in trout following an intra-arterial injection of ³H-cortisol were tetrahydrocortisone, 20 β -cortolone, tetrahydrocortisol, 5 β -dihydrocortisone and cortisone (Truscott, 1979; Mommsen et al., 1999). In stressed rainbow trout, tetrahydrocortisone, tetrahydrocortisol, cortisone, cortisol and β -cortolone were detected in the bile (Pottinger et al., 1992; Mommsen et al., 1999). The liver is thought to be the key organ for cortisol catabolism with the biliary system being the main route for cortisol excretion, whereas the kidney and gills have been found to play a smaller role in cortisol excretion (Idler and Truscott, 1972; Vijayan and Leatherland, 1990).

Figure 1.1 A schematic representation of cortisol synthesis in the interrenal cell. Reproduced with permission from the PhD thesis of JD Jeffrey. See text for detailed descriptions of pathways. *ACTH*, adrenocorticotrophic hormone, *MC2R*, melanocortin 2 receptors, *AC*, adenylate cyclase, *ATP*, adenosine triphosphate, *cAMP*, cyclic adenosine monophosphate, *PKA*, protein kinase A, *StAR*, steroidogenic acute regulatory protein, *P450scc*, cytochrome P450 side chain cleavage enzyme, *11 β -hyd*, 11 β -hydroxylase.



1.1.2 Roles of cortisol

Cortisol has widespread functions in fish that include the regulation of energy metabolism and salt and water balance, with impacts on immune function, growth and reproduction (Wendelaar Bonga, 1997; Mommsen et al., 1999; McCormick et al., 2008). Fish lack the tetrapod mineralocorticoid hormone aldosterone and therefore cortisol acts both as the main glucocorticoid and the main mineralocorticoid (Prunet et al., 2006). For instance, tilapia (*Sarotherodon mossambicus*) that were injected with cortisol experienced an increase in the number of ion-transporting chloride cells (Foskett et al., 1981), and cortisol has been found to induce an increase in $\text{Na}^+ - \text{K}^+$ -ATPase activity in the gill (Forrest et al., 1973). These results suggest that cortisol acts as a mineralocorticoid. Cortisol mobilizes energy resources to deal with increased energy demand during an acute stressor (Schreck, 1982). The increased energy demand is apparent from measurements of the rate of oxygen consumption; stressed fish exhibited a higher rate of oxygen consumption than unstressed fish and cortisol levels and oxygen consumption were correlated (Barton and Schreck, 1987). The role of cortisol in meeting this elevated energy demand was demonstrated by feeding cortisol to juvenile rainbow trout, which resulted in a reduction in liver glycogen content and an increase in resting plasma glucose concentrations (Barton et al., 1987). This increase in energy metabolism to cope with a stressor results in less energy being available for other necessary physiological processes, such as growth, immune function and reproduction (Barton and Iwama, 1991). For example, cortisol-fed fish exhibited poor growth (Barton and Schreck, 1987) and exogenous cortisol treatment increased the incidence of disease (Pickering and Duston, 1983). Similarly, fish fed cortisol experienced a reduction in lymphocytes (Barton and Schreck, 1987). Cortisol has also been found to act negatively on reproduction (Barton and Iwama, 1991). For instance, mature male

brown trout (*Salmo trutta* L.) that were chronically stressed by confinement for 1 month exhibited elevated cortisol levels and suppression of plasma testosterone and 11-ketotestosterone levels (Pickering et al., 1987).

Cortisol exerts its effects primarily by binding to corticosteroid receptors (CR) in target tissues and acting as a transcription factor (Mommsen et al., 1999); some evidence suggests that cortisol also can act in a non-genomic fashion by binding to membrane-associated receptors and/or via intercalation into the lipid bilayer to affect membrane fluidity (Dindia et al., 2012). The CR are ligand-activated transcription factors and they include two main forms, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Colombe et al., 2000; Bury et al., 2003; Greenwood et al., 2003; Sturm et al., 2005; Prunet et al., 2006). From what is known in mammals, in the absence of ligand, GR is present in the cytoplasm as part of a complex that involves heat shock protein 90 (HSP90), other HSPs, HSP70/HSP90 organising protein and immunophilins (Tissing et al., 2005), whereas MR is present in the cytoplasm and nucleus (Fejes-Tóth et al., 1998). Once bound to cortisol, CR dissociate from this complex and transfer to the nucleus (Htun et al., 1996; Kumar et al., 2006) where they recognise and bind to glucocorticoid response elements (GRE), eventually modifying transcription (Glass and Rosenfeld, 2000; Bury and Sturm, 2007). Teleost fish have two different GR paralogs, GR1 and GR2, with the exception of zebrafish (*Danio rerio*) that has only one paralog (Prunet et al., 2006; Schaaf et al., 2008), and GR are expressed in several tissues including the brain, head kidney, heart, spleen, gills, intestine, skin, liver, muscle and gonads (Teitsma et al., 1998; Greenwood et al., 2003; Alderman et al., 2012; Jeffrey et al., 2012; Teles et al., 2013a; Teles et al., 2013b). Interestingly, the GR2 has been found to be more sensitive to cortisol than the GR1, with transactivation activity at lower cortisol levels than GR1 (Bury et al., 2003). However, the

specific roles of GR1 and GR2, as well as MR, are still unknown (Bury et al., 2003; Prunet et al., 2006; Li et al., 2012).

1.1.3 Interaction between the HPI axis and other factors

The preceding sections described the anatomy of the HPI axis as well as the functions of cortisol. It is important to keep in mind, however, that the activity of the HPI axis is regulated by a variety of factors that remain imperfectly understood. For example, cortisol itself has been suggested to regulate all levels of the HPI axis through negative feedback (Bradford et al., 1992; Mommsen et al., 1999). Thus, exposure to stressors that tend to elevate cortisol in a chronic fashion would be expected to modulate HPI axis activity, such as environmental toxicants (Hontela et al., 1997; Hontela., 2005), parasite infection (Madison et al., 2013), repeated stressors (Flos et al., 1988; Barton, 2002), or social stressors (Sloman et al., 2002; Gilmour et al., 2005). Genetic factors (i.e. species differences) (McDonald et al., 1993; Ruane et al., 1999; Barton, 2002), developmental factors (i.e. the developmental stage of the fish) (Barton, 2002; Alsop and Vijayan, 2008), and environmental factors such as temperature (Davis and Parker, 1990; Barton, 2002) also may affect the magnitude of the cortisol response to a stressor. Interestingly, the HPI axis has also been found to regulate other axes such as the melanotropic axis (van den Burg et al., 2005; Bernier et al., 2009), the thyrotropic axis (Redding et al., 1986; Bernier et al., 2009; Subhash Peter, 2011) and the serotonergic system (Ou et al., 2001; Herculano and Maximino, 2014) and in turn, products of these axes may modulate HPI axis activity. For example, urotensin I which is part of the same family as CRF (Kelsall and Balment, 1998; Bernier et al., 2009), thyrotropin-releasing hormone which is part of the thyrotropic axis (Rotllant et al., 2000a; Bernier et al., 2009), dopamine (Metz et al., 2004; Bernier et al., 2009)

and 5-HT (Winberg et al., 1997; Bernier et al., 2009) which are part of the brain monoaminergic system, and α -MSH (Lamers et al., 1992; Bernier et al., 2009) and α -N-acetyl- β -endorphin (Balm et al., 1995a; Bernier et al., 2009) which are part of the melanotropic axis all have been found to affect HPI axis activity. Even beyond these factors, a host of additional compounds has been shown to interact with the HPI axis, including, melanocyte-concentrating hormone (Baker et al., 1986; Bernier et al., 2009), arginine vasotocin and isotocin (Fryer et al., 1985; Baker et al., 1996; Bernier et al., 2009), angiotensin II (Perrott and Balment, 1990; Bernier et al., 2009), as well as the reproductive axis (Bry, 1985; Milla et al., 2009; Schreck, 2010), and the immune system (Balm et al., 1995b; Yada and Nakanishi, 2002). Although the list of factors that can affect HPI axis activity is long, in most cases our understanding of the interactions is incomplete. Thus, the present thesis will focus on negative feedback and 5-HT specifically as modulators of the stress axis and attempt to identify and characterize the mechanisms underlying their effects. These two factors were chosen for investigation because both are thought to play a role in regulation of the stress axis during chronic social stress. Chronic social stress leads to high circulating cortisol values (Gilmour et al., 2005) that would be expected to inhibit stress axis activity through negative feedback. Also, subordinate fish experience high brain serotonergic activity (Winberg and Nilsson, 1993), suggesting the possibility of serotonergic regulation of the stress axis.

1.2 5-HT modulates the HP-adrenal (HPA) axis in mammals

Insight into how activity of the HPI axis may be modulated by 5-HT in fish can be gained from examining the literature on serotonergic modulation of HPA axis activity in mammals.

1.2.1 The serotonergic system

Serotonin is a monoamine found in the blood (primarily platelets), peripheral tissues (particularly the gastrointestinal tract), and central nervous system of mammals (Rand and Reid, 1951; Erspamer and Asero, 1952; Hagen and Cohen, 1966; Steinbusch, 1981; Mück-Seler and Pivac, 2011). The peripheral compartment contains 95% of 5-HT whereas the central nervous system contains only 5% of 5-HT (Mück-Seler and Pivac, 2011; Olivier, 2015). Serotonin is involved in the regulation of several physiological (body temperature, sleep, vomiting, sexuality, appetite), behavioural (aggression, mood) and cognitive (learning, memory) functions, but it also plays a role in the growth of the central nervous system during development as a growth factor in the immature brain (Lucki, 1998; Mück-Seler and Pivac, 2011). The essential amino acid L-tryptophan is the main precursor of 5-HT (Mück-Seler and Pivac, 2011) and it, together with 5-hydroxytryptophan, the other precursor, are transported from blood to brain through the blood-brain barrier (BBB) by a carrier (Chaouloff, 1993; Mück-Seler and Pivac, 2011). To synthesize 5-HT, the first and rate-limiting step is the conversion of tryptophan to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH) (Savelieva et al., 2008; Mück-Seler and Pivac, 2011). Tryptophan hydroxylase type 1 (TPH1) is involved in the synthesis of peripheral 5-HT, which occurs primarily in enterochromaffin cells of the gastrointestinal tract, whereas

tryptophan hydroxylase type 2 (TPH2) is involved in the synthesis of 5-HT in the serotonergic neurons of the brain (Liu et al., 2008; Mück-Seler and Pivac, 2011). Then, 5-HTP is converted to 5-HT by the enzyme aromatic amino acid decarboxylase in both enterochromaffin cells and serotonergic neurons (Mück-Seler and Pivac, 2011; McCorvy and Roth, 2015).

Although 5-HT can be synthesized in the brain, evidence suggests that 5-HT itself may cross the BBB using serotonin transporters (SERT) (Marc et al., 2011; Mück-Seler and Pivac, 2011). The 5-HT produced in the gastrointestinal tract is released into the blood stream and is mainly stored in blood platelets with lymphocytes, monocytes, macrophages and mast cells of the immune system playing a smaller role in 5-HT storage (Toh, 1954; Cass et al., 1958; Hagen and Cohen, 1966; Mück-Seler and Pivac, 2011). Peripheral 5-HT is metabolized in the liver by the enzyme monoamine oxidase-A (MAO-A) to 5-hydroxyindole acetic acid (5-HIAA) and then is excreted by the kidney (Mück-Seler and Pivac, 2011). Firing of a serotonergic neuron in the central nervous system releases 5-HT into the synaptic cleft where it binds to pre- and postsynaptic serotonergic receptors (Olivier, 2015). Reuptake of 5-HT occurs via SERT which is located on the presynaptic and somatodendritic membranes of serotonergic neurons (Olivier, 2015). Following reuptake, 5-HT is taken up by the vesicular monoamine transporter 2 and stored in synaptic vesicles for reuse (Olivier, 2015). Alternatively, 5-HT may be taken up by glial cells and degraded by MAO-A to its metabolite 5-HIAA (Youdim et al., 2006; Olivier, 2015).

1.2.2 5-HT receptors

Serotonin exerts its actions by binding to serotonergic receptors, of which 14 different subtypes exist (5-HT_{1A,1B,1D,1E,1F,2A,2B,2C,3,4,5A,5B,6} and 7) encompassing seven families (5-HT₁ to 5-HT₇) (Mück-Seler and Pivac, 2011; Göthert, 2013; McCorvy and Roth, 2015; Olivier, 2015). Serotonin receptors are G protein-coupled receptors with the exception of the 5-HT₃ receptor, which is a ligand-gated ion channel that induces neuronal depolarization when activated (Zifa and Fillion, 1992; Contesse et al., 2000; Lanfumey et al., 2008; Mück-Seler and Pivac, 2011; McCorvy and Roth, 2015; Olivier, 2015). The 5-HT₁ and 5-HT_{5A} receptors are coupled to an AC inhibitor G protein (G α_i), and this subunit inhibits the production of cAMP from adenosine triphosphate (ATP) (Contesse et al., 2000; Lanfumey et al., 2008; McCorvy and Roth, 2015). The 5-HT₂ receptors are coupled to a G protein subunit that activates phospholipase C (PLC) (G $_{q/11}$) (Contesse et al., 2000; McCorvy and Roth, 2015); in turn, PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacyl glycerol and inositol triphosphate, leading to an increase in cytosolic calcium levels (McCorvy and Roth, 2015). However, there is also evidence that the 5-HT₂ receptor may inhibit cAMP, possibly through G α_i activation (McCorvy and Roth, 2015). The 5-HT₄, 5-HT₆ and 5-HT₇ receptors are coupled to a G protein subunit that stimulates AC (G α_s), and this subunit activates the production of cAMP from ATP (Hegde and Eglen, 1996; McCorvy and Roth, 2015). The 5-HT₄ receptor also has been found to increase calcium currents, possibly through G $_{q/11}$ activation (McCorvy and Roth, 2015). The 5-HT_{1A}, 5-HT_{2A/2C} and 5-HT₄ receptors have been implicated in regulation of the HPA axis and will therefore be the focus of the next section.

1.2.3 5-HT and the HPA axis

Serotonin has been shown to regulate all levels of the HPA axis in mammals and amphibians, including the CRF-producing neurons of the paraventricular nucleus (PVN) of the hypothalamus, the corticotropic cells of the anterior pituitary, and the corticosteroid-producing cells of the adrenal cortex (Contesse et al., 2000). *In vivo* studies have shown that administration of 8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT), a 5-HT_{1A} agonist, and meta chlorophenylpiperazine (MCPPE) or 1- (2,5-dimethoxy-4-iodophenyl) 2-aminopropane (DOI), which are 5-HT₂ receptor agonists, stimulates ACTH release and cortisol/corticosterone production in humans and rats (Calogero et al., 1990; Chaouloff, 1993; Contesse et al., 2000; Klaassen et al., 2002). These results support the idea that 5-HT regulates the HPA axis; however, the level(s) of the HPA axis activated is/are not clear with *in vivo* experiments.

At the cellular level, nerve terminals containing 5-HT form synapses with CRF-containing cell bodies of the PVN and have been detected in the intermediate lobe of the rat pituitary, suggesting neuronal 5-HT regulation at these two levels of the HPA axis (Westlund and Childs, 1982; Liposits et al., 1987; Chaouloff, 1993; Contesse et al., 2000; Lanfumey et al., 2008). Furthermore, 5-HT stimulates the release of CRF and ACTH from the hypothalamus isolated from the rat and from primary cultures of the anterior pituitary, respectively (Spinedi and Negro-Vilar, 1983; Calogero et al., 1989; Contesse et al., 2000). Also, 5-HT_{1A} (8-OH-DPAT) and 5-HT₂ receptor agonists (DOI) stimulate CRF release from the isolated rat hypothalamus (Calogero et al., 1989; Contesse et al., 2000). Both 5-HT_{1A} (8-OH-DPAT) and 5-HT₂ (DOI) receptor agonists also can stimulate CRF-independent ACTH release in treated rats, which indicates that 5-HT may stimulate corticotropes directly through 5-HT_{1A} and 5-HT₂ receptors (Calogero et al., 1990).

In the adrenal gland, 5-HT is present in the catecholamine-producing chromaffin cells of rat, frog, pig, and mouse, and in mast cells of humans, suggesting that 5-HT may play a local, paracrine role at the level of the adrenal gland (Verhofstad and Jonsson, 1983; Kent and Coupland, 1984; Delarue et al., 1988; Kong et al., 1989; Lefebvre et al., 1992; Contesse et al., 2000). In support of this possibility, 5-HT enhances steroid release by rat adrenal glands that were perfused *in situ* but isolated from the systemic circulation, and by human adrenocortical fragments in a perfusion set-up (Hinson et al., 1989; Lefebvre et al., 1992; Contesse et al., 2000). The available evidence suggests that these effects of 5-HT are exerted through 5-HT₄ receptors. For example, the selective 5-HT₄ receptor agonists zacopride (4-amino-N-(1-azabicyclo [2.2.2] oct-3-yl)-5-chloro-2-methoxybenzamide), (R,S)-zacopride, BIMU 1 (endo-N-(8-methyl-8-azabicyclo-[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazole-1-carboxamide, HCl) and BIMU 8 (endo-N-(8-methyl-8-azabicyclo-[3.2.1]oct-3-yl)-2,3-dihydro-(1-methyl) ethyl-2-oxo-1H-benzimidazole-1-carboxamide, HCl) stimulated corticosterone/cortisol production from perfused frog adrenal slices and human adrenal gland, and the increased secretion rate was blocked by non-selective and selective 5-HT₄ receptor antagonists [ICS 205930 ((3 α -tropanyl)-1H-indole-3-carboxylic acid, ester), GR 113808 ([1-[2 (methylsulphonylamino) ethyl]-4-piperidiny] methyl 1-methyl-1H-indole-3-carboxylate, maleate), and DAU 6285 (endo-(8-methyl-8 azabicyclo-[3.2.1]oct-3-yl)-2,3-dihydro-6-methoxy-2-oxo-1H-benzimidazole-1-carboxylate, HCl)] (Lefebvre et al., 1992; Contesse et al., 1994; Contesse et al., 2000).

Studies have also been conducted to determine whether 5-HT is produced locally or taken up from the blood at the adrenal level. In the frog adrenal gland, there is evidence that chromaffin cells exhibit TPH activity, implying local synthesis (Lefebvre et al., 1992; Contesse

et al., 2000). However, in the rat adrenal gland, 5-HT is thought to be taken up directly from the circulation or to be formed by decarboxylation from 5-HTP (Verhofstad and Jonsson, 1983; Contesse et al., 2000). The metabolite 5-HIAA has also been found in frog and human adrenal tissue, which suggests that adrenal cells can metabolize 5-HT (Delarue et al., 1988; Lefebvre et al., 1992; Contesse et al., 2000). The detection of MAO in human adrenal gland supports this possibility (Lefebvre et al., 1996; Contesse et al., 2000).

In addition to studies that have focused on how the 5-HT system regulates the HPA axis, there is evidence that the 5-HT system can be regulated by activation of the HPA axis (Chaouloff, 1993). For example, activation of the HPA axis in mammals led to changes in brain 5-HT turnover (Lanfumeey et al., 2008). Indeed, increases in the synthesis, turnover and metabolism of 5-HT have been shown in different areas of the brain of rats following stressful conditions including electrical foot shocks, exposure to a cold environment, immobilization sessions or tail pinches (Pei et al., 1990; Inoue et al., 1994; Lanfumeey et al., 2008). There is also evidence that corticosteroids control 5-HT synthesis and metabolism during stress through GR-mediated stimulation of TPH activity (Boadle-Biber et al., 1989; Singh et al., 1990; Chaouloff, 1993; Chaouloff, 1995). The 5-HT_{1A} receptor is thought to be the receptor that is the most affected by the activation of the HPA axis (Lanfumeey et al., 2008). For example, GR activation in the dorsal raphe nucleus (DRN) of rats results in a decrease in the effectiveness of 5-HT_{1A} receptor agonists to inhibit the discharge of serotonergic neurons via autoregulation (Laaris et al., 1995; Lanfumeey et al., 2008). Similarly, an increase in corticosterone levels induced by moderate stress in rats or chronic mild stress in mice decreases the effectiveness of 5-HT_{1A} receptor agonists in inhibiting the electrical activity of serotonergic neurons in the DRN (Laaris et al., 1999; Lanfumeey et al., 1999; Lanfumeey et al., 2008). Administration of high doses of

corticosterone or the synthetic GR agonist dexamethasone to rats resulted in an increase in 5-HT_{2A} receptor binding in the cortex, a region where both MR and GR are present, suggesting stimulatory control of the 5-HT_{2A} receptor through GR (Kuroda et al., 1992; Herman, 1993; Kuroda et al., 1993; Chaouloff, 1995). The observation that stress-induced corticosterone levels correlate with changes in 5-HT_{2C} receptor mRNA expression in the hippocampus of rats similarly supports the possibility of regulation of the serotonergic system by glucocorticoids (Holmes et al., 1995; Lanfumey et al., 2008).

To summarize, four 5-HT receptor subtypes, 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} and 5-HT₄, have been implicated in serotonergic regulation of the stress axis in mammals and amphibians. At least two receptor subtypes (5-HT_{1A} and/or 5-HT_{2A/2C}) mediate the effects of 5-HT on pituitary-adrenocortical secretions in mammals, whereas the 5-HT₄ receptor subtype mediates the effects of 5-HT on glucocorticoid production (Contesse et al., 2000). This work provides a basis for investigating serotonergic regulation of the HPI axis in fish. The serotonergic system is well conserved in the brain of teleost fish (Lillesaar, 2011). In different fish species, 5-HT has been found in the POA (the equivalent of the PVN) and/or pituitary (Kah and Chambolle, 1983; Ritchie et al., 1983; Sano et al., 1983; Ekström and Van Veen, 1984; Groves and Batten, 1985; Margolis-Kazan et al., 1985; Corio et al., 1991; Batten et al., 1993; Khan and Thomas, 1993; Adrio et al., 1999; Chiba, 1999; Rodríguez-Gómez et al., 2000). Also, 5-HT_{1A} receptors have been found in the POA of zebrafish (Norton et al., 2008), which is the main site of CRF expression in teleosts (Alderman and Bernier, 2007; Bernier et al., 2009). Binding of [³H] ketanserin, a 5-HT₂ antagonist, was detected in the POA of rainbow trout which suggests the presence of 5-HT₂ receptors (Agrawal and Omeljaniuk, 2000). Furthermore, a correlation between hypothalamic serotonergic activity and plasma cortisol concentrations was found in

socially subordinate rainbow trout (Winberg and Lepage, 1998). These results suggest a role for 5-HT in regulation of the HPI axis.

Relatively few studies in teleost fish have been conducted to determine the role of 5-HT or 5-HT receptors in regulation of the HPI axis. Rainbow trout given a bolus injection of 5-HT exhibited increases in plasma cortisol levels, suggesting that 5-HT can influence HPI axis activity (L. Dionne-Wilson, MSc thesis, uOttawa). Similarly, administration of the 5-HT_{1A} receptor agonist 8-OH-DPAT provoked an increase in plasma cortisol concentrations in unstressed Gulf toadfish (*Opsanus beta*), goldfish (*Carassius auratus*) and rainbow trout (Winberg et al., 1997; Medeiros et al., 2010; Lim et al., 2013), implicating this 5-HT receptor subtype in mediating the effects of 5-HT on the HPI axis. However, administration of the 5-HT_{1A} receptor agonist 8-OH-DPAT to Arctic charr (*Salvelinus alpinus*) stressed by handling resulted in a lower plasma cortisol response compared to saline-injected fish, which suggests an inhibitory effect of this receptor in stressed fish (Höglund et al., 2002). In Gulf toadfish (Medeiros and McDonald, 2012), goldfish (Lim et al., 2013) and rainbow trout (L. Dionne-Wilson, MSc thesis, uOttawa), 5-HT also has been shown to stimulate cortisol production directly at the level of the head kidney. The receptors involved remain uncertain, although there are strong indications of 5-HT₄ receptor involvement. Thus, one goal of the present thesis was to test the hypothesis that 5-HT₄ receptors mediate effects of 5-HT on cortisol biosynthesis in interrenal cells. A second goal was to investigate possible sources of 5-HT that could regulate cortisol biosynthesis at the head kidney level, keeping in mind the paracrine model outline for the mammalian adrenal gland.

1.3 Regulation of the HPI axis by negative feedback

Previous work on negative feedback in the HPI axis provides a framework within which to examine negative feedback in the context of social stress, with the latter being the focus of the present project. The first section will cover negative feedback within the HPI axis, which likely occurs via CR and may be affected by 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2). The second section will cover negative feedback in the context of social stress which is known to be a disruptor of the HPI axis. These concepts are important to understand how elevated cortisol levels in the context of social status may modulate HPI axis activity.

1.3.1 Overview of negative feedback within the HPI axis

To regulate activity of the HPI axis in teleost fish, cortisol acts through long and/or ultra-short negative feedback mechanisms that are not yet well understood (Mommsen et al., 1999; Vijayan et al., 2010). Long-loop negative feedback occurs at the hypothalamus and pituitary to regulate CRF and ACTH release, respectively (Mommsen et al., 1999). For example, cortisol implants placed in the POA of goldfish inhibited the cortisol response to a sham-injection stress compared to the response observed in animals given blank implants, which is indicative of negative feedback (Fryer and Peter, 1977). Also, the presence of cortisol implants in the brain of goldfish resulted in a decrease in CRF mRNA levels in the telencephalon-preoptic region, which suggests that cortisol regulates CRF gene expression (Bernier et al., 1999). Interestingly, however, cortisol treatment in rainbow trout was linked to increased CRF mRNA levels in some studies (Jeffrey et al., 2012; Madison et al., 2015). At the level of the pituitary, addition of

cortisol to the buffer perfusing goldfish pituitary cells *in vitro* reduced ACTH secretion (Fryer et al., 1984) and cortisol treatment *in vivo* reduced ACTH secretion in rainbow trout (Jeffrey et al., 2012; Madison et al., 2015). However, cortisol treatment reduced CRF mRNA levels in isolated trout but not in confined trout, and both conditions increased ACTH levels (Doyon et al., 2006). These results demonstrate the complexity of negative feedback at the POA and pituitary of the HPI axis.

Ultra-short-loop negative feedback occurs in the head kidney of coho salmon (*Oncorhynchus kisutch*) (Bradford et al., 1992). Specifically, incubation with ACTH initially stimulated cortisol production by head kidney tissue *in vitro*, but after 3 to 6 h of ACTH-exposure, cortisol production returned to resting rates (Bradford et al., 1992). On the other hand, the rate of cortisol production for head kidney tissue that was transferred to fresh media with ACTH every 6 h did not return to resting levels but remained elevated for at least 24 h (Bradford et al., 1992). These results imply that the presence of cortisol in the incubation medium inhibited cortisol production. To confirm this conclusion, cortisol production in response to ACTH did not increase in tissues that were incubated with exogenous cortisol, and this effect was both reversible and dose-dependent (Bradford et al., 1992). Thus, cortisol can affect its own production in interrenal cells through ultra-short-loop negative feedback.

Whereas cortisol clearly regulates HPI axis through negative feedback, increasing evidence suggests that cortisol signalling through GR and MR also is required to maintain HPI axis activity. As noted previously, GR and MR are the receptors through which cortisol exerts its effects on target tissues, but GR and MR also may be implicated in regulation of the HPI axis as they are detected in the brain and head kidney of teleost fish (Teitsma et al., 1998; Sturm et al., 2005; Teles et al., 2013a; Teles et al., 2013b). For instance, addition of dexamethasone, a GR

agonist, resulted in the down-regulation of cortisol binding capacity in both liver and brain receptors of rainbow trout (Lee et al., 1992). Similarly, cortisol incubated *in vitro* or *in vivo* significantly decreased GR protein content in the liver of rainbow trout, suggesting a negative feedback loop for GR gene regulation by cortisol (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). Whether cortisol has similar effects on GR within the HPI axis has yet to be confirmed. To investigate the role of GR in the functioning of the HPI axis during stress in fish, trout were fed with mifepristone, a well-established antagonist of GR (Alderman et al., 2012). Mifepristone treatment resulted in a decrease in brain GR protein abundance together with an attenuated cortisol response to a handling stress *in vivo* as well as to ACTH *in vitro* by head kidney preparations (Alderman et al., 2012). These data show that GR signalling is crucial to HPI axis activity following an acute stressor. Furthermore, in trout fed with mifepristone, CRF mRNA levels were reduced compared to control fish, suggesting that GR signalling also has a role in maintaining CRF gene expression (Alderman et al., 2012).

In addition to the possible role of GR and MR signalling in maintenance of HPI axis activity, there is also evidence that 11 β HSD2 may contribute to regulation of HPI axis activity. The enzyme 11 β HSD2 converts cortisol to its inactive form, cortisone (Edwards et al., 1988). This enzyme is transcribed in many tissues of teleost fish, including the gill, heart, pyloric caeca, intestine, liver, kidney, spleen, testis, ovary, muscle, skin and eye (Kusakabe et al., 2003; Alderman and Vijayan, 2012). It is also transcribed in the brain and head kidney of teleost fish, suggesting that 11 β HSD2 may play a role in the regulation of the HPI axis through negative feedback (Kusakabe et al., 2003; Alderman and Vijayan, 2012). Interestingly, 11 β HSD2 mRNA levels and enzymatic activity consistently increase following a stressor (Nematollahi et al., 2009; Fuzzen et al., 2010; Alderman and Vijayan, 2012). Furthermore, 11 β HSD2 expression is

probably regulated by cortisol because three putative GREs have been found upstream of the 11 β HSD2 gene (Alderman and Vijayan, 2012). Collectively, these results suggest that cortisol may regulate 11 β HSD2 mRNA expression, which in turn can regulate cortisol concentrations and, by affecting the effectiveness of negative feedback, modulate cortisol production. To support this possibility, changes in cortisol levels during and after confinement were found to correlate in a time-delayed relationship with increased and decreased mRNA levels of 11 β HSD2, respectively (Nematollahi et al., 2009). Furthermore, inhibition of 11 β HSD2 activity by 18 β -glycyrrhetic acid (18 β -GA) in zebrafish resulted in higher cortisol concentrations, as well as higher CRF and MR mRNA levels following an acute stressor, suggesting that 11 β HSD2 regulates cortisol production by altering the effectiveness of negative feedback (Alderman and Vijayan, 2012). It should be briefly noted that the enzyme 11 β -hydroxysteroid dehydrogenase type 1, which converts inactive cortisone to the active form cortisol, does not seem to be present in fish (Baker, 2004).

1.3.2 Chronic stressors disrupt the HPI axis

Fish exposed to chronic stressors exhibit chronically elevated cortisol levels (Doyon et al., 2003; DiBattista et al., 2006; Alderman et al., 2008; Bernier et al., 2008; Castillo et al., 2008; Jeffrey et al., 2012). The chronic elevation of cortisol that occurs in response to chronic stress suggests that normal activity of the stress axis has been disrupted, because normally cortisol levels should rise and then decrease fairly quickly (Pickering and Pottinger, 1989). Continual activation of the HPI axis may explain the prolonged elevation of cortisol levels during chronic stress. For example, the mRNA abundance of CRF was found to be higher in the POA of subordinate trout compared to dominant fish for interactions of 8 h to 3 d duration (Doyon et al.,

2003; Bernier et al., 2008) although not for longer, 5 d interaction periods (Jeffrey et al., 2012). Juvenile rainbow trout form social hierarchies as a result of competition for limited resources (Stringer and Hoar, 1955; Chapman, 1966). The subordinate fish, the losers of these competitive interactions, are chronically stressed as indicated by chronically elevated cortisol levels (Jeffrey et al., 2012). Repeated periods of physical disturbance in rainbow trout also led to mRNA abundances of CRF1 in the POA and pituitary CRF-BP that were higher than those of controls or animals subjected to one period of physical disturbance (Doyon et al., 2005), again supporting a role for continuing activation of the HPI axis. At the level of the head kidney, gilthead seabream (*Sparus aurata*) chronically stressed by high density holding conditions exhibited an increase in head kidney StAR mRNA levels (Castillo et al., 2008). However, no increase in StAR was detected in subordinate trout (Jeffrey et al., 2014), suggesting that this response is species- and/or stressor-specific. Although CRF and CRF-BP are higher in chronic stress and so are StAR and cortisol, ACTH has been found to be lower in chronically stressed fish. Indeed, subordinate and cortisol-treated trout exhibited lower ACTH levels compared to control, fasted and dominant trout (Jeffrey et al., 2012). In a different study, cortisol-treated trout also exhibited lower levels of ACTH than control fish (Balm and Pottinger, 1995). Furthermore, after 96 h of confinement, ACTH levels in trout returned to control levels even though cortisol levels remained elevated (Balm and Pottinger, 1995). Similarly, in cortisol-fed gilthead seabream, plasma ACTH levels were lower than in control fish (Rotllant et al., 2000b). These results are consistent with modulation of ACTH levels by cortisol through negative feedback. In contrast to these findings, however, plasma ACTH levels were elevated in subordinate Arctic charr compared to dominant/sham animals suggesting that this response is species-specific (Höglund et al., 2000). Collectively, these results suggest that negative feedback could be dysregulated during chronic

stress. The conflicting observations that CRF is elevated but ACTH is reduced may suggest that on one hand, chronic stress is continually activating the HPI axis whereas on the other hand, negative feedback is trying to attenuate HPI axis activity. However, evidence to support this hypothesis remains sparse and therefore the function of the stress axis during chronic stress remains unclear. Thus, chronic social stress will be used in the present study to investigate stress axis activity at the level of the head kidney. The head kidney was chosen as a starting point because it is the source of cortisol production.

1.4 The structure and study of the head kidney

In teleost fish, the head kidney tissue consists mostly of lymphoid tissue with pigment and haemopoietic cells, but thyroid follicles (Chavin, 1956; Chester et al, 1980), catecholamine-producing chromaffin cells and interrenal cells also are present (Yoakim and Grizzle, 1980; Gallo and Civinini, 2003). Chromaffin and interrenal cells are well described in the literature. Chromaffin cells contain granules, are innervated by cholinergic nerve fibres, are present in clusters and are mainly composed of noradrenaline-releasing cells although a subpopulation of chromaffin cells that exhibits adrenaline release also has been found (Mastrolia et al., 1984; Gallo et al., 1993; Reid et al., 1995; Gallo and Civinini, 2003). Teleost interrenal cells consist of groups, cords or strands of cells that are found around the posterior cardinal vein (PCV) and its branches (Chester et al, 1980); in rainbow trout, interrenal cells account for approximately 1 in 8,000 cells within the head kidney (Hontela et al., 2008). The most prominent cytoplasmic constituents of interrenal cells in rainbow trout are mitochondria and SER (Jung et al., 1981;

Gallo and Civinini, 2003). Four main types of interrenal cells exist: type 1 comprises interrenal cells that surround the PCV or its largest branches; type 2 is composed of interrenal cells that surround small or medium-sized branches of the PCV and therefore are widely present throughout the head kidney; type 3 comprises interrenal cells that are associated with venous sinuses of the head kidney and form strands of cells that could either be scattered throughout the lymphoid and haemopoietic tissue or replace it; and type 4 consists of interrenal cells that form a mass of cells in a localized area (Nandi et al, 1962; Chester et al, 1980; Gallo and Civinini, 2003).

The complexity of the HPI axis and the many factors that can modulate HPI axis regulation provide challenges to the study of HPI axis activity that can be overcome in part by using reduced preparations. At the level of the head kidney, several different approaches have been used including homogenates of head kidney tissue *in vitro* (Vijayan and Leatherland, 1990; Benguira and Hontela, 2000; Lacroix and Hontela, 2001; Leblond et al., 2001; Aluru and Vijayan, 2004), *in situ* saline-perfused PCV preparations (Sloman et al., 2002), superfused head kidney preparations (Fuzzen et al., 2011; Lim et al., 2013) and isolated interrenal cells derived from head kidney homogenates using a Percoll gradient (Hontela et al., 2008). Arguably, homogenates of head kidney tissue *in vitro* have been used most widely, at least for larger bodied species such as rainbow trout. For example, this approach has been used to look at the effect of environmental contaminants on cortisol production (Benguira and Hontela, 2000; Leblond et al., 2001; Aluru and Vijayan, 2004). In the present study, *in vitro* head kidney preparations were used to investigate 5-HT and negative feedback as possible regulators of cortisol biosynthesis.

CHAPTER 2

REGULATION OF CORTISOL PRODUCTION BY 5-HT IN A TELEOST

FISH, RAINBOW TROUT

2.1 Introduction

Cortisol, the main glucocorticoid hormone in teleost fish, is synthesized in the interrenal cells of the head kidney as the end product of activation of the HPI axis (Chester et al, 1980; Wendelaar Bonga, 1997). Activation of the HPI axis stimulates the release of CRF from the POA of the brain, which then triggers the release of ACTH from corticotropes of the anterior pituitary (Bernier et al., 2009). Binding of ACTH to MC2R on the plasma membrane of interrenal cells (Aluru and Vijayan, 2008) activates AC, which in turn stimulates a cAMP-signalling cascade that has two main effects. First, it results in the movement of cholesterol to the inner mitochondrial membrane which is facilitated by the transport protein StAR (Aluru et al., 2005; Hagen et al., 2006; Aluru and Vijayan, 2008). Once in the mitochondria, cholesterol is converted to pregnenolone by P450scc, which also is activated by the cAMP-signalling cascade (Aluru et al., 2005; Hagen et al., 2006; Aluru and Vijayan, 2008). The transport of cholesterol into mitochondria by StAR and the conversion of cholesterol to pregnenolone catalyzed by P450scc are the rate-limiting steps in cortisol production (Sewer and Waterman, 2003).

Although the synthesis of cortisol in fish interrenal cells has been described, our understanding of how this biosynthetic pathway is regulated remains incomplete. Cortisol is thought to regulate its biosynthesis through an ultra-short-loop negative feedback pathway (Bradford et al., 1992). In addition, recent evidence suggests that cortisol signalling through the GR may be needed to maintain the biosynthetic capacity for cortisol, because when GR protein levels were reduced by treatment with mifepristone, StAR mRNA expression as well as cortisol production were reduced (Alderman et al., 2012). Several lines of evidence suggest that the monoamine 5-HT also may play a role in regulating cortisol biosynthesis.

First, 5-HT receptors have been found at all levels of the HPI axis in several teleost fish. For instance, the Gulf toadfish expresses the 5-HT_{1A} receptor in the POA, pituitary and head kidney (Medeiros et al., 2010), as does the goldfish (Lim et al., 2013). In rainbow trout, mRNA for 5-HT_{1A}, 5-HT₂ and 5-HT₄ receptors was detected at all three levels of the HPI axis (L. Dionne-Wilson, MSc thesis, uOttawa). However, it is important to note that the POA, pituitary and head kidney are heterogeneous tissues; that is, cell types other than the ones of interest (i.e. CRF-expressing neurons of the POA, corticotropes of the anterior pituitary, and interrenal cells of the head kidney) are also present and may express 5-HT receptors. To date, cellular localization of 5-HT receptors in the cells of interest of the HPI axis has not yet been reported.

Second, 5-HT has been shown to stimulate cortisol production by the head kidney of Gulf toadfish, goldfish and rainbow trout, albeit with some variation among species. In goldfish, head kidney tissue exposed to ACTH, 5-HT and ACTH, the 5-HT_{1A} receptor agonist 8-OH-DPAT or the 5-HT₄ receptor agonist cisapride exhibited increases in cortisol synthesis (Lim et al., 2013). Incubation of Gulf toadfish head kidney tissue with 5-HT, the 5-HT₄ receptor agonist RS67506 (1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-2-methylsulphonylamino)ethyl-4-piperidinyl]-1-propanone HCl) or the 5-HT₂ receptor agonist α -methylserotonin (3-(2-aminopropyl)-1H-indol-5-ol) resulted in an increase in cortisol synthesis, whereas incubation with 8-OH-DPAT did not (Medeiros and McDonald, 2012). Finally, incubation of rainbow trout head kidney tissue with 5-HT or cisapride resulted in an increase in cortisol synthesis, whereas incubation with 8-OH-DPAT or α -methylserotonin did not (L. Dionne-Wilson, MSc thesis, uOttawa). Collectively, these data suggest that 5-HT acting through 5-HT₄ receptors may play a role in regulating cortisol synthesis in the interrenal cells of teleost fish. Interestingly, incubation of the head kidney from crowded (stressed) Gulf toadfish with 5-HT resulted in lower cortisol production

than in control fish, suggesting the possibility of interaction between cortisol and 5-HT at the level of the head kidney (Medeiros and McDonald, 2012). It is also possible, however, that crowding stress causes downregulation of cortisol production, and so incubation with 5-HT then results in less cortisol being produced.

The likelihood that 5-HT regulates cortisol biosynthesis raises questions concerning the source of the 5-HT that would exert this effect. In mammals, 5-HT plays a paracrine role in the adrenal gland. Serotonin has been found in the chromaffin cells of several species such as rat, pig and mouse, but also has been found in the mast cells of human (Verhofstad and Jonsson, 1983; Kent and Coupland, 1984; Kong et al., 1989; Lefebvre et al., 1992; Contesse et al., 2000). Furthermore, 5-HT has been found to stimulate steroid production in the adrenal glands of rat and adrenocortical fragments from humans (Hinson et al., 1989; Lefebvre et al., 1992; Contesse et al., 2000). A similar situation may exist in teleost fish because the presence of 5-HT in the catecholamine-producing chromaffin cells of the head kidney has been reported in goldfish, European eel (*Anguilla anguilla*) and Atlantic cod (*Gadus morhua*) (Reid et al., 1995; Lim et al., 2013). Thus, activation of chromaffin cells could release 5-HT at the same time as catecholamines, and 5-HT could then act on the interrenal cells to stimulate cortisol synthesis (Lim et al., 2013). However, unlike the situation in eel and cod, Reid et al. (1995) found little evidence for localization of 5-HT to the chromaffin cells of rainbow trout, even though 5-HT was detected in good abundance in the head kidney of this species ($44.6 \pm 5.96 \mu\text{g g}^{-1}$ tissue) (Fritsche et al., 1993). In agreement with these observations, the cholinergic agonist carbachol (2-[(Aminocarbonyl)oxy]-N,N,N-trimethylethanaminium chloride), which stimulates catecholamine release in perfused head kidney preparations of rainbow trout, did not stimulate the release of 5-HT (Fritsche et al., 1993). Although 5-HT was detected in head kidney by

immunohistochemistry, it was concentrated in an unidentified population of cells (Reid et al., 1995).

With this background in mind, the goal of the present study was to investigate the role of 5-HT in the regulation of cortisol synthesis in the head kidney of rainbow trout. The hypothesis underlying the present study was that 5-HT acts as a paracrine regulator of cortisol synthesis in the interrenal cells of rainbow trout, with the 5-HT₄ receptor mediating its effects. If 5-HT acts as a paracrine regulator of cortisol biosynthesis by interrenal cells, then 5-HT would be predicted to be located in cells that are in proximity to the interrenal cells of the head kidney. Thus, immunohistochemical approaches were used to investigate the distribution of 5-HT in trout head kidney tissue. In addition, the 5-HT₄ receptor would be predicted to be localized to interrenal cells. *In situ* hybridization was used to investigate the distribution of the 5-HT₄ receptor mRNA in rainbow trout head kidney tissue, and an *in vitro* head kidney preparation was used to confirm the involvement of the 5-HT₄ receptor in mediating the effects of 5-HT on cortisol production. Finally, a limited set of experiments was carried out on Atlantic salmon (*Salmo salar*) to allow comparisons to be made within salmonid fish.

2.2 Materials and methods

2.2.1 Experimental animals

Female juvenile rainbow trout (237 ± 13 g, $N = 34$) obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) were held at the University of Ottawa Aquatic Facility in 1275 L

fibreglass tanks. Female juvenile Atlantic salmon (326 ± 27 g, $N = 11$) obtained from National Cold Water Marine Aquaculture Centre in Franklin (Maine, USA) were held at the University of Ottawa Aquatic Facility in 1275 L fibreglass tanks. Tanks were supplied with flowing, aerated dechloraminated city of Ottawa tap water at 13°C under a 12h:12h light:dark photoperiod. Fish were fed to satiation with commercial fish pellets three times a week. Fish were acclimated to these conditions for at least two weeks before experiments were carried out. Experimental protocols were approved by an institutional animal care committee (BL-2118) and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching.

2.2.2 Localization of 5-HT within the head kidney

Immunohistochemistry was carried out on sections of head kidney tissue to investigate the cell type(s) containing 5-HT. Rainbow trout (193 ± 21 g, $N = 13$) or Atlantic salmon (413 ± 14 g, $N = 2$) chosen at random from holding tanks were euthanized by immersion in a solution of benzocaine (0.5 mg L^{-1} ethyl-4-aminobenzoate; Sigma Aldrich, Oakville, ON). Heparinized saline solution (40 mL of 0.9% NaCl containing 2500 IU mL^{-1} heparin sodium salt; Sigma Aldrich) was then injected into the PCV to clear the blood from the head kidney tissue. To conserve the basic organization of the tissue, the head kidney tissue and surrounding muscle were removed together and washed in phosphate-buffered saline (PBS; $0.01 \text{ mol L}^{-1} \text{ PO}_4^{3-}$ buffer, $0.0027 \text{ mol L}^{-1} \text{ KCl}$, $0.137 \text{ mol L}^{-1} \text{ NaCl}$, pH 7.4). The tissue was then fixed by immersion in 4% paraformaldehyde (PFA) in PBS at 4°C overnight. Following fixation, the tissue was rinsed 4 x 1 min in PBS and the head kidney was isolated from the surrounding tissues and cryoprotected by immersion in a 30% sucrose solution at 4°C overnight. After embedding in

Cryomatrix (Shandon Cryomatrix, ThermoFisher Scientific, Burlington, ON), the tissue was sectioned at 12 μm using a cryostat (Leica CM3050S), and sections were mounted on Superfrost® Plus microscope slides (ThermoFisher Scientific).

To carry out immunohistochemistry, slides were incubated for 5 min in 10 mmol L⁻¹ boiled (for 45 s) sodium citrate buffer in 0.05% Tween-20 (BioShop, Burlington, ON). The slides were washed 3 times with PBS and then were permeabilized using 1% Triton-X in PBS-Tween (PBST; 0.1% Tween-20) for 2 h at room temperature. Primary antibodies diluted in PBS (Table 2.1) were added and slides were incubated overnight at room temperature in a humidified chamber. Specifically, interrenal cells were identified using one of three different antisera, all of which were raised against enzymes involved in cortisol production; an antibody raised against rainbow trout P450_{scc}, the rate-limiting enzyme of cortisol production (Sewer and Waterman, 2003), an antibody raised against trout 3 β -hydroxysteroid dehydrogenase/ Δ 5-4 isomerase (3 β HSD), or an antibody raised against frog 11 β -hyd. Three markers were used for nerves, the zebrafish neuron-specific surface antigen (ZN-12) (Metcalf et al., 1990), an antibody raised against *Ommata* synaptic vesicle protein type 2 (SV2), that is found in neuronal and endocrine cells (Buckley and Kelly, 1985) and an antibody raised against sea urchin (*Strongylocentrotus purpuratus*) acetylated tubulin (AT). Trials with AT were carried out by a collaborator, Tina E. Suntres using methods developed during her research (T. Suntres, MSc thesis, University of Windsor) and head kidney sections sent from uOttawa. Finally, chromaffin cells were identified using an antibody raised against the rate-limiting enzyme of catecholamine production, tyrosine hydroxylase (TH) (Levitt et al., 1965). As a negative control, primary antibody was omitted. Following incubation with primary antibodies, slides were washed 3 times in PBS to remove unbound primary antibody and then incubated with fluorescently-labelled secondary antibodies

diluted in PBS (Table 2.1) at room temperature overnight in the dark. The slides were washed 3 times in PBS to remove unbound secondary antibody and mounted with cover slips (ThermoFisher Scientific) and Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) as a cell nucleus marker (Vector Laboratories, Burlingame, CA, USA). The slides were stored in an opaque box at 4°C until viewed using a confocal scanning system (Nikon A1R+ A1+ confocal microscope; Nikon Instruments, Melville, NY, USA).

2.2.2.1 Confirmation of the specificity of heterologous antibodies for rainbow trout proteins

Western blotting was used to examine the specificity of primary antibodies for rainbow trout proteins. Rainbow trout were terminally anaesthetized as described above. Head kidney tissue was dissected on ice and added to 4 volumes of radioimmunoprecipitation assay (RIPA) buffer (150 mmol L⁻¹ NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mmol L⁻¹ Tris-HCl, 1 mmol L⁻¹ ethylenediaminetetraacetic acid, 1 mmol L⁻¹ phenylmethanesulphonyl fluoride) containing a protease inhibitor cocktail (ThermoFisher Scientific). The tissue was homogenized by passing the tissue and RIPA buffer through needles of two different sizes (18G 1½”; 23G 1”) with a 3 mL syringe until all of the solution easily passed through the smaller gauge needle. The solution was then centrifuged at 16,000 *g* for 10 min at 4°C and the supernatant containing extracted protein was stored at -20°C until used for western blotting. A bicinchoninic acid assay (Sigma Aldrich) was used to assess protein concentration.

Table 2.1 Details of primary and secondary antibodies used for immunohistochemistry.

Antibody	Dilution	Source	Host	Secondary
Primary				
5-HT	1:200 or 1:400	Sigma-Aldrich	rabbit	Alexa Fluor 633 or 488
5-HT	1:400	Immunostar	goat	Alexa Fluor 488
ZN-12	1:200	The Developmental Studies Hybridoma Bank	mouse	Dylight 488
TH	1:1000 or 1:3000	Immunostar	mouse	Alexa Fluor 546
SV2	1:3000	The Developmental Studies Hybridoma Bank	mouse	Alexa Fluor 546
P450scc	1:1000	A kind gift of Graham Young (Kobayashi et al, 1997)	rabbit	Alexa Fluor 488, 568 or Dylight 488
3 β HSD	1:1600	A kind gift of Graham Young (Kobayashi et al, 1997)	rabbit	Dylight 488
11 β -hyd	1:500	A kind gift of Hiroshi Takemori (Nonaka et al, 1995)	rabbit	Dylight 488
Secondary				
Alexa Fluor 633	1:400	Invitrogen	rabbit	-----
Alexa Fluor 488	1:400	Life Technologies	rabbit	-----
Alexa Fluor 488	1:400	Life Technologies	goat	-----
Dylight 488	1:400	Jackson Immuno Research Laboratories, Inc.	mouse	-----
Alexa Fluor 546	1:400	Invitrogen	mouse	-----

5-HT, serotonin, *ZN-12*, zebrafish neuron-specific surface antigen, *TH*, tyrosine hydroxylase, *SV2*, synaptic vesicle protein type 2,

P450scc, cytochrome P450 side chain cleavage enzyme, *3 β HSD*, 3 β -hydroxysteroid dehydrogenase/ Δ 5-4 isomerase, *11 β -hyd*, 11 β -hydroxylase.

Prior to electrophoresis (10% resolving gel and 4% stacking gel for SDS polyacrylamide gel electrophoresis (SDS-PAGE)), either head kidney or gonad (collected and prepared as described for head kidney tissue and used as a positive control for the P450scc antibody) tissue proteins were mixed 1:1 with Laemmli buffer (Sigma Aldrich) containing 10% 2-mercaptoethanol (Sigma Aldrich) and heated for 10 min (see Table 2.2). Samples as well as Page ruler™ prestained protein ladder (ThermoFisher Scientific) were loaded into wells and the gel was run for 40 min at 200 V. Separated proteins were transferred (Trans-Blot SD transfer system; Bio-Rad, Mississauga, ON) to an Immun-blot® low fluorescence polyvinylidene fluoride membrane (Bio-Rad) for 75 min at 15 V. Membranes were incubated overnight with shaking at 4°C with 5% powdered milk in Tris-buffered saline containing 0.05% Tween-20 (TBST; 152 mmol L⁻¹ Tris-HCl, 46 mmol L⁻¹ Tris base, 1505 mmol L⁻¹ NaCl, pH to 7.5) to block non-specific binding. Next, membranes were probed overnight at 4°C with shaking with primary antibody (Table 2.2) diluted in TBST containing 2% powdered milk. The next day, membranes were washed 3 x 5 min in TBST and incubated with secondary antibody (Table 2.2) for 1 h at room temperature while shaking. Membranes were washed 3 x 15 min in TBST at room temperature with shaking and Luminata Classic™ Western Horseradish Peroxidase (HRP) substrate (Millipore, Etobicoke, ON) was used to detect bands. Membranes were visualized using Image lab software 5.2 (Bio-Rad) and a Molecular Imager® Chemi Doc™ XRS+ (Bio-Rad). An anti-β-actin antibody (Table 2.2) was used as a positive control.

Table 2.2 Details of primary and secondary antibodies used for western blotting.

Antibody	Dilution	Source	Host	Secondary	Heat (°C)	Protein loaded (µg)
Primary						
Actin	1:4000	Sigma-Aldrich	rabbit	Rabbit HRP	65	150
ZN-12	1:4000	The Developmental Studies Hybridoma Bank	mouse	Mouse HRP	65	150
P450scc	1:4000	A kind gift of Graham Young (Kobayashi et al, 1997)	rabbit	Rabbit HRP	95	250
3βHSD	1:4000	A kind gift of Graham Young (Kobayashi et al, 1997)	rabbit	Rabbit HRP	95	250
11β-hyd	1:4000	A kind gift of Hiroshi Takemori (Nonaka et al, 1995)	rabbit	Rabbit HRP	65	150
Secondary						
Rabbit HRP	1:4000 or 1:5000	Bio-Rad	rabbit	-----		
Mouse HRP	1:4000	Thermo Scientific	mouse	-----		

ZN-12, zebrafish neuron-specific surface antigen, *P450scc*, cytochrome P450 side chain cleavage enzyme, *3βHSD*, 3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase, *11β-hyd*, 11β-hydroxylase, *HRP*, horseradish peroxidase.

To confirm the specificity of the anti-P450scc rainbow trout antibody, immunoprecipitation was used to isolate the protein of interest. Head kidney or gonad tissue proteins from one fish were mixed 1:1 with Laemmli buffer and heated to 95°C for 10 min in order to denature proteins. A 1:50 dilution of P450scc antibody was added to the sample, which was incubated overnight at 4°C with gentle rocking. Next, protein A agarose beads (Cell Signalling Technology, Whitby, ON) were added to the samples (10:1 v/v of 50% bead slurry in RIPA buffer) and the mixture was incubated overnight at 4°C with gentle rocking. Samples were centrifuged at 3800 *g* for 30 s at 4°C and the pellet containing the beads bound to the protein of interest was washed 6 times with 500 µL of ice-cold RIPA buffer. The pellet was resuspended in 20 µL of 2X Laemmli buffer and the sample was heated to 95°C for 10 min. Samples and Page ruler™ prestained protein ladder (ThermoFisher Scientific) were loaded into the wells of a 10% SDS-PAGE gel, and the gel was run for 40 min at 200 V. The gel was cast using the TGX™ and TGX Stain-Free™ FastCast™ Acrylamide kit (Bio-Rad) in order to activate the gel to determine the size of the protein of interest. The gel was visualized using Image lab software 5.2 (Bio-Rad) and a Molecular Imager® Chemi Doc™ XRS+ (Bio-Rad).

2.2.3 Localization of 5-HT₄ receptor mRNA in the head kidney

In situ hybridization was used to investigate the cellular location of rainbow trout 5-HT₄ receptor mRNA in head kidney tissue. A riboprobe against the rainbow trout 5-HT₄ sequence reported by L. Dionne-Wilson (MSc thesis, uOttawa) was generated and interrenal cells were identified by immunohistochemistry using P450scc as a marker.

2.2.3.1 Generation of a riboprobe against the rainbow trout 5-HT₄ receptor

A previous study (L. Dionne-Wilson, MSc thesis, uOttawa) reported that 5-HT₄ receptor transcript levels were high in the POA relative to pituitary and head kidney tissues. Therefore, POA tissue from one rainbow trout was collected to use in generation of a riboprobe against the 5-HT₄ receptor. The fish was terminally anaesthetized as described above and POA tissue was collected on ice, immediately flash frozen in liquid N₂, and stored at -80°C for later analysis.

Total RNA was extracted from 100 mg of POA tissue using TRIzol reagent (Invitrogen, Burlington, ON) according to the manufacturer's protocol. The tissue was homogenized by passing the solution of tissue and TRIzol through 18G and 23G needles with a 3 mL syringe until the solution passed easily through the smaller needle. The resultant RNA was washed with 1 mL of cold 75% ethanol to ensure RNA purity and quantified using a Nanodrop® 2000 Spectrophotometer (ThermoFisher Scientific). To generate complementary deoxyribonucleic acid (cDNA), a QuantiTect® Reverse Transcription kit (Qiagen, Toronto, ON) was used according to the manufacturer's protocol with 1 µg of RNA. The resulting cDNA was stored at -20°C. To check for genomic DNA contamination, "no reverse transcriptase (RT)" control samples were generated in the same fashion but RT was omitted from the reaction.

Primers were designed using a partial sequence of the rainbow trout 5-HT₄ receptor that was reported in an earlier study (L. Dionne-Wilson, MSc thesis, uOttawa). Primer –BLAST (NCBI) was used for primer design and primer quality (i.e. presence of hairpin structures, unwanted dimerization, etc.) was assessed using OligoAnalyzer 3.1 (IDT). For the purpose of *in situ* hybridization, the T7 sequence was included in the primers (Table 2.3). The resulting primers were used for polymerase chain reactions (PCR), which were carried out on a Bio-Rad S1000 Thermal Cycler using the following cycling conditions: 95°C (30 s), 68°C (30 s) and

72°C (45 s) for 40 cycles. Template (2 µL of cDNA) was added to 2.5 µL of 10X Dream Taq Green Buffer (ThermoFisher Scientific), 0.5 µL of 10 mmol L⁻¹ deoxynucleotide (Invitrogen), 0.5 µL each of forward and reverse primers (10 µmol L⁻¹), 18.875 µL of autoclaved water and 0.125 µL of Dream Taq DNA polymerase (ThermoFisher Scientific). The sizes of PCR products were verified by gel electrophoresis and the PCR products were purified using the QIAquick® PCR purification kit (Qiagen) according to the manufacturer's protocol and quantified using a Nanodrop® 2000 Spectrophotometer (ThermoFisher Scientific). The identities of the purified PCR fragments were verified by DNA sequencing which was carried out either by Genscript USA Inc., or by the Core Molecular Biology and Genomics Laboratory at the University of Ottawa.

The purified PCR products were used as template for *in vitro* transcription to create either antisense or sense riboprobes using T7 RNA polymerase. Template (400 ng of purified PCR product) was added to 2 µL of 10X transcription buffer (Roche, Laval, QC), 2 µL of 10X digoxigenin (DIG) RNA labelling mix (Roche), 1 µL of RNase out recombinant ribonuclease inhibitor (Invitrogen), 2 µL of T7 RNA polymerase (Roche) and RNase-free water (Qiagen) to a final volume of 20 µL. This mixture was incubated at 37°C for 4 h. Turbo DNase (1µL; Ambion, Burlington, ON) was then added and the mixture was incubated for another 15 min at 37°C. The mixture was purified using the RNeasy® mini kit (Qiagen) according to the manufacturer's protocol, and quantification of RNA was conducted using a Nanodrop® 2000 Spectrophotometer (ThermoFisher Scientific). The sizes of the riboprobe products were confirmed by gel electrophoresis.

Table 2.3 Oligonucleotide primer pairs used in generation of a riboprobe against the rainbow trout (*Oncorhynchus mykiss*) 5-HT₄ receptor.

Gene	Primers	Product length (base pairs)	Accession No.
5-HT₄ anti-sense	F TTCTGCCTGGTCCGAACATC T7-R TAATACGACTCACTATAGGGGGAACACGTCAGAGCGTAGG	341	KP334157
5-HT₄ sense	T7-F TAATACGACTCACTATAGGGTTCTGCCTGGTCCGAACATC R GGAACACGTCAGAGCGTAGG	341	KP334157

Primer sequences are given in the 5' to 3' direction.

2.2.3.2 Combined *in situ* hybridization and immunohistochemistry for the cellular localization of 5-HT₄ receptor mRNA

Rainbow trout ($N = 2$) were euthanized and head kidney tissue was collected, fixed and sectioned as described above. Adjacent cryosections were permeabilized with 1% Triton-X in PBST for 2 h at room temperature and then hybridized at 65°C in a humidified chamber overnight with the sense or antisense probe diluted to a final concentration of 0.67 ng μL^{-1} in hybridization buffer (47% formamide, 4.7X saline-sodium citrate, 0.09% Tween-20, 9 mmol L^{-1} citric acid) supplemented with 47 $\mu\text{g mL}^{-1}$ heparin (Sigma Aldrich) and 470 $\mu\text{g mL}^{-1}$ yeast transfer RNA (Invitrogen). Following hybridization, slides were washed 2 x 30 min at 65°C in hybridization buffer and 2 x 30 min at room temperature in PBST. In a humidified chamber, slides were blocked using 10% fetal bovine serum (FBS) in PBST for 90 min at room temperature, and then incubated with anti-DIG Fab fragments (Roche) in PBST containing 10% FBS overnight at 4°C. Slides were then washed 5 x 20 min in PBST and 2 x 10 min in alkaline Tris buffer (100 mmol L^{-1} Tris-HCl, 50 mmol L^{-1} MgCl_2 , 100 mmol L^{-1} NaCl and 0.1 % Tween-20, pH 9.5) and incubated in the dark at room temperature with a staining solution of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt in a humidified chamber until the desired intensity of colouration was obtained. To stop the colour reaction, slides were washed 3 x 5 min in distilled water and fixed in 4% PFA for 20 min.

Immunohistochemical localization of P450scc, as a marker for interrenal cells, was then conducted on these slides. The protocol described in section 2.2.2 was followed with the exception that permeabilization occurred prior to *in situ* hybridization (see above). Slides were visualized using an Axiophot microscope (Zeiss; Toronto, ON) and images were captured using Image pro v 6_0.

2.2.4 Involvement of the 5-HT₄ receptor in mediating the effects of 5-HT on cortisol production

To investigate the role of the 5-HT₄ receptor in interrenal cells, cortisol production was measured in the presence of 5-HT and the selective 5-HT₄ receptor blocker 5-fluoro-2-methoxy-[1-[2-[(methylsulphonyl) amino] ethyl]-4-piperidinyl]-1h-indole-3-methylcarboxylate sulphamate (GR125487) using an *in vitro* head kidney preparation similar to that of Aluru et al. (2005) and Jeffrey et al. (2014). Rainbow trout ($N = 12$) and Atlantic salmon ($N = 9$) were euthanized by terminal anaesthesia as described above. A ventral midline incision was used to expose the PCV, which was perfused with 40 mL of cold (4°C) Hanks' solution (92 mmol L⁻¹ NaCl, 3.6 mmol L⁻¹ KCl, 0.5479 mmol L⁻¹ MgSO₄, 0.3996 mmol L⁻¹ KH₂PO₄, 0.2296 mmol L⁻¹ Na₂HPO₄, 7.479 mmol L⁻¹ (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid)(HEPES), 2.77 mmol L⁻¹ NaHCO₃, 0.85 mmol L⁻¹ CaCl₂, 0.3 g L⁻¹ bovine serum albumin; pH 7.15) to remove blood from the head kidney. In brief, head kidney tissue was weighed, minced with a razor blade (to ~1 mm³) and incubated on a shaker with gentle rocking for 1.5 h at 13°C in 75 mL g⁻¹ tissue of incubation medium [modified Hanks' solution supplemented with minimum essential medium (MEM) amino acid solution (50x; 2 mL per 100 mL medium; Sigma Aldrich), MEM non-essential amino acid solution (100x; 1 mL per 100 mL medium; Sigma Aldrich), and antibiotic-antimycotic solution (100x; 1 mL per 100 mL medium; Sigma Aldrich)]. The tissue was then distributed evenly into the wells of a 48-well microplate (~25 mg of tissue per well) and incubated in duplicate with 250 µL of one of the following solutions; fresh medium (blank; negative control), 1 IU mL⁻¹ of porcine ACTH (Sigma Aldrich) as a positive control, 100 nmol L⁻¹ 5-HT (Sigma Aldrich), or a combination of 100 nmol L⁻¹ 5-HT and 2 x 10⁻⁶ mol L⁻¹ GR125487 (Tocris Bioscience, Burlington, ON). Agonist/antagonist concentrations were

selected based on previous experiments using the same approach (L. Dionne-Wilson, MSc thesis, uOttawa). The tissue was incubated for 1 h at 13°C on a shaker with gentle rocking after which the tissue suspensions were transferred to microcentrifuge tubes and centrifuged at 14,000 *g* for 10 min. The head kidney tissue was weighed, and the supernatant was flash frozen in liquid N₂ and stored at -80°C for later analysis of cortisol concentrations. Cortisol concentrations were measured using a commercially-available radioimmunoassay (RIA) (MP Biomedicals, Solon, OH) that was previously validated for trout plasma (Gamperl et al., 1994) and has been used for head kidney preparations (Jeffrey et al., 2014). The intra-assay coefficient of variation was 8.1%, and the inter-assay coefficient of variation was 21.4%.

2.2.5 Statistical analyses

SigmaPlot v. 11.0 (Systat Software) was used to perform statistical analyses. Data are presented as mean values $\pm$ one standard error of the mean (SEM). For all analyses, the level of statistical significance (α) was set to $p < 0.05$. One-way repeated measures (RM) analysis of variance (ANOVA) was used to test the involvement of the 5-HT₄ receptor in mediating the effects of 5-HT on cortisol production in rainbow trout and salmon head kidney preparations. A Holm-Sidak *post hoc* test for multiple comparisons was used as appropriate to identify the source of statistically-significant differences among treatment groups.

2.3 Results

2.3.1 Localization of 5-HT within the head kidney

Immunohistochemical approaches were used to investigate the distribution of 5-HT within the head kidney. Three different antibodies, against P450scc, 3 β HSD, and 11 β -hyd were used to identify interrenal cells. Immunoblot analysis in which rainbow trout tissues were probed with rabbit anti-trout P450scc (a kind gift from Graham Young) revealed a single band at ~38 kDa in head kidney tissue whereas two bands, one at ~38 kDa and a second at the expected size of ~54 kDa (Kobayashi et al., 1998) were detected in gonadal tissue, which was used as a positive control (Fig. 2.1A). Further analysis of trout head kidney tissue using immunoprecipitation followed by gel analysis revealed a single band at ~59 kDa (Fig. 2.1B). Immunoblot analyses in which trout head kidney tissue was probed with rabbit anti-trout 3 β HSD (a kind gift from Graham Young) or rabbit anti-frog 11 β -hyd (a kind gift from Hiroshi Takemori) showed single bands at the expected sizes of ~42 kDa (Fig. 2.2A) and ~49.5 kDa (Fig. 2.2B), respectively. Immunohistochemical analysis of rainbow trout head kidney tissue using these antibodies as well as an antibody raised against TH to identify chromaffin cells revealed that chromaffin cells and interrenal cells were in close proximity to each other and typically clustered around the PCV; the three interrenal cell markers identified the same clusters of cells near the PCV (Fig. 2.3).

In rainbow trout, 5-HT was detected in low amount near but never within interrenal cells (Fig. 2.4) and chromaffin cells (Fig. 2.5). Serotonin also did not colocalize to any substantial extent with nerves using antibodies against ZN-12 (Fig. 2.6), SV2, a synaptic vesicle protein (Fig. 2.7) and AT (Fig. 2.8) as nerve markers. Specificity of the ZN-12 antibody was confirmed

by immunoblot analysis of trout head kidney tissue, in which a band was detected at the expected size of ~125 kDa (Fig. 2.9). By contrast, 5-HT colocalized with chromaffin cells around the PCV in Atlantic salmon (Fig. 2.10).

2.3.2 Localization of 5-HT₄ receptor mRNA in the head kidney

To investigate whether 5-HT₄ receptor mRNA is localized to interrenal cells, *in situ* hybridization was combined with immunostaining. Using a riboprobe designed against the trout 5-HT₄ receptor, 5-HT₄ receptor mRNA was detected in interrenal cells. The staining appeared to be associated with cell nuclei as well as the cytoplasm (Figs. 2.11, 2.12 and 2.13). The signal for 5-HT₄ receptor mRNA seemed weaker in interrenal cells than in surrounding cells (Figs. 2.11A and D, 2.12A, B and D). No signal was detected in sections that were hybridized with digoxigenin-labelled sense riboprobe (Fig. 2.11E).

2.3.3 Stimulation of head kidney tissue by 5-HT *in vitro*

In vitro head kidney preparations were used to confirm the involvement of the 5-HT₄ receptor in mediating effects of 5-HT on cortisol production. In rainbow trout preparations, incubation of head kidney tissue with 5-HT significantly increased cortisol production compared to that of unstimulated tissue (Fig. 2.14; RM ANOVA, $p < 0.001$). The increase in cortisol production elicited by 5-HT was blocked when head kidney tissue was incubated with a combination of 5-HT and the selective 5-HT₄ receptor antagonist GR125487 (Fig. 2.14; RM ANOVA, $p < 0.001$). In Atlantic salmon preparations, incubation of head kidney tissue with ACTH but not 5-HT significantly increased cortisol production compared to that of unstimulated

tissue (Fig. 2.15A; RM ANOVA, $p < 0.001$). However, scrutiny of the data for individual preparations suggested that a subset of fish responded to 5-HT by increasing cortisol production above the baseline value. Within this group, the response to 5-HT was blocked when the head kidney tissue was incubated with a combination of 5-HT and GR125487 (Fig. 2.15B; RM ANOVA, $p = 0.001$).

Figure 2.1 Representative western blots illustrating the bands detected in rainbow trout (*Oncorhynchus mykiss*) head kidney and gonad using a rabbit anti-rainbow trout P450scc antibody (A) and following immunoprecipitation carried out using the same antibody on head kidney tissue (B). *HK*, head kidney.

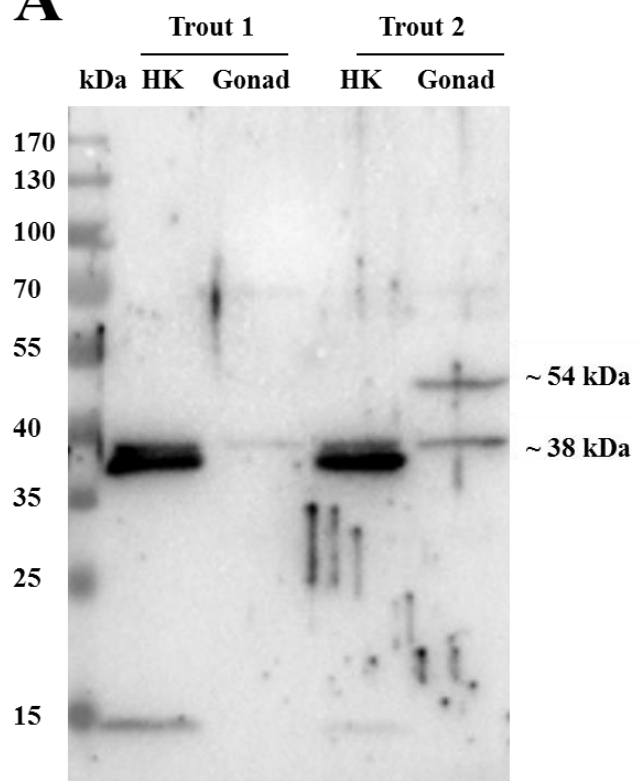
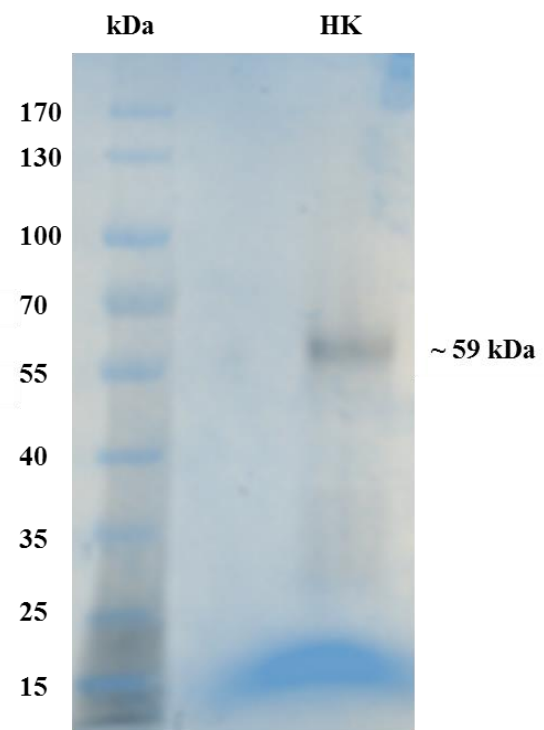
A**B**

Figure 2.2. Representative western blots illustrating the bands detected in rainbow trout (*Oncorhynchus mykiss*) head kidney tissue using a rabbit anti-rainbow trout 3 β HSD antibody (A) and a rabbit anti-frog 11 β -hyd antibody (B). *HK*, head kidney.

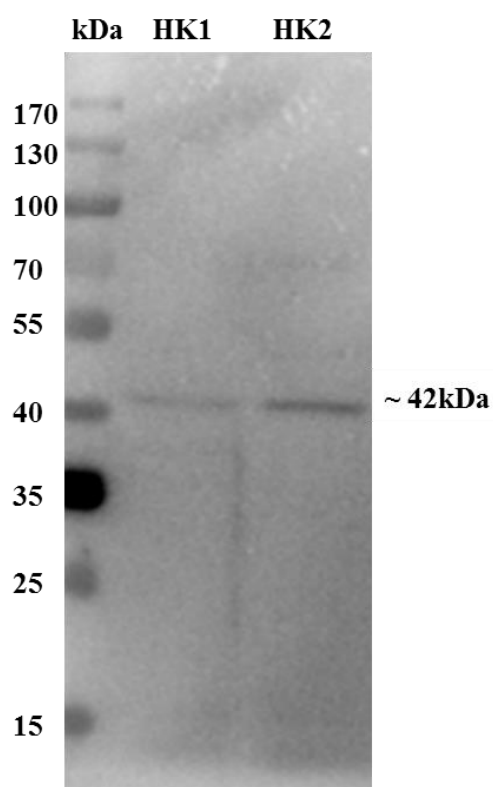
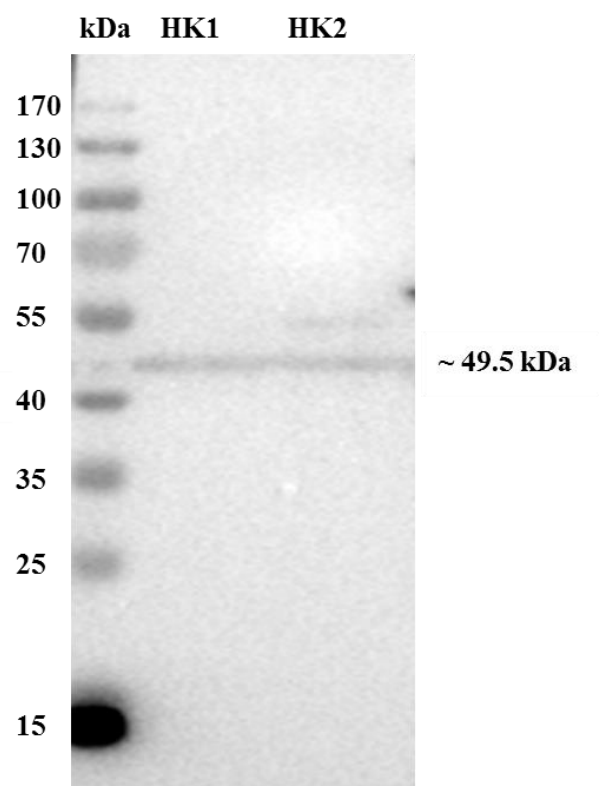
A**B**

Figure 2.3. Representative transverse sections of rainbow trout (*Oncorhynchus mykiss*) head kidney tissue showing fluorescent immunolabelling of interrenal cells (green), chromaffin cells (TH, red) and cell nuclei (DAPI, blue). Three different interrenal cell markers were used in adjacent sections from the same head kidney tissue: a 3 β HSD antibody in panel (A); an 11 β -hyd antibody in panel (B); and a P450scc antibody in panel (C). PCV, posterior cardinal vein.

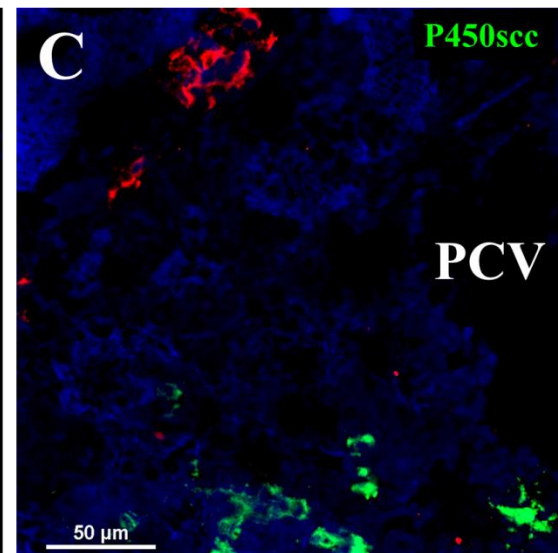
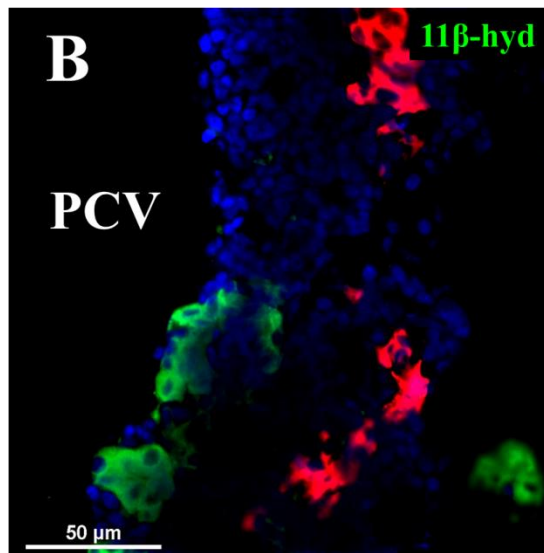
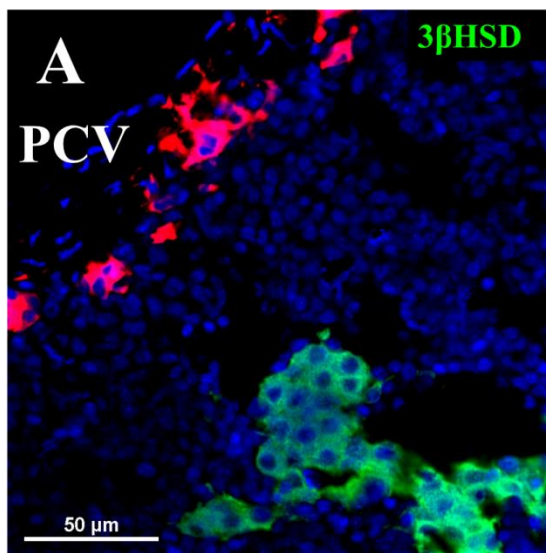


Figure 2.4. Representative transverse sections of rainbow trout (*Oncorhynchus mykiss*) head kidney tissue showing fluorescent immunolabelling of serotonin (5-HT), interrenal cells (P450scc) and cell nuclei (DAPI, blue). Panel (B) presents a close-up of the area bounded by the yellow box in panel (A); in these images, goat anti-5-HT was used in combination with rabbit anti-trout P450scc. Panels (C) and (D) present the same area in adjacent sections using rabbit anti-5-HT and rabbit anti-trout P450scc. No 5-HT or P450scc immunofluorescence was detected in control sections from which the primary antibodies were omitted (white box in panel A). PCV, posterior cardinal vein.

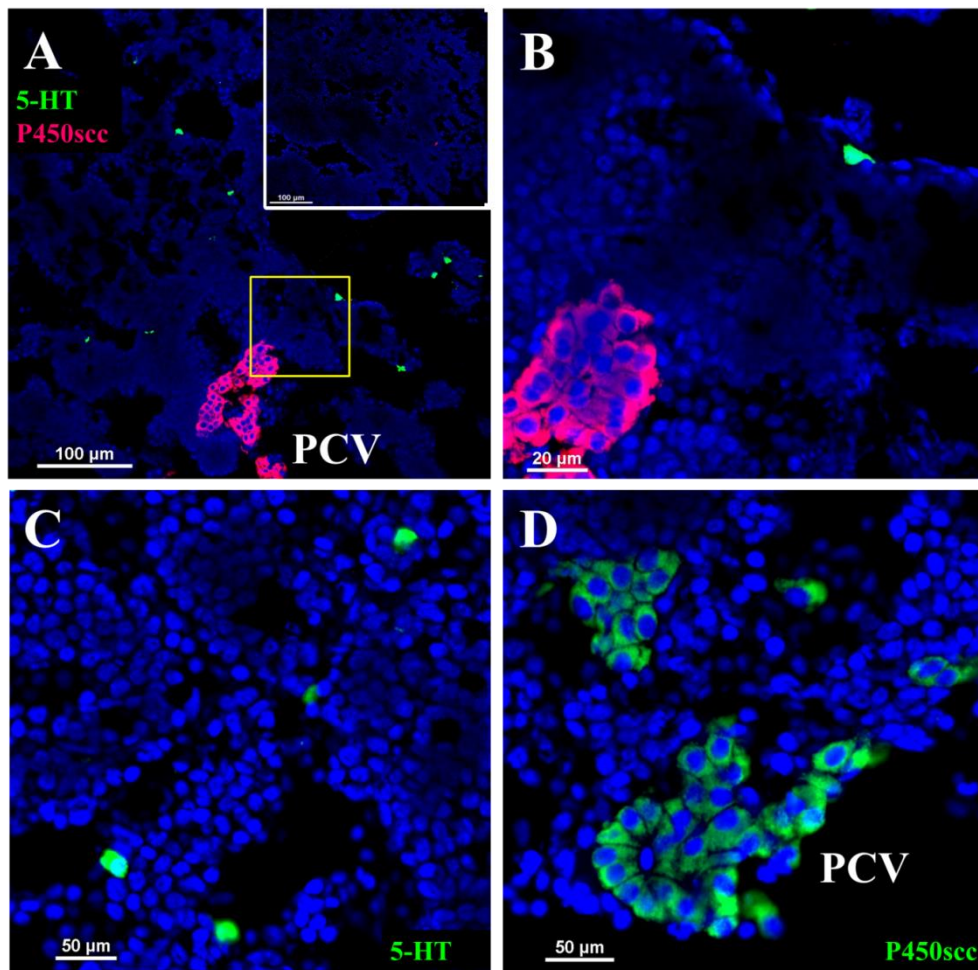


Figure 2.5. Representative transverse sections of rainbow trout (*Oncorhynchus mykiss*) head kidney tissue showing fluorescent immunolabelling of serotonin (5-HT, green), chromaffin cells (TH, red) and cell nuclei (DAPI, blue). Panel (B) represents a close-up of the area bounded by the yellow box in panel (A). No 5-HT or TH immunofluorescence was detected in control sections from which the primary antibodies were omitted (white box in panel A). PCV, posterior cardinal vein.

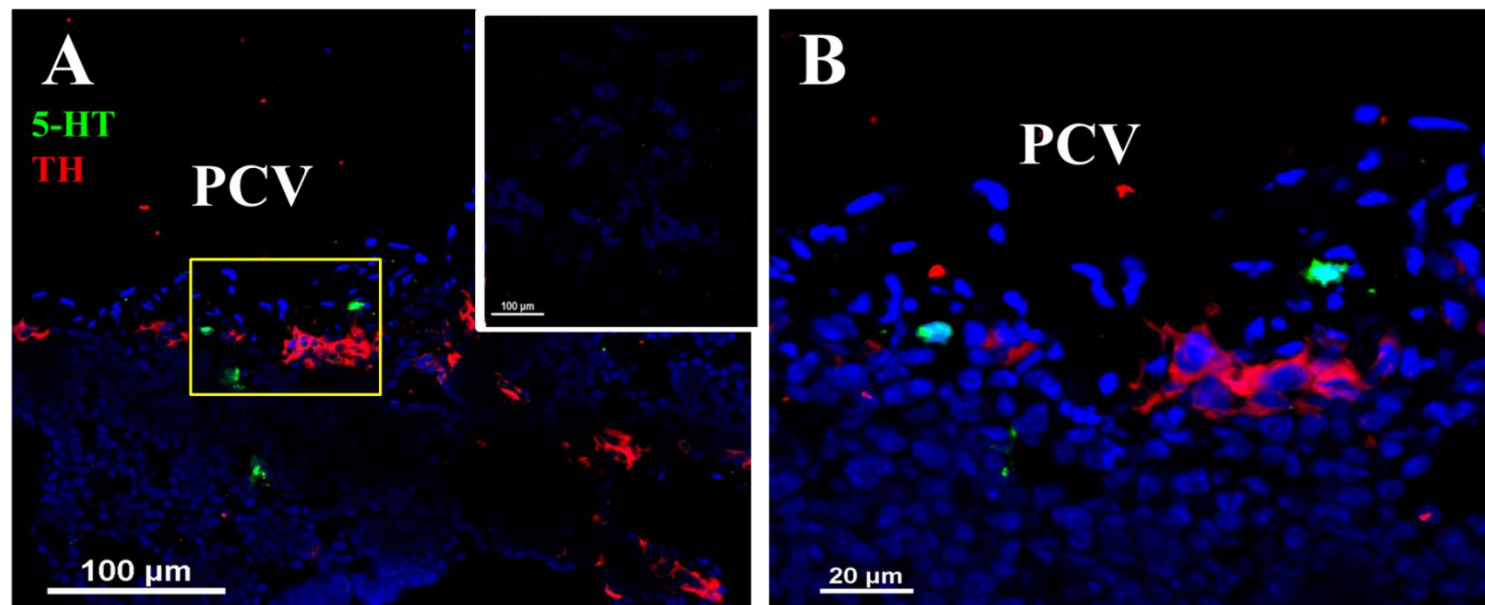


Figure 2.6. Representative transverse sections of rainbow trout (*Oncorhynchus mykiss*) head kidney tissue showing fluorescent immunolabelling of serotonin (5-HT, green), nerves (ZN-12, red), and cell nuclei (DAPI, blue). No 5-HT or ZN-12 immunofluorescence was detected in control sections from which the primary antibodies were omitted (white box in panel A). PCV, posterior cardinal vein.

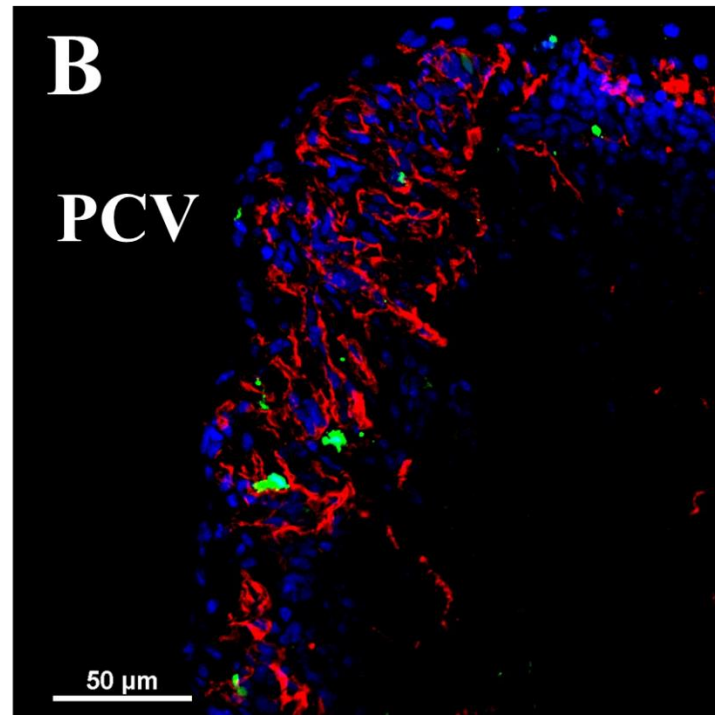
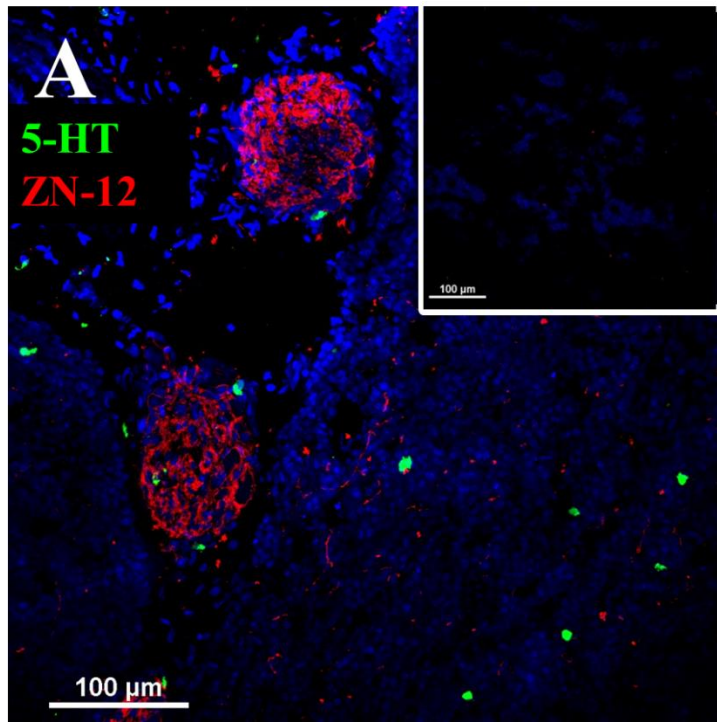


Figure 2.7. Representative transverse sections of rainbow trout (*Oncorhynchus mykiss*) head kidney tissue showing fluorescent immunolabelling of serotonin (5-HT, green), synaptic vesicles of nerve endings (SV2, red) and cell nuclei (DAPI, blue). A representative negative control section from which the primary antibodies were omitted is shown in the white box in panel (A). Some non-specific staining for SV2 was detected. *PCV*, posterior cardinal vein.

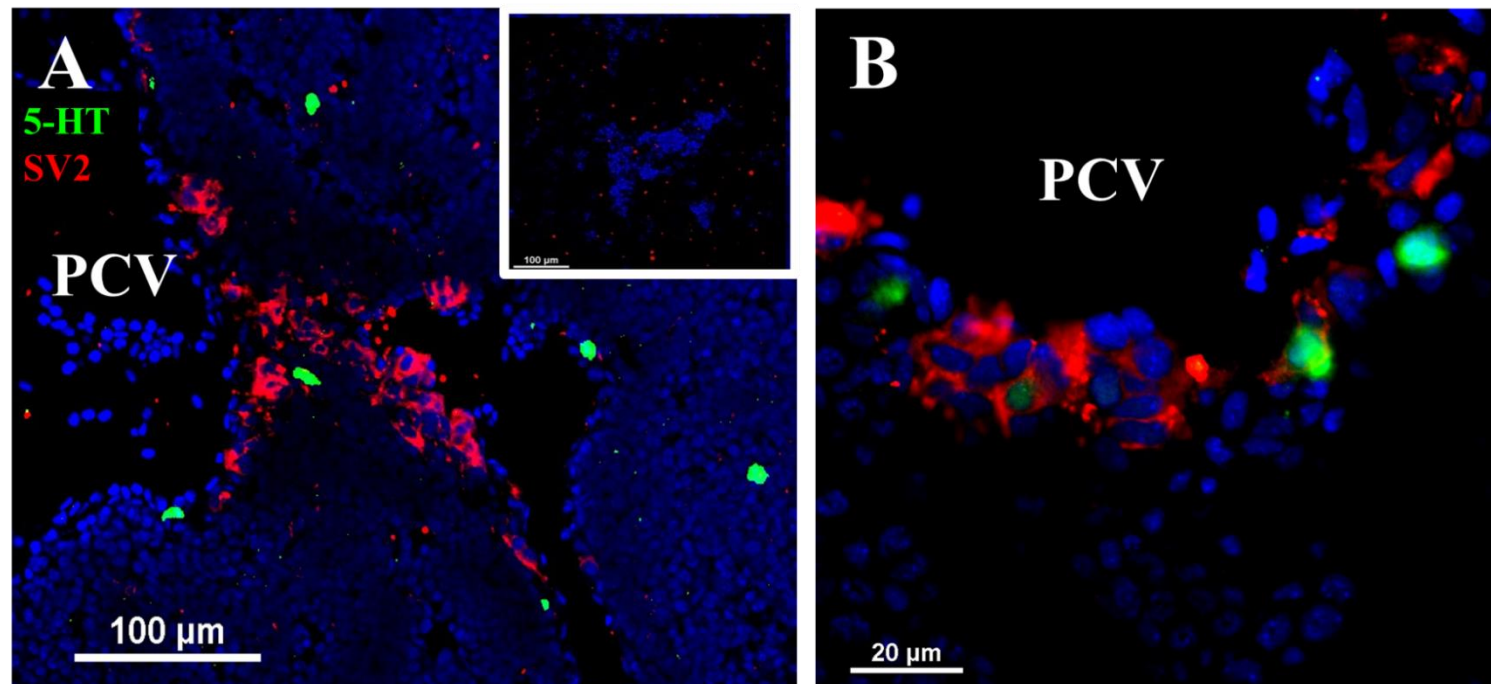


Figure 2.8. Representative transverse sections of rainbow trout (*Oncorhynchus mykiss*) head kidney tissue showing fluorescent immunolabelling of serotonin (5-HT, red), acetylated tubulin (AT, green) and cell nuclei (DAPI, blue). *PCV*, posterior cardinal vein.

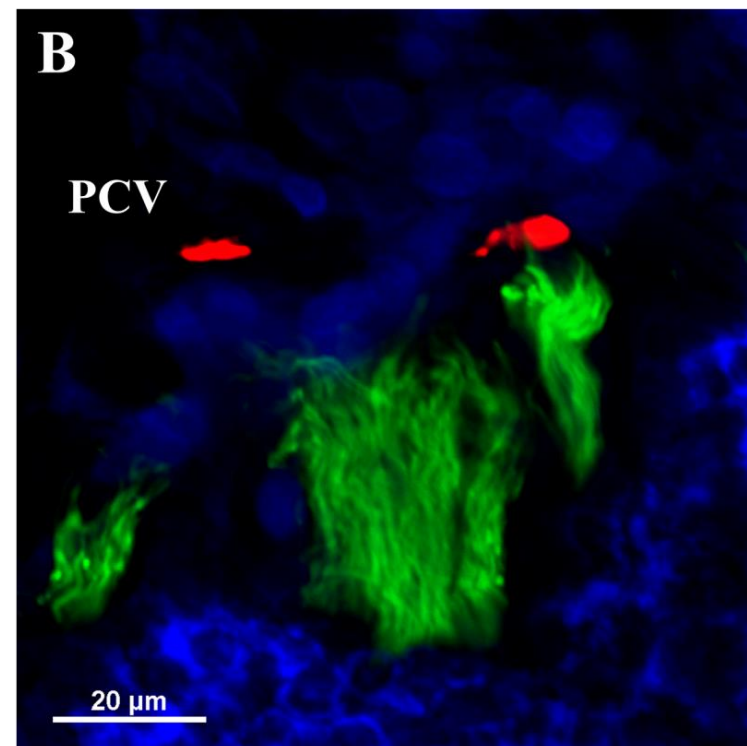
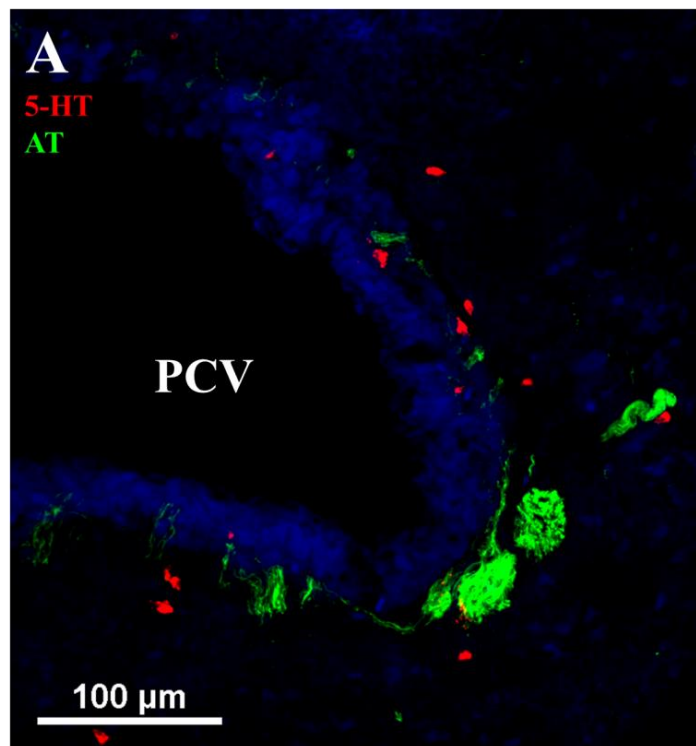


Figure 2.9. Representative western blot illustrating the bands detected in rainbow trout (*Oncorhynchus mykiss*) head kidney tissue using a mouse anti-zebrafish ZN-12 antibody. *HK*, head kidney.

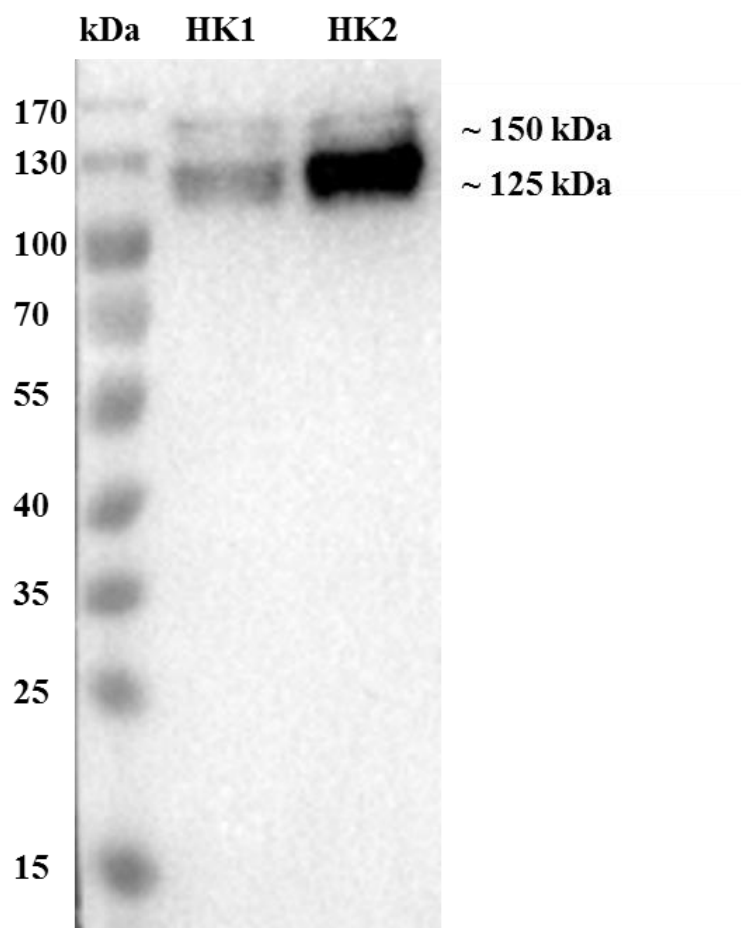


Figure 2.10. Representative transverse sections of Atlantic salmon (*Salmo salar*) head kidney tissue showing fluorescent immunolabelling of serotonin (5-HT, green), chromaffin cells (TH, red) and cell nuclei (DAPI, blue). Co-localization of 5-HT and TH is represented in yellow in panels (B) and (C); panel (C) presents a close-up of the area bounded by the white box in panel (B). PCV, posterior cardinal vein.

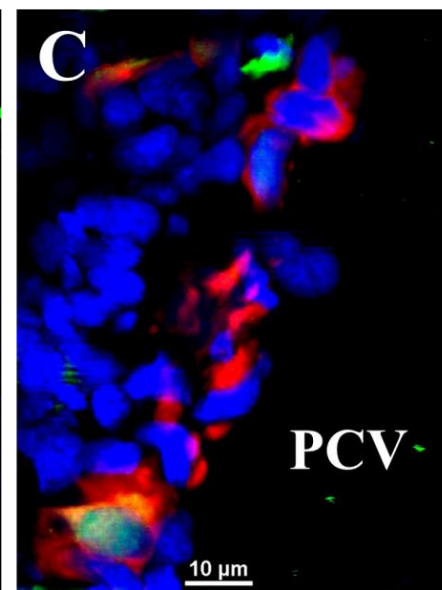
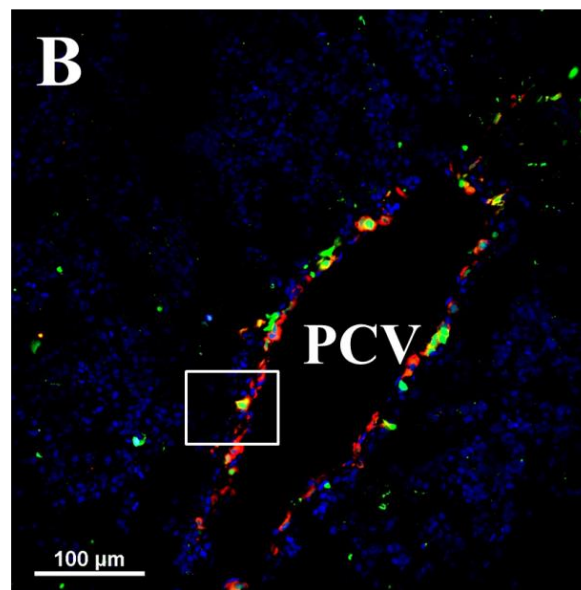
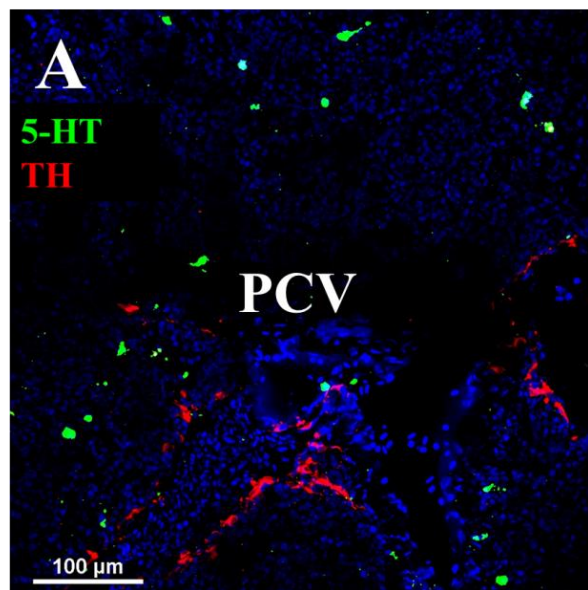


Figure 2.11. Representative images showing staining associated with *in situ* hybridization of 5-HT₄ receptor mRNA and P450_{scc} immunolabelling of interrenal cells in transverse sections of rainbow trout (*Oncorhynchus mykiss*) head kidney tissue. Panels A to D present the same section, viewed under light microscopy to visualize staining associated with 5-HT₄ receptor mRNA (purple; arrowheads) (A) and under fluorescence to visualize staining associated with interrenal cells (red) (B), or interrenal cells and cell nuclei (DAPI, blue) (C). Panel (D) presents an overlay of the images in panels A and B, showing the presence of staining for 5-HT₄ receptors in interrenal cells. Panel (E) presents a representative section that was hybridized with sense riboprobe as a negative controls; no colour staining is visible. Arrowheads identify interrenal cells that express 5-HT₄ receptor mRNA and arrows indicate melanomacrophages. PCV, posterior cardinal vein.

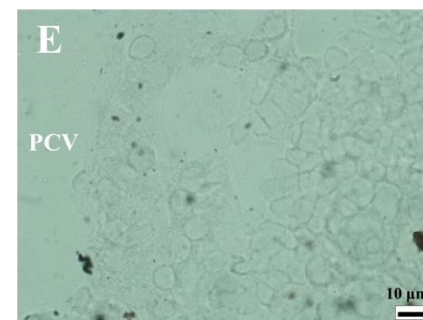
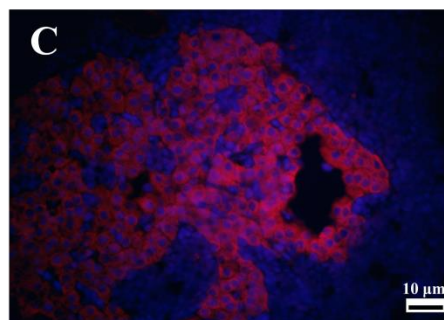
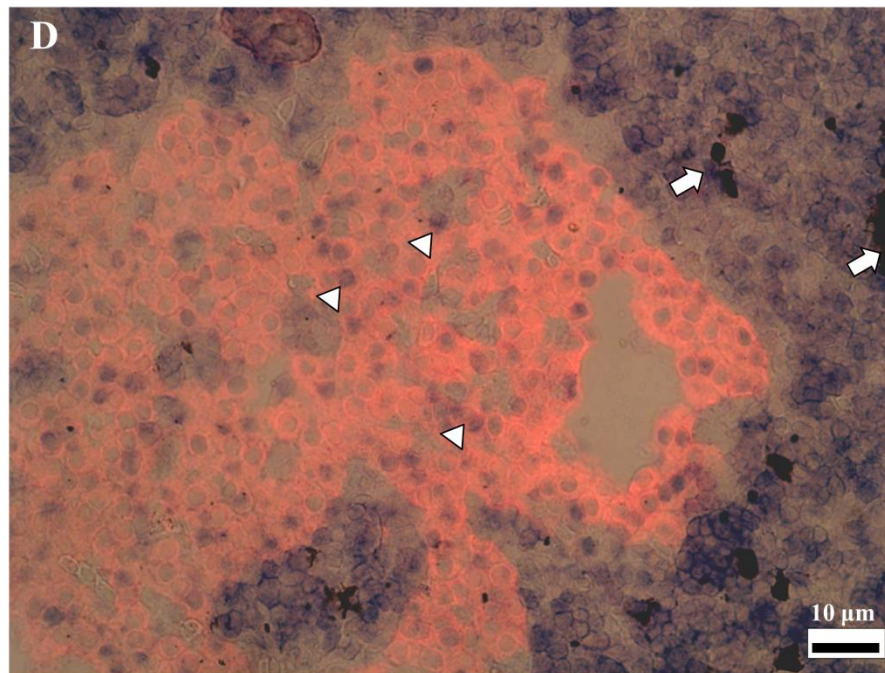
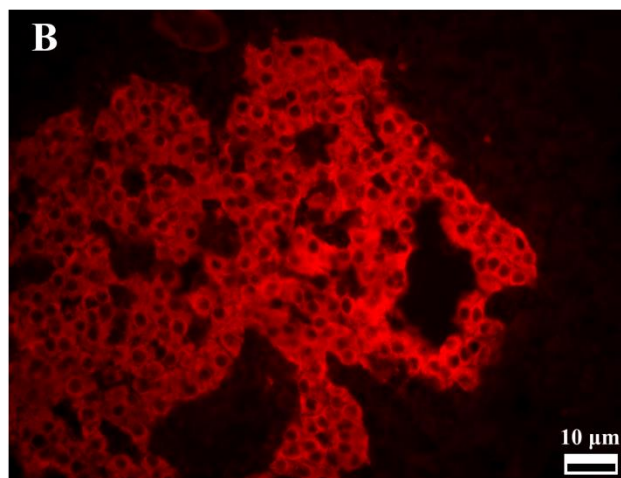
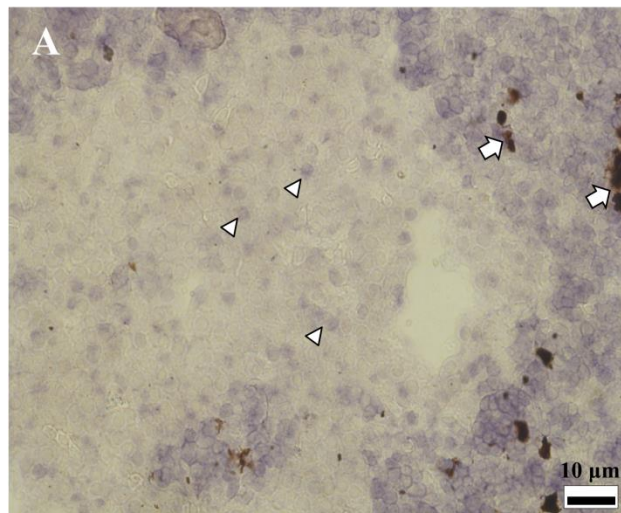


Figure 2.12. Representative images showing staining associated with *in situ* hybridization of 5-HT₄ receptor mRNA and P450_{scc} immunolabelling of interrenal cells in transverse sections of rainbow trout (*Oncorhynchus mykiss*) head kidney tissue. Panels A to D present the same section viewed under different conditions. Panel (A) presents an image collected under transmitted light conditions to identify staining associated with 5-HT₄ receptor mRNA (purple), while in panel B, this image was overlaid with one collected under phase contrast conditions to identify cell nuclei. Panel C was collected under fluorescent conditions to detect P450_{scc} immunolabelling of interrenal cells (red), and panel D presents this image overlaid with panel A to illustrate staining associated with 5-HT₄ receptor mRNA in interrenal cells. Arrowheads identify interrenal cells that express 5-HT₄ receptor mRNA. Arrows identify melanomacrophages.

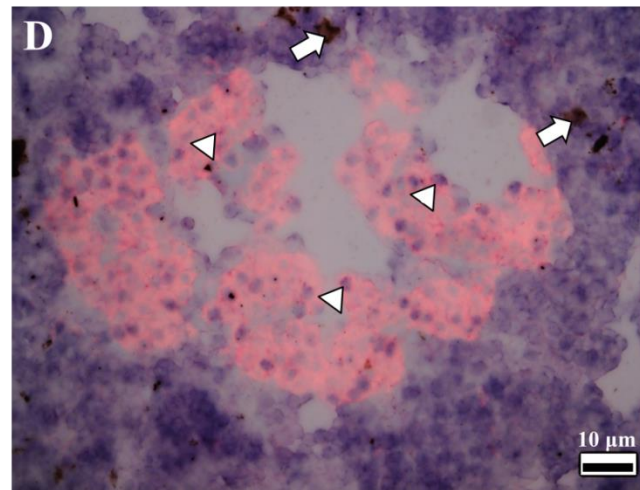
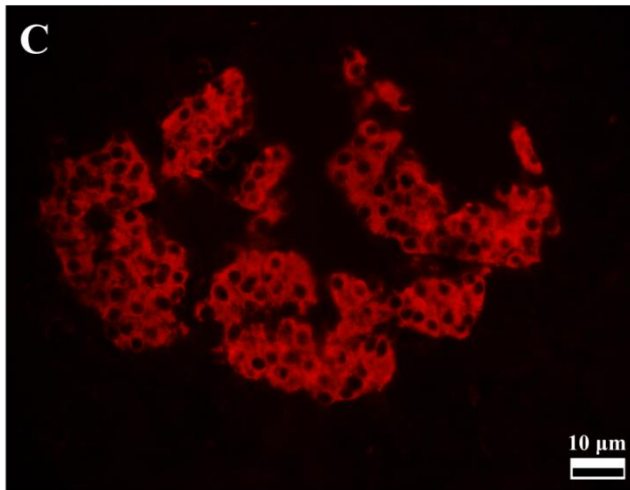
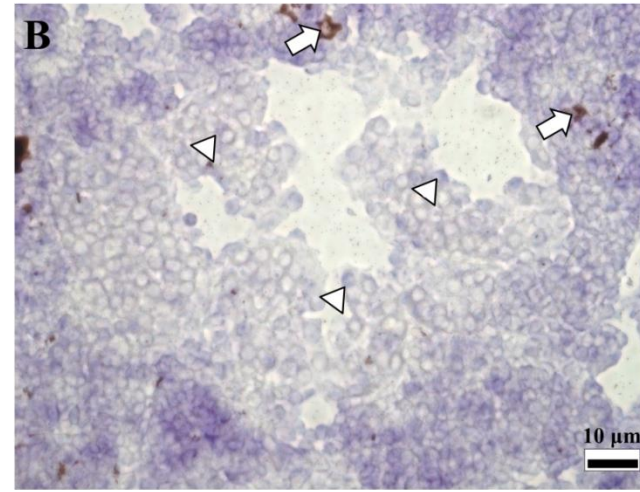
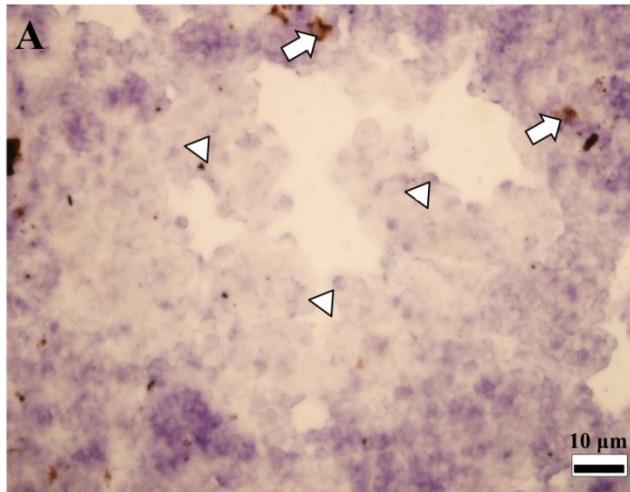


Figure 2.13. Representative images showing staining associated with *in situ* hybridization of 5-HT₄ receptor mRNA and P450_{scc} immunolabelling of interrenal cells in transverse sections of rainbow trout (*Oncorhynchus mykiss*) head kidney tissue. Panels A to D present the same section viewed under different conditions. Panel (A) presents an image collected under transmitted light conditions to identify staining associated with 5-HT₄ receptor mRNA (purple), while in panel B, this image was overlaid with one collected under phase contrast conditions to identify cell nuclei. Panel C was collected under fluorescent conditions to detect P450_{scc} immunolabelling of interrenal cells (red), and panel D presents this image overlaid with panel A to illustrate staining associated with 5-HT₄ receptor mRNA in interrenal cells. Arrowheads identify interrenal cells that express 5-HT₄ receptor mRNA.

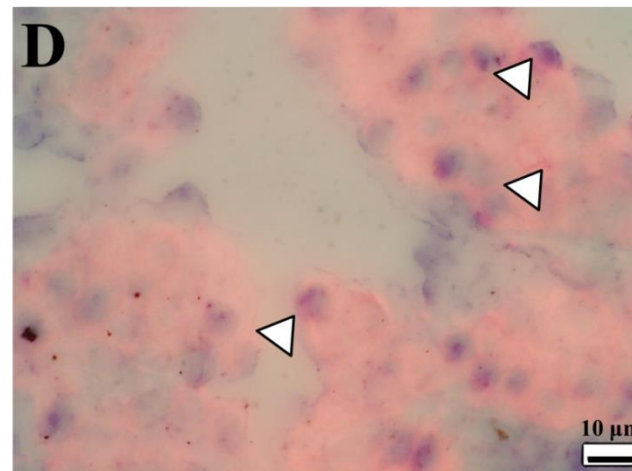
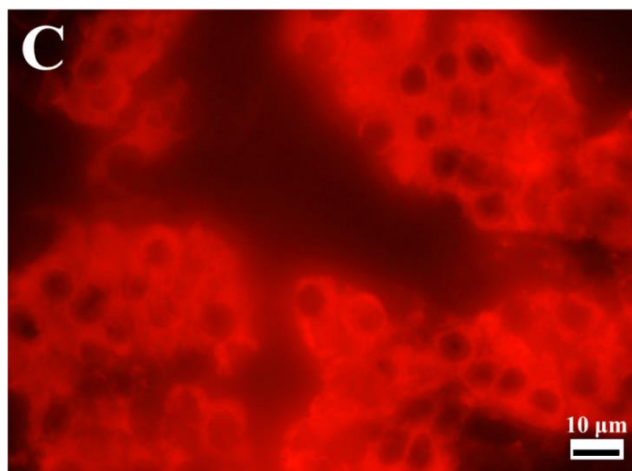
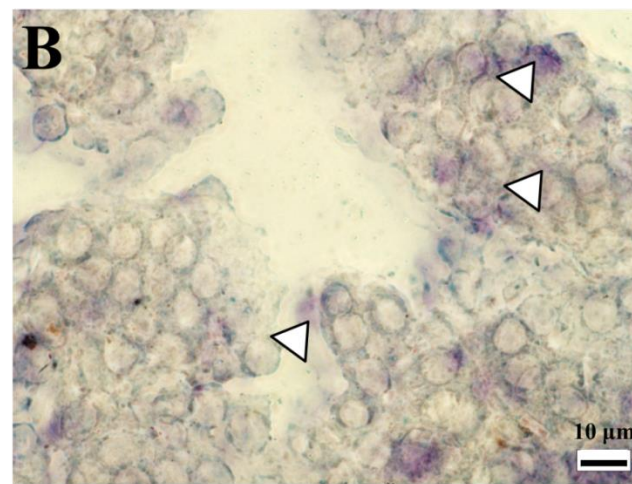
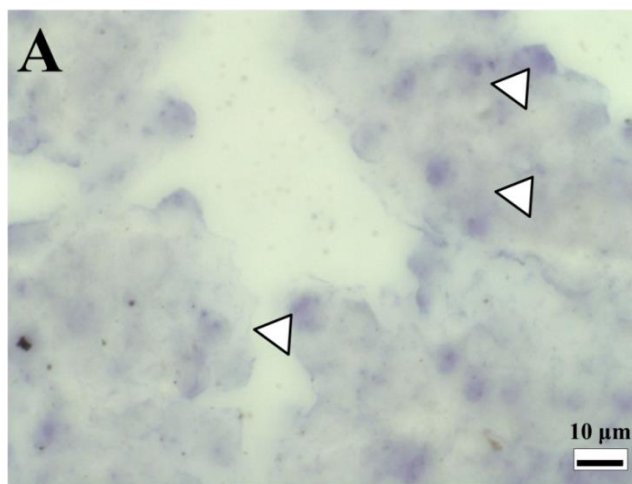


Figure 2.14. Rate of cortisol production for *in vitro* head kidney preparations from rainbow trout (*Oncorhynchus mykiss*, $N = 12$) incubated with saline (blank), 100 nmol L⁻¹ 5-HT, or 100 nmol L⁻¹ 5-HT in combination with 2×10^{-6} mol L⁻¹ GR125487. Values are means + SEM. Treatment groups that share a letter do not differ significantly from one another (RM ANOVA, $p < 0.001$).

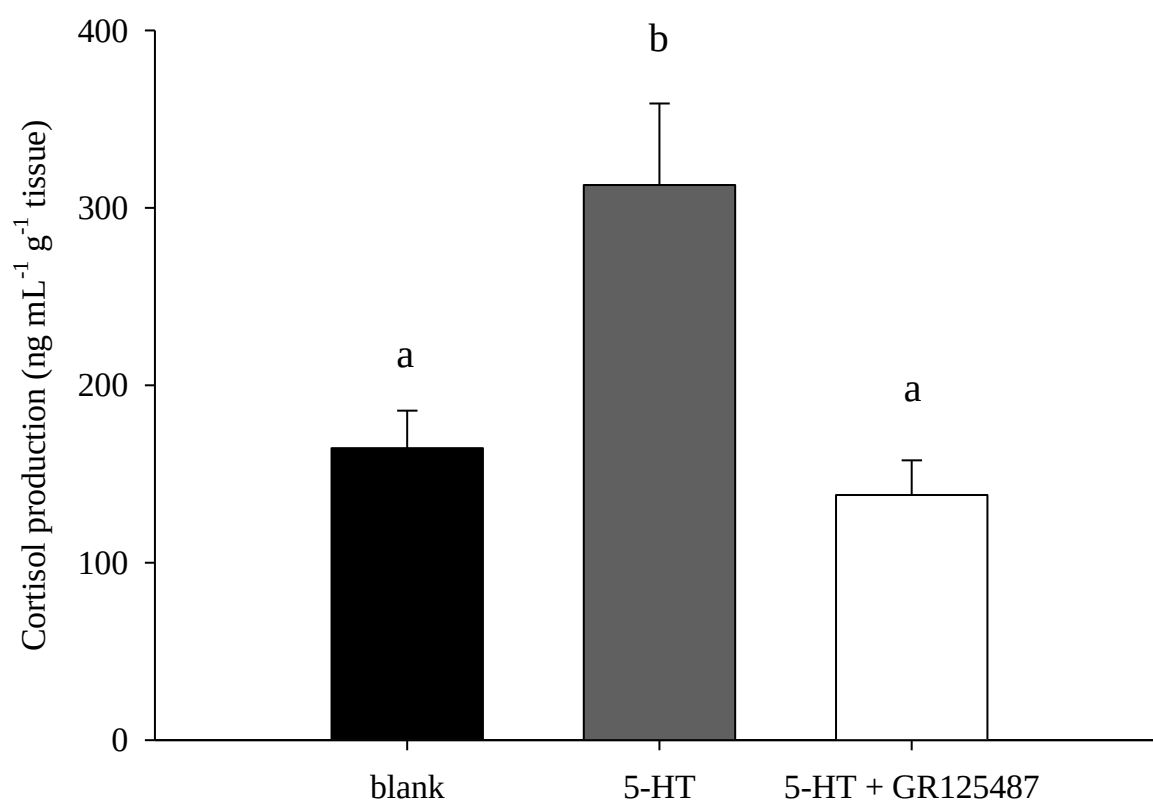
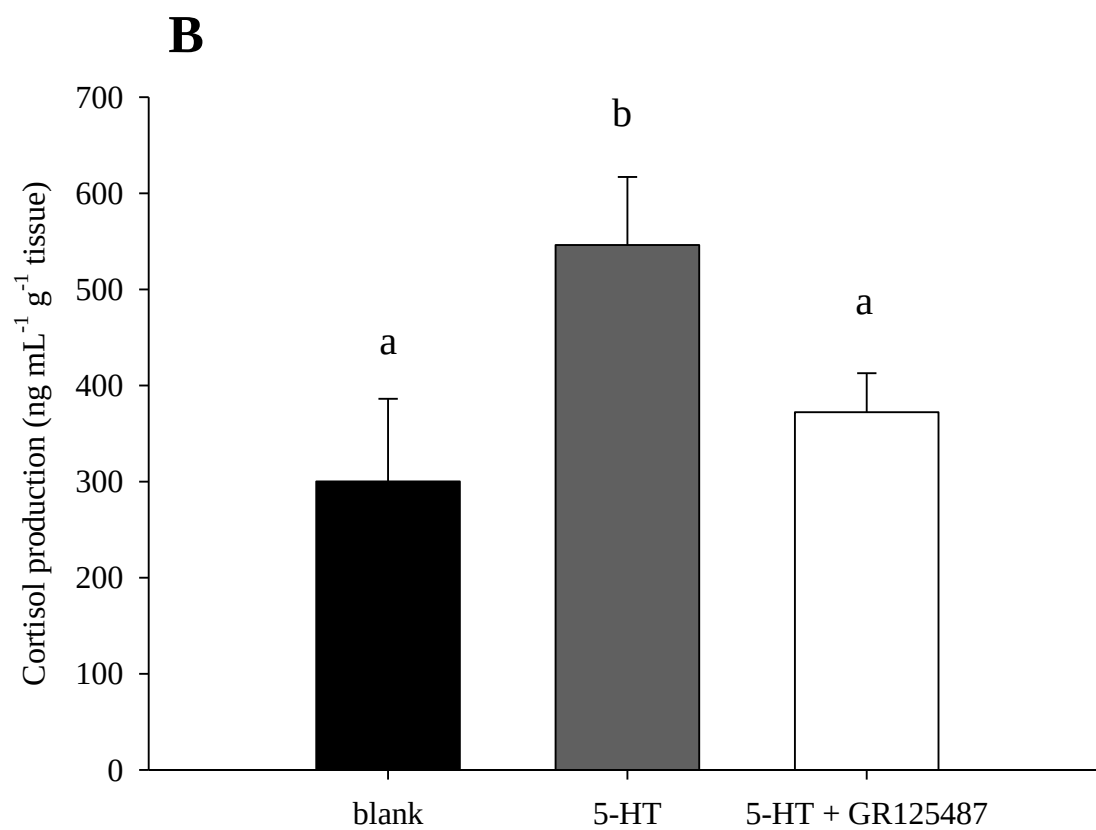
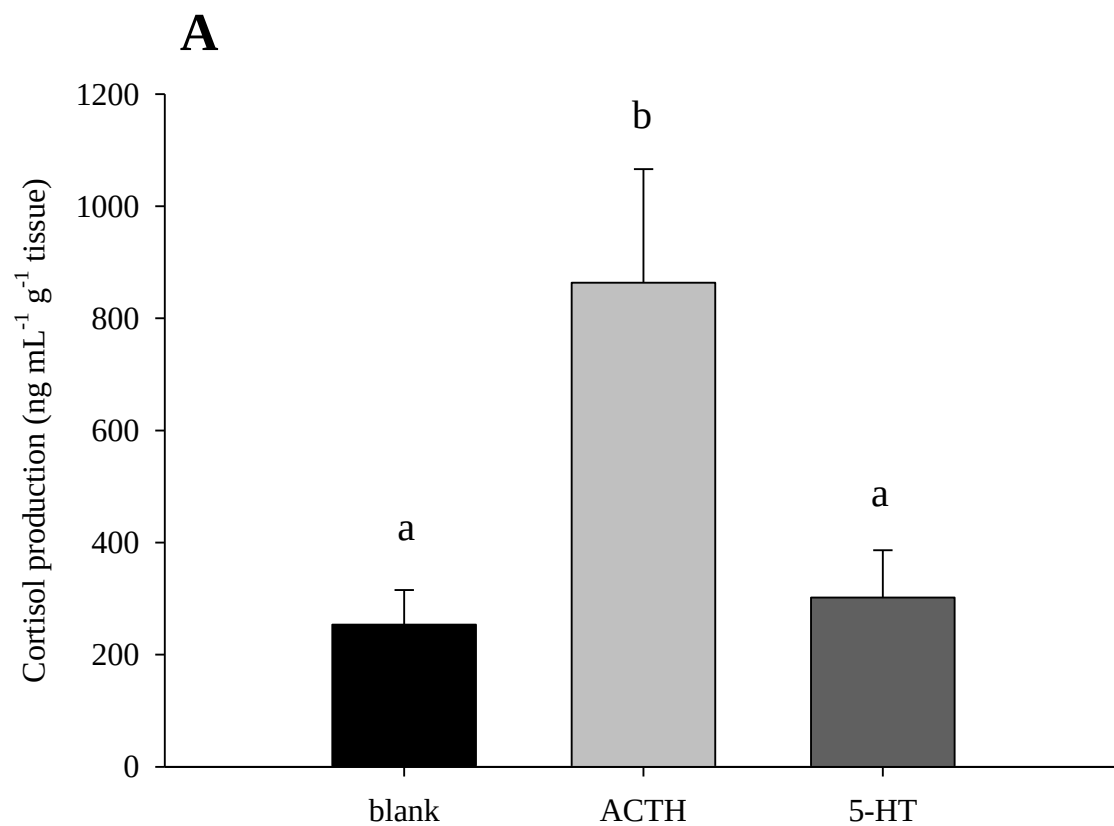


Figure 2.15. Rate of cortisol production for *in vitro* head kidney preparations from Atlantic salmon (*Salmo salar*). Panel (A) presents cortisol production for head kidney tissue ($N = 9$) that was incubated with saline (blank), 1 IU mL^{-1} ACTH or 100 nmol L^{-1} 5-HT. Panel (B) presents cortisol production for a subset of the preparations illustrated in panel A ($N = 4$) that appeared to respond to 5-HT; these preparations were incubated with saline (blank), 100 nmol L^{-1} 5-HT, or 100 nmol L^{-1} 5-HT in combination with $2 \times 10^{-6} \text{ mol L}^{-1}$ GR125487. Values are means + SEM. Treatment groups that share a letter do not differ significantly from one another (RM ANOVA, $p < 0.001$ and $p = 0.001$ for panels A and B, respectively).



2.4 Discussion

The goal of the present study was to investigate the role of 5-HT in the regulation of cortisol production in the head kidney of rainbow trout. Specifically, experiments were carried out to test the hypothesis that 5-HT acts as a paracrine regulator of cortisol production in the interrenal cells of rainbow trout, with its effects being mediated via the 5-HT₄ receptor. The results provide some support for this hypothesis. Serotonin was detected near interrenal cells, but the cell type that stores 5-HT is still unknown and the abundance of 5-HT was, perhaps, lower than might be expected for a paracrine regulator. *In situ* hybridization placed mRNA for the 5-HT₄ receptor in interrenal cells. This result was supported by data obtained using *in vitro* head kidney preparations, which showed that 5-HT stimulated cortisol production in the head kidney of rainbow trout, an effect that was blocked by the 5-HT₄ receptor antagonist GR125487.

2.4.1 Serotonin is present near interrenal cells

In mammals, experimental evidence supports a paracrine role for 5-HT in the adrenal gland (Contesse et al., 2000). In fish, 5-HT has been detected in the head kidney of several species (Fritsche et al., 1993; Reid et al., 1995; Lim et al., 2013) and has been reported to regulate cortisol production (Medeiros and McDonald, 2012; Lim et al., 2013), suggesting the possibility of a paracrine mechanism. To play a paracrine role, 5-HT is expected to be located in cells that are in close proximity to the interrenal cells. Using immunohistochemistry, 5-HT was detected near interrenal cells in the present study, as was also reported by Reid et al. (1995), but the cell type containing 5-HT remains uncertain. In cod, European eel and goldfish, 5-HT was

reported to be present in chromaffin cells (Reid et al., 1995; Lim et al., 2013), which are in close proximity to interrenal cells (Nandi, 1961). In the present study, double labelling for 5-HT and TH also revealed the presence of 5-HT in chromaffin cells of Atlantic salmon. However, no immunohistochemical evidence of localization of 5-HT to chromaffin cells was detected in rainbow trout in the present study, in agreement with the work of Reid et al. (1995). Furthermore, rainbow trout *in situ* PCV preparations perfused with carbachol, a cholinergic receptor agonist that stimulates catecholamine secretion from chromaffin cells, did not exhibit secretion of 5-HT, further suggesting that 5-HT is not stored in chromaffin cells (Fritsche et al., 1993). In contrast, in hagfish (*Myxine glutinosa*), perfusion of the PCV and heart with high $[K^+]$ saline to depolarize excitable cells elicited the release of 5-HT (Bernier and Perry, 1996). This result suggests that 5-HT is stored in the chromaffin cells of hagfish, which are found in the heart, and supporting this conclusion, 5-HT was detected in the hagfish heart (Reid et al. 1995). Thus, rainbow trout may be unusual in the absence of 5-HT from chromaffin cells.

Although 5-HT has been shown to be present in the chromaffin cells of several mammalian species as well as an amphibian (Verhofstad and Jonsson, 1983; Kent and Coupland, 1984; Delarue et al., 1988; Kong et al., 1989), in human adrenal glands, 5-HT is found in mast cells (Lefebvre et al., 1992). Preliminary attempts in the present study to identify mast cells histologically were unsuccessful, probably because mast cells are sparse or absent from the head kidney of rainbow trout (Victor Mulero, personal communication). Mast cells in rainbow trout also differ from those in humans because at least those of the intestine and gills do not contain histamine, nor is it likely that they express 5-HT (Mulero et al., 2007). Serotonin is also known as a neurotransmitter and therefore, it is possible that 5-HT is stored in nerve endings of the head kidney in rainbow trout. However, data collected in the present study are not consistent with

localization of 5-HT to nerve endings or synaptic vesicles of nerve endings because double-labelling did not reveal co-localization of 5-HT with ZN-12, SV2 or AT in the head kidney of rainbow trout. Cumulatively, these results confirm the presence of 5-HT in the head kidney of rainbow trout, but the cell type that stores 5-HT remains uncertain.

2.4.2 Presence of the 5-HT₄ receptor in interrenal cells and its involvement in mediating the effects of 5-HT on cortisol production

Work in mammals and amphibians indicated that 5-HT acts as a paracrine regulator of cortisol or corticosterone production via the 5-HT₄ receptor (Lefebvre et al., 1992; Contesse et al., 1994; Contesse et al., 2000). Similarly, in the fish species studied to date (goldfish, Gulf toadfish and rainbow trout), different receptors seem to be involved in the regulation of cortisol production by 5-HT, but the 5-HT₄ receptor has been shown to stimulate cortisol production in each fish species studied (Medeiros and McDonald, 2012; Lim et al., 2013; L. Dionne-Wilson, MSc thesis, uOttawa). Accordingly, the 5-HT₄ receptor is expected to be present in interrenal cells of rainbow trout. Two promising mammalian 5-HT₄ receptor antibodies were tested for use in investigating the presence of 5-HT₄ receptors in trout interrenal cells; ab10190 (Abcam, Cambridge, MA) which has 81% identity to the rainbow trout sequence for the immunogen, and sc-376158 (Santa Cruz Biotechnology, Dallas, TX) which has 76% identity to the rainbow trout sequence for the immunogen. However, western analysis to confirm the specificity of these antibodies in trout tissues did not yield encouraging results. Therefore, *in situ* hybridization was used to detect mRNA for the 5-HT₄ receptor. Staining was detected in interrenal cells, suggesting that 5-HT₄ receptor mRNA is present. Somewhat unexpectedly, while staining was present in the cytoplasm of interrenal cells, the cell nuclei seemed to stain most intensely. While unusual, other

studies have reported nuclear staining with *in situ* hybridization (Shyu et al., 2005). In mammals (Bender et al., 2000; De Maeyer et al., 2008), several splice variants are found for the 5-HT₄ receptor, and a similar situation may exist in trout (L. Dionne-Wilson, MSc thesis, uOttawa), which may explain why it is difficult to generate an appropriate probe and to detect mRNA for this receptor by *in situ* hybridization. As mentioned by Shyu et al. (2005), only the spliced products have, in principle, the potential to leave the nucleus and be transported to the cytoplasm to be translated into protein. Therefore, it is possible that the probe that was designed in the present study includes the region that is spliced and could explain why the cell nuclei were stained most intensely. Notably, staining for mRNA of the 5-HT₄ receptor was not restricted to interrenal cells but also was observed in surrounding cells. Serotonin has been reported to stimulate catecholamine secretion in *in situ* perfused PCV preparations of rainbow trout (Fritsche et al., 1993) but the 5-HT receptor subtype or subtypes involved in mediating this effect have received little attention to date (Perry and Capaldo, 2011).

Further support for the presence of the 5-HT₄ receptor in interrenal cells was obtained from the measurement of cortisol production *in vitro* in head kidney preparations. Previously, cortisol production was stimulated by the selective 5-HT₄ receptor agonist cisapride and this increase was blocked when cisapride was used in combination with the selective 5-HT₄ receptor antagonist GR125487 (L. Dionne-Wilson, MSc thesis, uOttawa). In the present study, these results were confirmed by blocking the stimulation of cortisol production elicited by 5-HT using GR125487. These results not only confirm a role for the 5-HT₄ receptor in stimulating cortisol production in the head kidney of rainbow trout, but also suggest that this receptor is the only or most important 5-HT receptor subtype involved in the response. This situation contrasts with that in goldfish or Gulf toadfish, where evidence from perfused or *in vitro* preparations suggests the

involvement in addition of 5-HT_{1A} or 5-HT₂ receptors, respectively. Preliminary studies were carried out on *in vitro* head kidney preparations from Atlantic salmon, to broaden the range of species examined and to provide a comparison species within salmonid fish. Interestingly, preparations from only a subset of the fish examined responded to 5-HT with an increase in cortisol production, but among these preparations, the response to 5-HT was blocked by GR125487. These data suggest that in Atlantic salmon, as in trout, the 5-HT₄ receptor plays a key role in mediating the effects of 5-HT on cortisol production. Further experiments are needed to support this conclusion.

In summary, the results of the present study provide further support for the involvement of 5-HT and the 5-HT₄ receptor in regulating cortisol biosynthesis in the interrenal cells of rainbow trout. Further experiments are needed to confirm whether 5-HT acts as a paracrine regulator of cortisol production, but the relatively sparse distribution of 5-HT in head kidney tissue was not encouraging in this regard. Identification of the cell type containing 5-HT is an important next step, together with identification of stimuli that promote 5-HT release. Although the source of head kidney 5-HT remains unclear, the findings of the present study support a role for 5-HT in regulating cortisol production by interrenal cells via a 5-HT₄ receptor.

CHAPTER 3

THE ROLE OF NEGATIVE FEEDBACK IN REGULATING CORTISOL PRODUCTION DURING CHRONIC SOCIAL STRESS IN A TELEOST FISH, RAINBOW TROUT

Notes on Chapter 3

Measurement of db-cAMP-stimulated cortisol production using *in vitro* head kidney preparations was carried out as part of the honours undergraduate thesis of Amanda Gour, which was mentored by M-È Bélair-Bambrick. Measurement of GR1, GR2 and 11 β HSD2 transcript abundance was carried out as part of the honours undergraduate thesis of Rebecca Robinson, which was mentored by M-È Bélair-Bambrick and C Best.

3.1 Introduction

In juvenile salmonid fish, social hierarchies are formed through aggressive interactions when resources, such as feeding territories, are limited (Stringer and Hoar, 1955; Chapman, 1966; Symons, 1968). Dominant fish monopolise limited food resources, are aggressive towards subordinate fish, and defend a territory (Stringer and Hoar, 1955; Chapman, 1966). In contrast, subordinate fish reduce their food intake and swimming activity, and exhibit prolonged elevation of circulating cortisol levels (Abbott et al., 1985; Øverli et al., 1998; Øverli et al., 1999a). This maintained elevation of cortisol concentrations in subordinate fish is indicative of chronic stress, and is accompanied by downstream effects (Gilmour et al., 2005) including reduced growth rates (Abbott and Dill, 1989; Winberg and Nilsson, 1993; DiBattista et al., 2006), immunosuppression (Peters et al., 1988), and an attenuation of the cortisol response to an acute stressor (Jeffrey et al., 2014; Culbert and Gilmour, 2016). Interestingly, separation of a subordinate fish from the dominant conspecific with which it was paired allowed cortisol levels in the subordinate fish to fall within 48 h to values that were comparable to those of dominant or sham (fish handled as paired fish were handled, but housed individually) fish, and under these conditions, the cortisol response to an acute stressor was re-established (Culbert and Gilmour, 2016). These observations suggest a role for negative feedback in the regulation of stress axis activity in subordinate fish experiencing chronic social stress.

Negative feedback could act at all levels of the HPI axis. Activation of the HPI axis stimulates the release of CRF produced by neurons of the POA onto corticotropes of the anterior pituitary, in turn stimulating ACTH release (Bernier et al., 2009). The binding of ACTH to MC2R on interrenal cell membranes activates a cAMP-signalling cascade that stimulates StAR

to facilitate the movement of cholesterol to the inner mitochondrial membrane (Stocco, 2000; Sewer and Waterman, 2003; Payne and Hales, 2004; Aluru and Vijayan, 2008) and P450_{scc} to catalyze the conversion of cholesterol to pregnenolone (Stocco, 2000); these processes are the rate-limiting steps for cortisol production (Sewer and Waterman, 2003). For negative feedback to act on this stress axis, CR, which are ligand-dependent transcription factors and include GR and MR (Mommensen et al., 1999; Prunet et al., 2006), should be present within stress axis tissues. Two GR paralogs, GR1 and GR2, are present in most teleost fish, including rainbow trout (Bury et al., 2003; Prunet et al., 2006; Schaaf et al., 2008). In rainbow trout, GR mRNA and/or protein have been detected in the POA, pituitary and head kidney (Teitsma et al., 1998; Alderman et al., 2012; Teles et al., 2013b), and MR in brain and head kidney (Sturm et al., 2005; Teles et al., 2013b). In addition, regulation of cellular cortisol levels is influenced by the enzyme 11 β HSD2, which converts cortisol to its inactive form cortisone (Edwards et al., 1988). The expression of 11 β HSD2 mRNA has been detected in rainbow trout brain, pituitary and head kidney (Kusakabe et al., 2003).

The presence of CR in tissues of the stress axis supports the possibility of negative feedback regulation, but relatively few studies to date have investigated negative feedback at the head kidney level. Using an *in vitro* preparation of head kidney tissue from coho salmon, evidence that elevated cortisol levels suppressed cortisol production directly has been reported, i.e. ultra-short-loop negative feedback (Bradford et al., 1992). Evidence for negative feedback actions on the head kidney of trout is mixed and largely indirect. Treatment of rainbow trout with cortisol failed to yield changes in head kidney MC2R, StAR or P450_{scc} mRNA abundance (Jeffrey et al., 2012). However, evidence of negative feedback action on cortisol biosynthesis was obtained for rainbow trout subjected to chronic social stress. Although circulating cortisol

concentrations are elevated in subordinate fish, cortisol synthesis at the level of the interrenal cells appears to be attenuated by low social status. For instance, *in vitro* head kidney preparations or head kidney tissue perfused *in situ* have shown that cortisol production following ACTH stimulation is reduced in subordinate rainbow trout compared to dominant fish (Sloman et al., 2002; Jeffrey et al., 2014). Head kidney MC2R mRNA relative levels were unaffected by social status, whereas StAR and P450scc mRNA relative abundances were higher in dominant than subordinate fish (Jeffrey et al., 2012). Furthermore, even though cortisol synthesis by head kidney preparations from subordinate trout was lower than that of preparations from dominant trout, social status did not affect the increases in mRNA relative abundances of MC2R, StAR and P450scc that occurred in response to an acute netting stressor (Jeffrey et al., 2014).

Given this background, the present study aimed to examine the effects of social status on cortisol production at the head kidney level. The hypothesis that chronic social stress regulates the expression of key proteins involved in cortisol biosynthesis as well as CR, resulting in a reduced capacity for cortisol production in fish experiencing chronic social stress, was tested in several ways. Cortisol production was assessed for head kidney preparations exposed to cortisol *in vivo* (by cortisol treatment of the fish) or *in vitro*. In addition, the head kidney of subordinate fish was examined. Under the hypothesis tested, GR1 and GR2 mRNA abundance would be predicted to decrease in the head kidney of subordinate fish. Similarly, the cAMP increase elicited by ACTH acting on MC2R would be predicted to be smaller in subordinate fish, as would db-cAMP-stimulated cortisol synthesis. Finally, 11 β HSD2 mRNA abundance would be predicted to increase in the head kidney of subordinate fish, to compensate for the increase in cortisol concentrations.

3.2 Materials and methods

3.2.1 Experimental animals

Female juvenile rainbow trout (122 ± 4 g, $N = 112$) that were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario) were held in the University of Ottawa Aquatic Facility in 1275 L fibreglass tanks. The fish were held in flowing and aerated dechloraminated tap water from the city of Ottawa. The temperature of the water was 13°C and a 12h:12h light:dark photoperiod was used. Fish were fed to satiation with commercial fish pellets every two days. Experiments were not carried out until at least two weeks after the arrival of new fish to let them acclimate to these conditions. All experimental protocols were approved by an institutional animal care committee (BL-2118) and were in agreement with the guidelines of the CCAC for the use of animals in research and teaching.

Social pairing experiments were conducted using procedures similar to those described by DiBattista et al. (2005, 2006) and Jeffrey et al. (2012). In brief, immersion in a solution of benzocaine (0.1 mg L^{-1} ethyl-4-aminobenzoate; Sigma Aldrich) was used to lightly anaesthetize juvenile rainbow trout chosen at random from holding tanks (103 ± 3 g, $N = 63$) and weight, fork length and fin damage were assessed (day 0). The right or left pectoral fin was clipped to recognize individual fish within a pair. A fish was paired with a conspecific of similar length (mean $\Delta\text{fork-length} = 0.4 \pm 0.1$ cm) and weight (mean $\Delta\text{mass} = 5.5 \pm 0.9$ g), and the individuals were placed on either side of an opaque, perforated divider in a 40 L flow-through observation tank to allow the fish to recover overnight. The next morning, the divider was removed (day 1) to let the fish within a pair interact for a period of 4 days. Fish were observed twice daily for 5 min each time, the first observation being in the morning and the second, at least 4 h later in the

afternoon. Fish were scored according to the number of acts of aggression, their position within the tank, and feeding behaviour during the observation period (Table 3.1). Fin damage was assessed after the fish were euthanized on the morning of day 5 (see below) and was compared to the fin damage observed on day 0 (Table 3.1). To calculate overall behaviour scores from the observations for individual fish, a principal component analysis (PCA) was used (Minitab v 16). Within a pair, the fish with the higher behaviour score was assigned dominant social status, whereas the fish with the lower behaviour score was assigned subordinate social status. Sham-treated fish served as a control and these fish were handled in the same way as paired fish but were housed individually.

At the end of the 4 day interaction period, fish were terminally anaesthetized with an overdose of benzocaine (0.5 mg L^{-1} ethyl-4-aminobenzoate; Sigma Aldrich). Blood samples were collected by caudal venipuncture using heparin-coated syringes (2500 IU mL^{-1} heparin, Sigma-Aldrich) and were centrifuged ($10,000 g$ for 2 min) to isolate plasma for analysis of cortisol concentrations. Cortisol concentrations were measured using a commercially available RIA according to the manufacturer's protocol. This RIA has been validated previously for rainbow trout plasma samples (Gamperl et al., 1994; Jeffrey et al., 2012). The intra-assay coefficient of variation was 8.1%, and the inter-assay coefficient of variation was 21.4%.

Table 3.1 Point system used to score observations of behaviour within paired rainbow trout (*Oncorhynchus mykiss*) to determine dominant and subordinate social status within the pair.

Behaviour	Description	Points awarded
Acts of aggression	Number of attacks initiated	>5 acts = 2 points 1-4 acts = 1 point No acts = 0 points
Position	Position in the tank	Patrolling = 10 points Bottom/Shelter = 5 points Top/Skulking = 0 points
Feeding	Fish who gets to the first pellet	Feeds = 1 point Does not feed = 0 points
Fin damage	Fin damage compared between day 0 and day 5	0% (healthy) = 3 points <30% = 2 points 30-70% = 1 point 70-100% = 0 points

Fin damage represents the % of fin damage and/or fin loss.

3.2.2 Experiment 1: Does chronic social stress impact ACTH-stimulated cAMP production in the head kidney of rainbow trout?

To investigate whether chronic social stress impacts the activity of MC2R in interrenal cells, an *in vitro* head kidney preparation was used to measure cAMP production in response to ACTH. At the end of the interaction period (see above), fish were euthanized and head kidney tissue was collected for *in vitro* preparations from dominant ($N = 5$), subordinate ($N = 6$) and sham ($N = 6$) fish. The *in vitro* head kidney preparations were carried out as described in section 2.2.4 with the exception that preparations were incubated with either fresh medium (blank; negative control) or 1 IU mL⁻¹ of porcine ACTH (Sigma Aldrich) for 5 or 10 min, respectively. The incubation times were chosen on the basis of pilot trials in which cAMP production was assessed at 5, 10 and 15 min. After incubation, tissue suspensions were centrifuged at 14,000 *g* for 2 min and the supernatant was collected, flash frozen in liquid N₂ and stored at -80°C for later analysis of cAMP concentrations using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (cAMP ELISA kit; Cayman Chemical, Ann Arbor, MI, USA).

3.2.3 Experiment 2: Does chronic social stress impact db-cAMP-stimulated cortisol production in the head kidney of rainbow trout?

To investigate whether chronic social stress impacts cortisol production in response to cAMP in interrenal cells, an *in vitro* head kidney preparation was used to measure cortisol production in response to the cell-permeable cAMP analogue db-cAMP (Sigma Aldrich). At the end of the interaction period (see above), fish were euthanized and head kidney tissue was collected for *in vitro* preparations from dominant ($N = 8$), subordinate ($N = 8$) and sham ($N = 6$)

fish. The *in vitro* head kidney preparations were carried out as described in section 2.2.4 with the exception that preparations were incubated with either fresh medium (blank; negative control) or 1 mmol L⁻¹ db-cAMP at 13°C with shaking for 2 h. The concentration of db-cAMP was chosen on the basis of values used in published studies (e.g. Patino et al., 1986) and pilot trials carried out using 0.5 to 2 mmol L⁻¹ db-cAMP. After incubation, tissue suspensions were centrifuged at 14,000 *g* for 10 min. Supernatants were collected, flash frozen in liquid N₂ and stored at -80°C until used for analysis of cortisol concentrations by RIA as previously described.

3.2.4 Experiment 3: Does chronic social stress impact GR1, GR2 and 11βHSD2 transcript abundance in the head kidney of rainbow trout?

To investigate the impact of chronic social stress on GR1, GR2 and 11βHSD2 expression, semi-quantitative real-time RT-PCR (qPCR) was carried out on the head kidney tissue of dominant (*N* = 8), subordinate (*N* = 7-8) and sham (*N* = 6-7) trout. At the end of the interaction period, fish were terminally anaesthetized as described above and the head kidney was collected on ice, flash-frozen in liquid N₂ and stored at -80°C for later analysis of mRNA abundance. The extraction of RNA was carried out using TRIzol reagent according to the manufacturer's instructions. Frozen head kidney tissue (40-60 mg) was homogenized by passing a solution of tissue and TRIzol through 18G and 23G needles with a 3 mL syringe until the solution passed easily through the smaller needle. The resultant RNA was quantified using a Nanodrop® 2000 Spectrophotometer (ThermoFisher Scientific). To synthesize cDNA, a QuantiTect® Reverse Transcription kit (Qiagen) was used with 0.5 µg of RNA according to the manufacturer's protocol, with the exception that half reaction volumes were used (10 µL). To

check for genomic DNA contamination, no RT control samples were generated in the same fashion but RT was omitted from the reaction.

Relative mRNA levels of GR1, GR2 and 11 β HSD2 were determined by qPCR using gene-specific primers (Table 3.2) and the ribosomal subunit 18S as a reference gene. Primers for 11 β HSD2 were developed using Primer-BLAST (NCBI) and primer quality (i.e. presence of hairpin structures, unwanted dimerization, etc.) was assessed using OligoAnalyzer 3.1 (IDT). Primer specificity was checked by sequencing the product at the McGill University and G  nome Qu  bec Innovation Centre. Standard curves were developed for each primer set using serial dilution of cDNA from the head kidney of a representative fish. To ensure the absence of genomic DNA contamination, no RT samples were included in all analyses. No template negative controls, in which water rather than cDNA was used as the template, also were included in all analyses. Reactions were carried out using a Rotor-Gene SYBR Green PCR Kit (Qiagen) and a Rotor-Gene Q System (Qiagen) with the following cycling conditions: 95  C (5 s) and 60  C (10 s) (annealing temperature) for 40 cycles. For all reactions, the manufacturer's instructions were followed with the exception that reaction volumes were scaled to 10 μ L; 5 μ L of 2X SYBR mix (Qiagen), 1 μ L (for 18S primers, the template was diluted 1000x) or 0.5 μ L (for GR1, GR2 and 11 β HSD2 primers) of template, 1 μ L (for 18S primers), 0.2 μ L (for GR1 and 11 β HSD2 primers) or 0.1 μ L (for GR2 primers) of combined forward and reverse primers (primer stock 10 μ mol L⁻¹) and 3 μ L (for 18S primers), 4.3 μ L (for GR1 and 11 β HSD2 primers) or 4.4 μ L (for GR2 primers) of water from the kit. Comparisons among social status categories (sham, dominant and subordinate) were made by expressing the mRNA abundance of each gene relative to that of the sham group using the modified delta-delta Ct method (Pfaffl, 2001) with 18S as a normalizing gene.

Table 3.2 Oligonucleotide primer pairs used for identification of GR1, GR2 and 11 β HSD2 by qPCR in rainbow trout (*Oncorhynchus mykiss*).

Gene	Primers	Product length (base pairs)	Accession No.	Source	Efficiency (%)
18S	F GGCGGCGTTATTCCCATGACC R GGTGGTGCCCTTCCGTCAATTC	115	AF309412.1	Jeffrey et al, 2012	92
GR1	F TTCCAAGTCCACCACATCAA R GGAGAGCTCCATCTGAGTCG	115	NM_001124730.1	Ings et al, 2011	105
GR2	F GGGGTGATCAAACAGGAGAA R CTCACCCACAGATGGAGAT	140	NM_001124482.1	Ings et al, 2011	106
11βHSD2	F AGGGCAAGGCTGTCTTCATC R CCTCCCCACACAGATCCAAC	117	NM_001124218.1		98

Primer sequences are given in the 5' to 3' direction. *GR1*, glucocorticoid receptor type 1, *GR2*, glucocorticoid receptor type 2, *11 β HSD2*, 11 β -hydroxysteroid dehydrogenase type 2.

3.2.5 Experiment 4: Does cortisol inhibit ACTH-stimulated cortisol production in the head kidney of rainbow trout?

To investigate the possibility that cortisol inhibits cortisol biosynthesis in interrenal cells via an ultra-short negative feedback loop, an *in vitro* head kidney preparation was used to measure cortisol production in the presence of cortisol. The *in vitro* head kidney preparations were carried out essentially as described in section 2.2.4 with the exception that the incubation conditions were varied as described below. Three different protocols were adopted. In the first protocol, half of the head kidney tissue was incubated with fresh medium (control) while the other half was incubated with medium containing 100 ng mL⁻¹ of cortisol (hydrocortisone 21-hemisuccinate, Sigma Aldrich). This cortisol concentration was chosen to mimic the circulating cortisol level typical of subordinate trout (e.g. Jeffrey et al., 2012). Incubation was carried out on a shaker at 13°C for 1 (*N* = 14), 3 (*N* = 8) or 7 (*N* = 8) h. Following the incubation period, the head kidney tissue was distributed into the wells of a microplate, the medium (control or cortisol-treated) was refreshed, and 1 IU mL⁻¹ porcine ACTH (Sigma Aldrich) was added to half of the wells for each treatment for a 1 h incubation period on a shaker at 13°C. After incubation, tissue suspensions were centrifuged at 14,000 *g* for 10 min. Supernatants were collected, flash frozen in liquid N₂ and stored at -80°C until analysis of cortisol concentrations by RIA. The second protocol also used head kidney tissue incubated with either control medium or medium containing 100 ng mL⁻¹ of cortisol for 7 h (*N* = 6). The procedure was the same as that described above except that the medium was refreshed every 2 h and cortisol was omitted from all solutions during the final 1 h incubation period.

The third protocol focused on the effects of cortisol treatment *in vivo* instead of *in vitro*. Rainbow trout (*N* = 8) were anaesthetized in a solution of benzocaine (0.1 mg L⁻¹) and given an

intraperitoneal injection of 10-20 mg kg⁻¹ body mass of cortisol (hydrocortisone 21-hemisuccinate, Sigma Aldrich) emulsified in 5 mL kg⁻¹ body mass of melted cocoa butter (NOW, Herb n' Spice Shop, Ottawa, ON). Fish were then held in individual, opaque experimental chambers supplied with flowing, aerated, dechloraminated city of Ottawa tap water at 13°C for 15 h. This dose of cortisol was chosen based on pilot experiments in which it yielded a circulating cortisol concentration of ~100 ng mL⁻¹, i.e. comparable to that of subordinate fish (e.g. Jeffrey et al., 2012). Fish sampled directly from the holding tank ($N = 5$) were used as the control group for this experiment. All fish were terminally anaesthetized as described above and a blood sample was collected by caudal venipuncture using a heparin-coated syringe (2500 IU mL⁻¹ heparin, Sigma-Aldrich). Blood samples were centrifuged (10,000 g for 2 min) to isolate plasma that was flash frozen in liquid N₂ and stored at -80°C for later analysis of circulating cortisol concentrations by RIA. The head kidney was collected for *in vitro* head kidney preparations. Head kidney preparations were incubated with fresh medium (blank) or 1 IU mL⁻¹ porcine ACTH (Sigma Aldrich) at 13°C on a shaker for 1 h. After incubation, tissue suspensions were centrifuged at 14,000 g for 10 min and supernatants were used for analysis of cortisol concentrations by RIA.

3.2.6 Statistical analyses

SigmaPlot v. 11.0 (Systat Software) was used to perform statistical analyses. Data are presented as mean values $\pm$ SEM. For all analyses, the level of statistical significance (α) was set to 0.05. Because rates of cortisol production by *in vitro* head kidney preparations can be variable, data for ACTH- or db-cAMP-stimulated preparations were expressed relative to the blank value for the same preparation (e.g. Benguira and Hontela, 2000; Aluru and Vijayan, 2004). One-way

ANOVA was used to assess the statistical significance of differences among dominant, subordinate and sham fish. A Holm-Sidak *post hoc* test for multiple comparisons was used as appropriate to identify the source of statistically-significant differences among treatment groups. Comparisons between control and cortisol-treated fish or *in vitro* head kidney preparations were carried out using Student's *t*-tests. For all analyses, data that did not meet the underlying assumptions of normality and equal variance were transformed to meet these assumptions, or if the assumptions could not be met through transformation, equivalent, non-parametric tests were used.

3.3 Results

3.3.1 Effects of chronic social stress on the head kidney (Experiments 1-3)

Rainbow trout confined in pairs for 4 days formed social hierarchies on the basis of mean behaviour scores, which were higher for dominant than subordinate fish (dominant 1.18 ± 0.16 , subordinate -1.18 ± 0.20 , $N = 22$ pairs), and elevated plasma cortisol concentrations in subordinate compared to dominant fish (dominant $5.4 \pm 1.9 \text{ ng mL}^{-1}$, subordinate $60.1 \pm 12.7 \text{ ng mL}^{-1}$, $N = 22$ pairs) (Table 3.3). To investigate how chronic social stress (i.e. subordinate social status) results in a reduced capacity for cortisol production (Sloman et al., 2002; Jeffrey et al., 2014), *in vitro* head kidney preparations were carried out using dominant, subordinate and sham fish. Incubation with ACTH resulted in cAMP levels that were significantly higher than the baseline value (paired Student's *t*-test on log-transformed data, $p < 0.001$) but no significant differences were detected among dominant, subordinate and sham fish in ACTH-stimulated

cAMP production (Fig. 3.1; ANOVA, $p = 0.295$). Similarly, no significant differences were detected among dominant, subordinate and sham fish in terms of cortisol production stimulated by incubation of head kidney preparations with 1 mmol L^{-1} db-cAMP (Fig. 3.2; ANOVA on log-transformed data, $p = 0.070$). However, a trend for db-cAMP-stimulated cortisol production to be significantly higher in dominant fish was detected. In addition, qPCR was used to determine whether head kidney GR1, GR2 and $11\beta\text{HSD2}$ relative mRNA levels differed among dominant, subordinate and sham-treated trout. No significant differences were detected for GR1 (Fig. 3.3A; ANOVA, $p = 0.718$), GR2 (Fig. 3.3B; ANOVA, $p = 0.069$) or $11\beta\text{HSD2}$ (Fig. 3.3C; ANOVA on log-transformed data, $p = 0.220$). However, a trend for transcript abundance for GR2 to be significantly higher in dominant fish was detected.

3.3.2 Does cortisol inhibit ACTH-stimulated cortisol production in the head kidney of rainbow trout (Experiment 4)?

One possible explanation for the effect of chronic social stress in lowering ACTH-stimulated cortisol production by the head kidney *in vitro* (Sloman et al., 2002; Jeffrey et al., 2014) is an ultra-short negative feedback mechanism in which cortisol inhibits cortisol production at the level of the head kidney as demonstrated for coho salmon (Bradford et al., 1992). To test this possibility in rainbow trout, cortisol production was assessed for *in vitro* head kidney preparations that were exposed to cortisol *in vitro*, or for preparations from trout that were exposed to cortisol *in vivo*. Head kidney tissue that was incubated with ACTH had significantly higher rates of cortisol production ($962 \pm 162 \text{ ng mL}^{-1}$ for $N = 41$ control preparations used in Experiment 4) than head kidney tissue that was incubated without ACTH (blank) ($171 \pm 20 \text{ ng mL}^{-1}$; Signed Rank *t*-test, $p < 0.001$). However, high variability in rates of

ACTH-stimulated cortisol production (note the SEM above), might have masked treatment effects and therefore the data were expressed as fold increases in cortisol production over the blank value.

Head kidney tissue that was incubated *in vitro* with cortisol for 1, 3 or 7 h and then stimulated with ACTH in the presence of cortisol showed significantly lower cortisol production than corresponding preparations incubated without cortisol (Fig. 3.4A, paired Student's *t*-test on log-transformed data, $p < 0.001$, B and C, paired Student's *t*-tests, $p = 0.017$ and 0.007 , respectively). However, when the head kidney was incubated *in vitro* with cortisol for 7 h and then treated with ACTH for the last hour in the absence of cortisol, no significant difference was detected (Fig. 3.5; paired Student's *t*-test, $p = 0.504$).

Trout that received cortisol *in vivo* exhibited significantly higher plasma cortisol levels ($69.4 \pm 9.6 \text{ ng mL}^{-1}$) than the control group ($1.1 \pm 0.2 \text{ ng mL}^{-1}$; Student's *t*-test, $p < 0.001$), but no significant difference in cortisol production by head kidney preparations *in vitro* was detected (Fig. 3.6; Student's *t*-test on log-transformed data, $p = 0.633$).

Table 3.3 Behaviour scores and plasma cortisol concentrations for dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) used in Experiments 1, 2 and 3.

Experiment	Plasma [cortisol] (ng mL ⁻¹)			P value	Behaviour score	
	Dominant	Subordinate	Sham		Dominant	Subordinate
Expt 1: ACTH-stimulated cAMP production	3.4 ± 2.6 ^b	53.5 ± 23.9 ^a	1.4 ± 0.4 ^b	0.001	1.25 ± 0.57	-1.25 ± 0.32
Expt 2: db-cAMP-stimulated cortisol production	9.0 ± 4.6	15.2 ± 6.9	24.3 ± 18.7	0.68	0.85 ± 0.05	-0.85 ± 0.44
Expt 3: GR1, GR2, 11βHSD2 transcript abundance	3.3 ± 1.2 ^b	109.3 ± 17.8 ^a	1.5 ± 0.3 ^b	<0.001	1.46 ± 0.14	-1.46 ± 0.26

Values are means ± SEM; *N* = 5-6 in Experiment #1, *N* = 4-8 in Experiment #2 and *N* = 7-8 in Experiment #3. The p value refers to the analysis of plasma cortisol concentrations only. Plasma cortisol concentrations that share a letter are not significantly different from one another (ANOVA on log-transformed data, significant p values indicated by bold font). Behaviour scores were generated using PCA (see text for details). *cAMP*, cyclic adenosine monophosphate, *ACTH*, adrenocorticotrophic hormone, *db-cAMP*, dibutyl cAMP, cyclic adenosine monophosphate, *GR1*, glucocorticoid receptor type 1 *GR2*, glucocorticoid receptor type 2, *11βHSD2*, 11-β-hydroxysteroid dehydrogenase type 2.

Figure 3.1. Mean ACTH-stimulated cAMP production for *in vitro* head kidney preparations of dominant ($N = 5$), subordinate ($N = 6$) and sham ($N = 6$) rainbow trout (*Oncorhynchus mykiss*). The production of cAMP in the presence of ACTH is presented as the percentage increase over cAMP production by unstimulated tissue (blank). Values are means + SEM. No statistically significant differences were detected (ANOVA, $p = 0.295$).

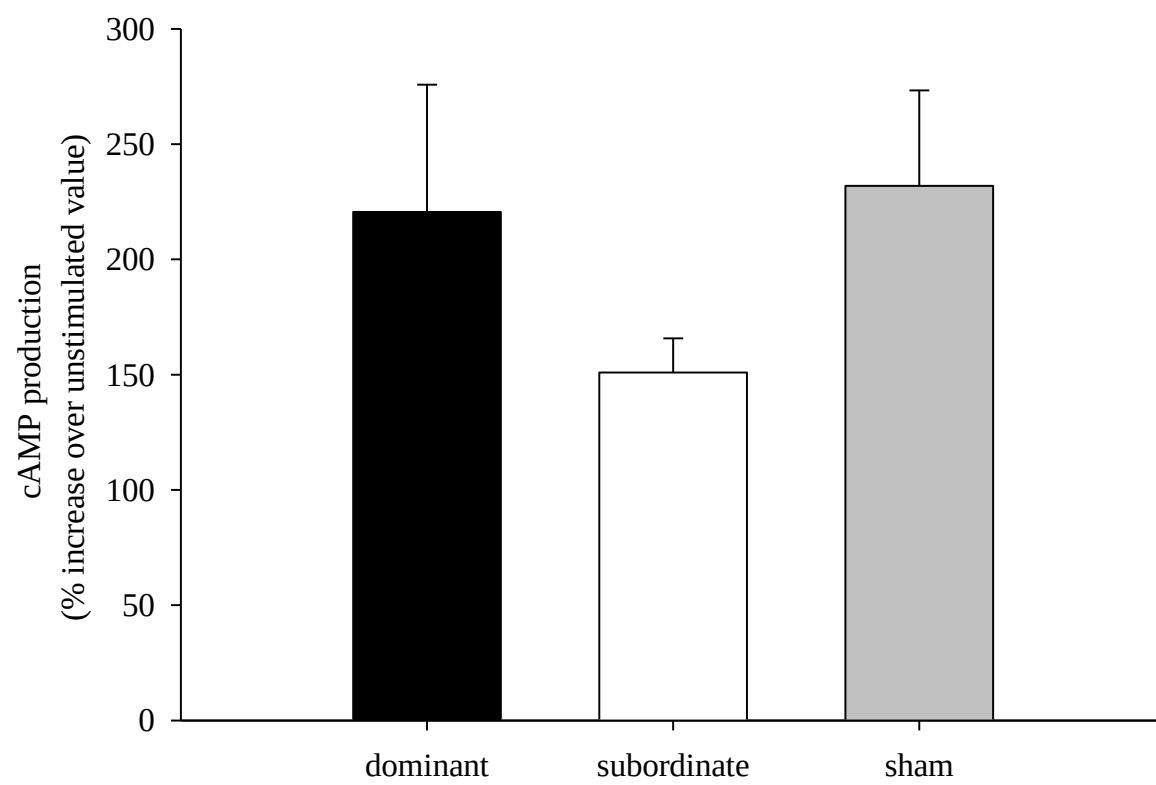


Figure 3.2. Cortisol production by *in vitro* head kidney preparations of dominant ($N = 8$), subordinate ($N = 8$) and sham ($N = 6$) rainbow trout (*Oncorhynchus mykiss*) stimulated with 1 mmol L⁻¹ db-cAMP. Cortisol production in the presence of db-cAMP is presented as a fold increase over the value for unstimulated tissue (blank). Values are means + SEM. No statistically significant differences were detected (ANOVA on log-transformed data, $p = 0.070$).

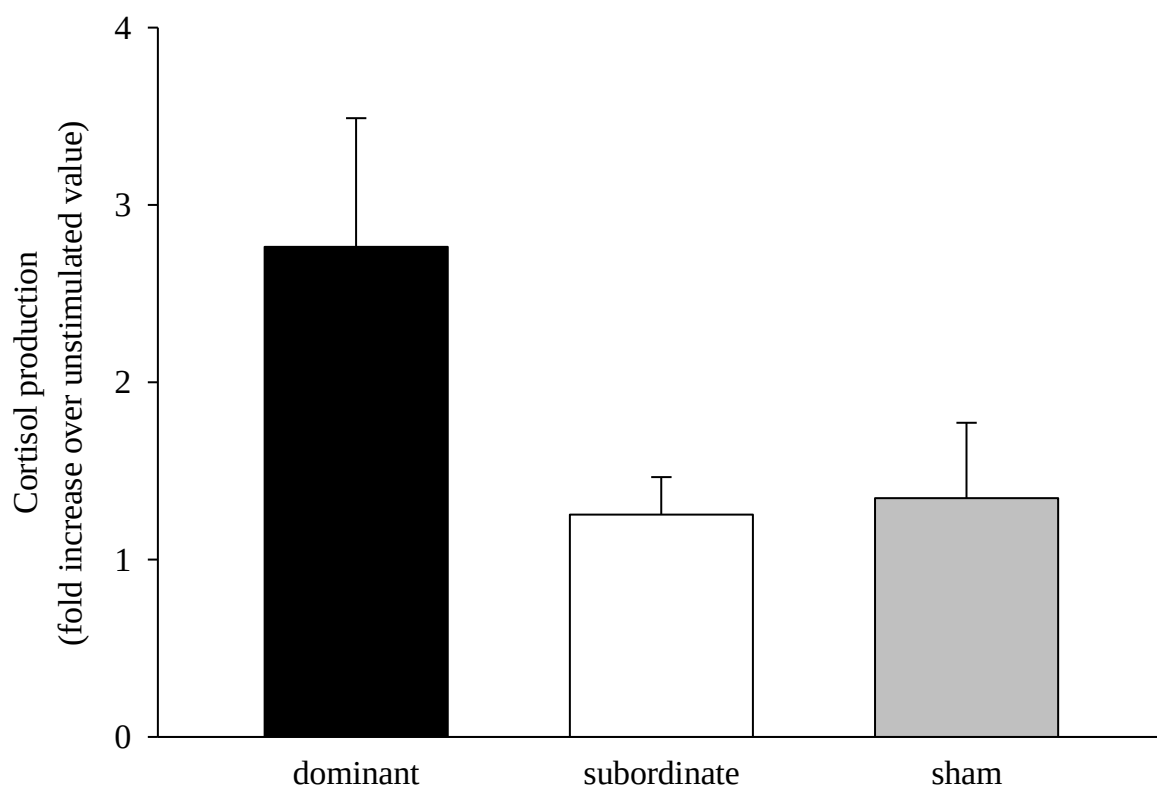


Figure 3.3. Relative mRNA abundance of GR1 (A), GR2 (B) and 11 β HSD2 (C) in the head kidney of dominant ($N = 8$), subordinate ($N = 7-8$) and sham ($N = 6-7$) trout (*Oncorhynchus mykiss*). All data are expressed relative to the sham group, and normalized to the mRNA abundance of 18S. Values are means + SEM. No statistically significant differences were detected (ANOVA, $p = 0.718$, $p = 0.069$, and ANOVA on log-transformed data, $p = 0.220$ for panels A, B and C, respectively).

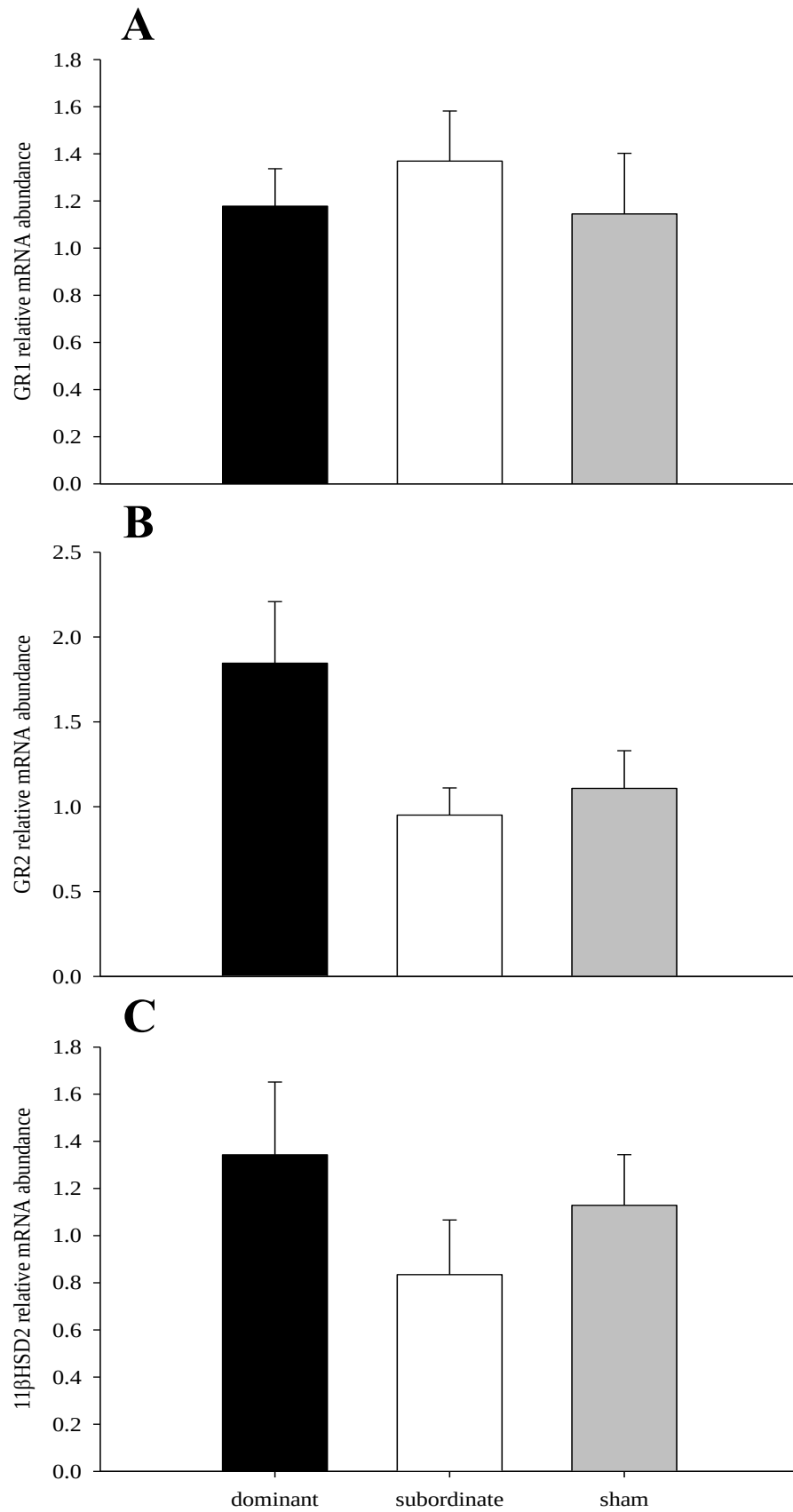


Figure 3.4. Cortisol production by *in vitro* head kidney preparations of rainbow trout (*Oncorhynchus mykiss*) incubated with or without cortisol for 2 (A), 4 (B) or 8 h (C), with cortisol production being measured during the final hour of the incubation period. Cortisol production stimulated by ACTH is expressed as the percent fold increase over cortisol production by unstimulated tissue. Values are means + SEM, with $N = 14$ (A) or 8 (B and C). An asterisk indicates a significant effect of incubation in the presence of cortisol (paired Student's *t*-test on log-transformed data, $p < 0.001$ for panel A; paired Student's *t*-tests, $p = 0.017$ and 0.007 for panels B and C, respectively).

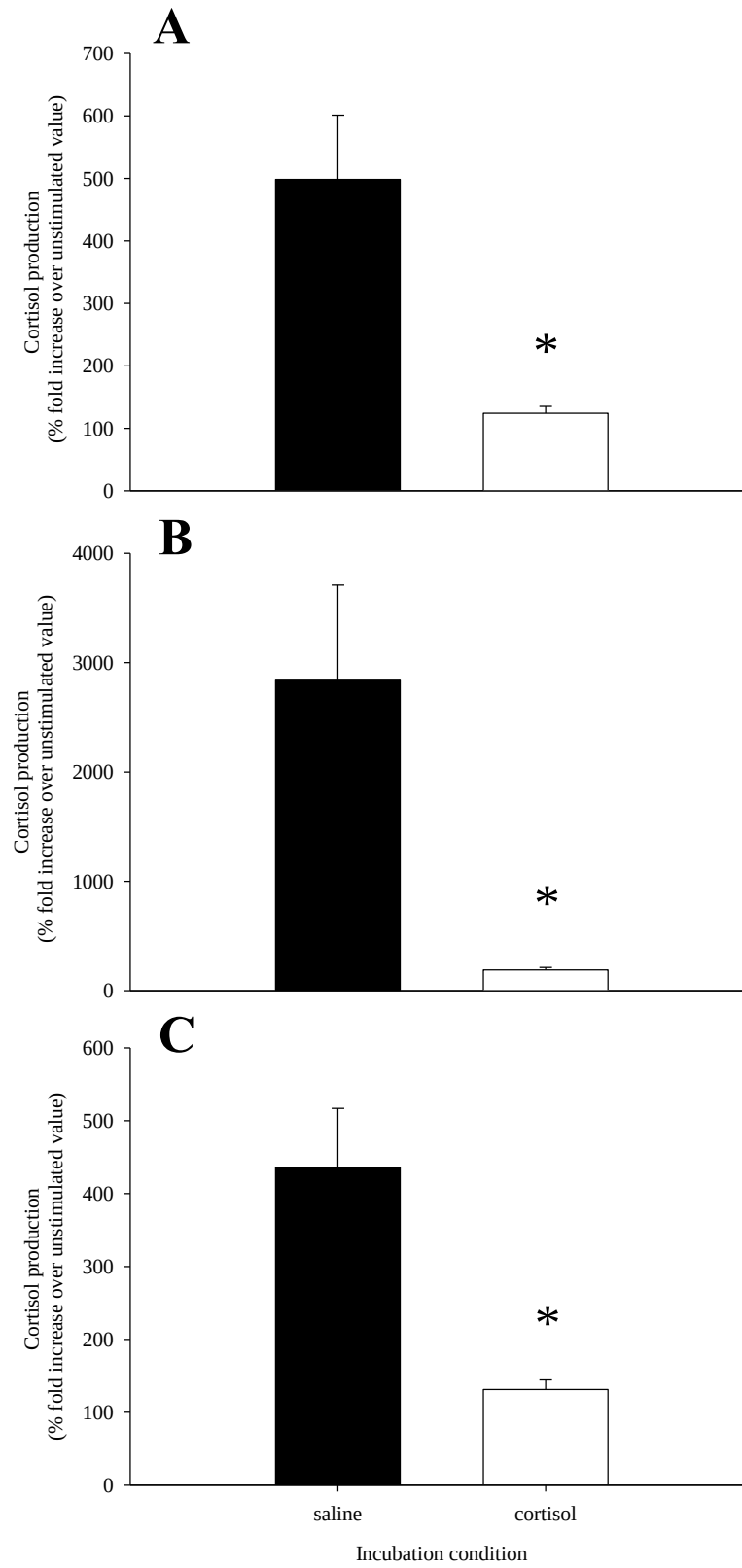


Figure 3.5. Cortisol production by rainbow trout (*Oncorhynchus mykiss*) *in vitro* head kidney preparations that were incubated with cortisol for 7 h but stimulated with ACTH (for 1 h) in the absence of exogenous cortisol. Cortisol production in the presence of ACTH is presented as the percent fold increase over cortisol production by unstimulated tissue (blank). Values are means + SEM, $N = 6$. No significant treatment effects were detected (paired Student's t -test, $p = 0.504$).

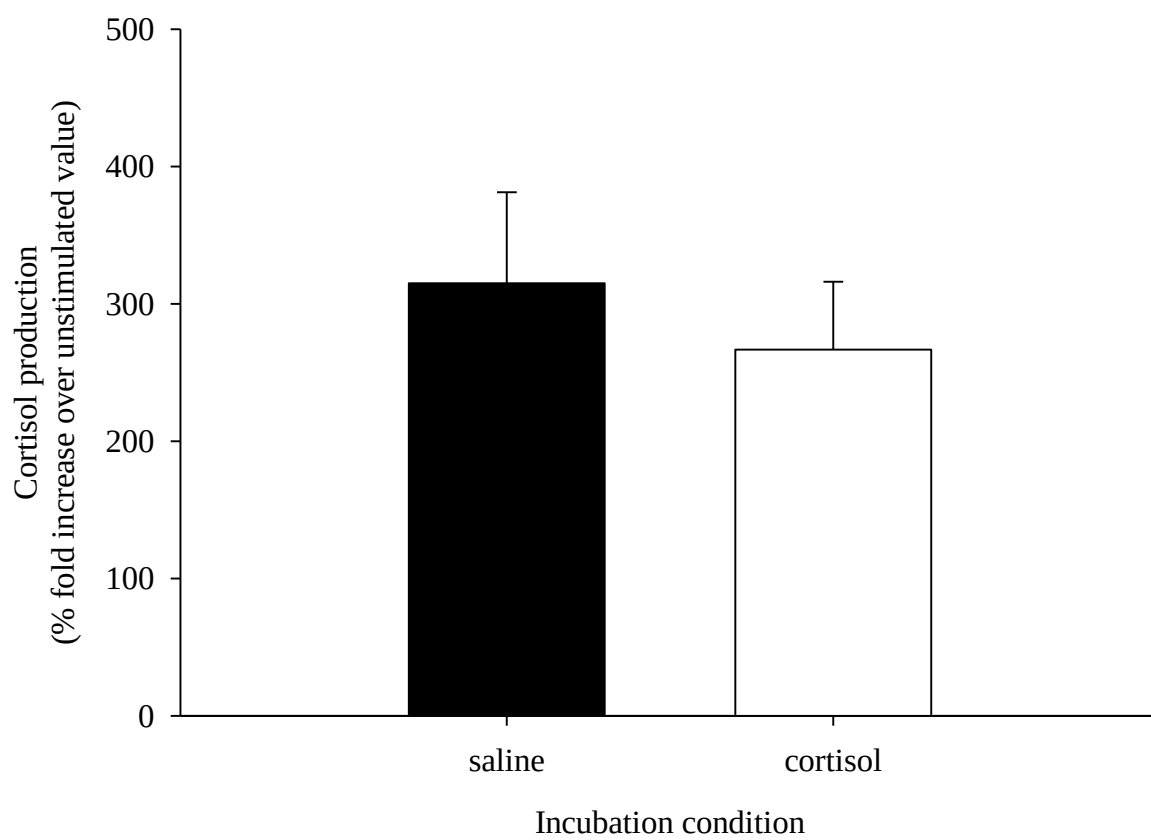
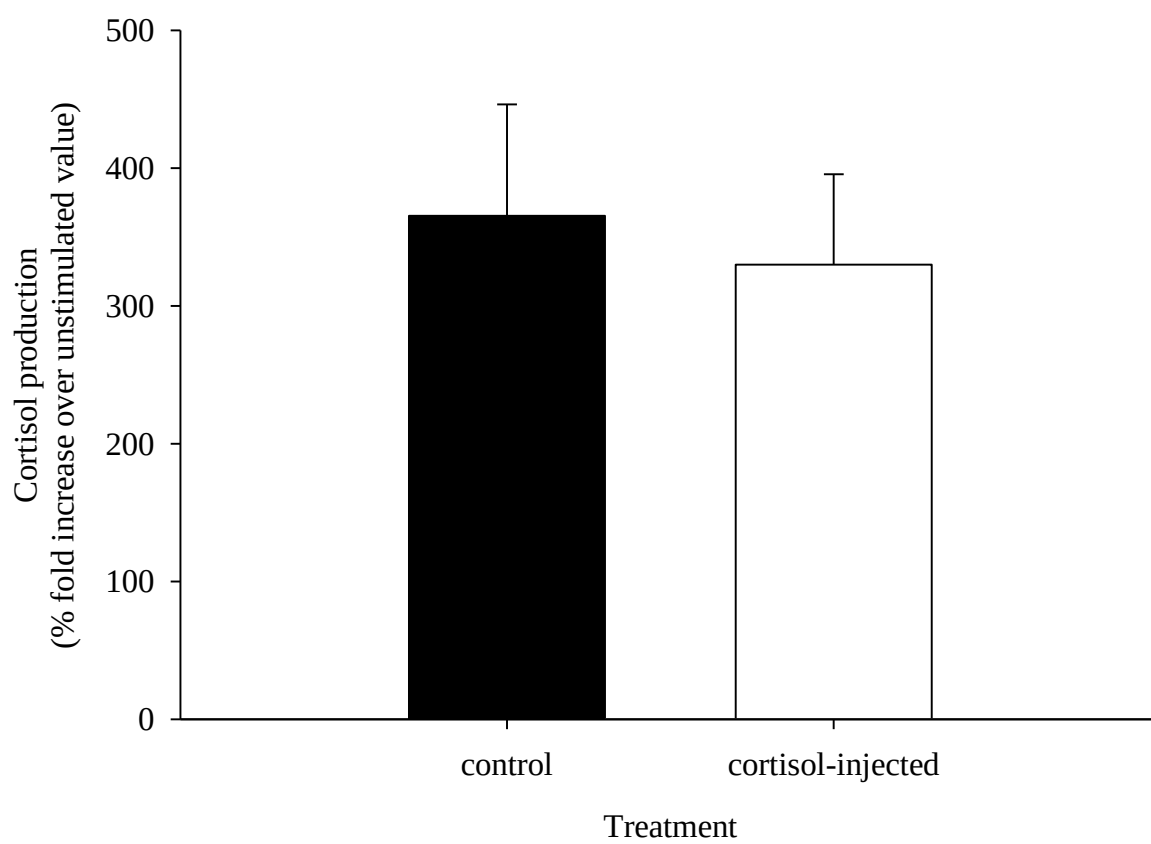


Figure 3.6. Cortisol production by rainbow trout (*Oncorhynchus mykiss*) *in vitro* head kidney preparations that were treated with cortisol *in vivo*. Cortisol production in the presence of ACTH is presented as the percent fold increase over cortisol production by unstimulated tissue (blank). Values are means + SEM, $N = 5$ control or 8 cortisol-treated fish. No significant differences were detected (Student's *t*-test on log-transformed data, $p = 0.633$).



3.4 Discussion

The goal of this study was to determine the effects of social status on cortisol production in the head kidney of rainbow trout. We hypothesized that chronic social stress modulates the expression of key proteins involved in cortisol production as well as CR, resulting in a reduced capacity for cortisol production. However, the absence of differences among dominant, subordinate, and sham fish in ACTH-stimulated cAMP production or db-cAMP-stimulated cortisol production by *in vitro* head kidney preparations provided little support for this hypothesis. Similarly, no differences in CR transcript abundance or transcript abundance of 11 β HSD2 were detected as a function of social status. The high cortisol levels experienced by subordinate fish might also reduce interrenal cell cortisol production through negative feedback, a hypothesis that was tested in the present study using *in vitro* head kidney preparations that were incubated in the presence of cortisol. Head kidney tissue incubated with cortisol for 2, 4 or 8 h showed a reduction in cortisol production compared to control preparations, providing evidence in support of an ultra-short-loop negative feedback mechanism operating at the level of the interrenal cells. The cellular mechanisms through which this negative feedback mechanism operates, however, remain to be determined.

3.4.1 Does chronic social stress disrupt the cortisol synthesis pathway?

Rainbow trout paired for 4 days formed strong social hierarchies as indicated by highly polarized behaviour scores in all experiments and elevated plasma cortisol concentrations in the subordinate fish of experiments 1 and 3. Subordinate fish of experiment 2 did not exhibit

elevated circulating cortisol concentrations which is somewhat unusual, but has been reported occasionally in previous studies (e.g. LeBlanc et al., 2011). The presence of a strong hierarchy results in subordinate fish experiencing chronic stress (Gilmour et al., 2005). Sloman et al. (2002) and Jeffrey et al. (2014) demonstrated that even though subordinate trout exhibit elevated plasma cortisol levels, cortisol production when the head kidney is stimulated *in vitro* with ACTH is lower for head kidney preparations from subordinate trout compared to dominant trout. At the whole-animal level, this lowering of cortisol production results in an attenuated cortisol response to an acute stressor (Jeffrey et al., 2014; Culbert and Gilmour, 2016). One objective of the present study was to investigate possible explanations for the attenuated cortisol production of subordinate fish.

Disruption of the cortisol synthesis pathway in interrenal cells of subordinate fish could account for lower cortisol production. Cortisol biosynthesis is initiated when circulating ACTH binds to MC2R on the plasma membrane of interrenal cells, activating a cAMP cascade (Sewer and Waterman, 2003; Aluru and Vijayan, 2008). Jeffrey et al. (2012) did not find a difference at the mRNA levels for MC2R between dominant and subordinate trout, suggesting that MC2R is not transcriptionally regulated by chronic social stress. As a functional test of MC2R abundance and activity, ACTH-stimulated cAMP levels were measured in the present study, with the prediction being that less cAMP would be produced in the head kidney of subordinate trout after activation of MC2R by ACTH. Despite the relatively low abundance of interrenal cells in the head kidney (Hontela et al., 2008), incubation of head kidney preparations with ACTH resulted in significant and detectable cAMP accumulation. However, no differences in ACTH-stimulated cAMP accumulation among dominant, subordinate and sham fish were detected, suggesting that MC2R activity does not change with chronic social stress. Activation of the MC2R and its cAMP

signalling pathway stimulates two key proteins in cortisol synthesis, StAR, which facilitates movement of cholesterol into the mitochondrion, and P450scc, which catalyzes the conversion of cholesterol to pregnenolone in the first and rate-limiting step in cortisol synthesis (Stocco, 2000; Sewer and Waterman, 2003; Payne and Hales, 2004; Aluru and Vijayan, 2008). Reduced abundance and/or activity of one or both of these proteins could account for the limitations on cortisol production in subordinate fish. Jeffrey et al. (2012) found that StAR and P450scc mRNA abundances in the head kidney of subordinate fish were not affected by chronic social stress, suggesting that these proteins are not transcriptionally regulated by chronic social stress. As a functional test of the activity of these proteins and cortisol biosynthesis more broadly, db-cAMP-stimulated cortisol production was measured in the present study using *in vitro* head kidney preparations, with the prediction being that subordinate fish would exhibit lower db-cAMP-stimulated rates of cortisol production. Although no statistically significant differences were detected among dominant, subordinate and sham fish, a trend for dominant fish to exhibit higher rates of cortisol production when head kidney preparations were stimulated with db-cAMP was present. Although this finding requires further investigation, it suggests that cortisol biosynthesis may not be affected by chronic social stress, but rather by the acute stress associated with the attainment of dominant status (see below).

Recent work suggests that cortisol itself may play a role in regulating cortisol biosynthesis through GR signalling (Alderman et al., 2012). In particular, Alderman et al. (2012) reported that decreases in GR protein abundance in the head kidney were accompanied by reductions in StAR transcript abundance and ACTH-stimulated cortisol production *in vitro*. Jeffrey et al. (2012) found that GR2 transcript abundance and GR protein levels were lower in the liver of subordinate relative to dominant and/or control trout, suggesting that down-

regulation of GR might serve as a protective mechanism to reduce stimulation of liver GR by the persistent cortisol elevation of chronic social stress. Evidence from cortisol-treated trout suggests that this down-regulation of GR protein is stimulated by cortisol itself in an auto-regulatory mechanism (Vijayan et al., 2003; Jeffrey et al., 2012). If the same auto-regulation occurs at the source of cortisol production, the head kidney, then this mechanism could explain the attenuated cortisol production observed in the head kidney of subordinate trout. However, the present study detected no difference in mRNA relative abundance for GR1 and GR2 among the head kidneys of dominant, subordinate and sham-treated fish. Similarly, Teles et al. (2013) failed to detect any effect of acute or chronic cortisol treatment on head kidney GR1 and GR2 mRNA abundance. The availability of cortisol to trigger auto-regulatory mechanisms within the cell may be affected by 11 β HSD2 activity, as this enzyme catalyzes the conversion of cortisol to cortisone (Edwards et al., 1988). In zebrafish, evidence suggests that transcriptional regulation of 11 β HSD2 by cortisol occurs (Alderman and Vijayan, 2012; Faught et al., 2016). If a similar system operates in rainbow trout, then the high cortisol levels associated with chronic social stress could increase expression of 11 β HSD2. The failure in the present study to detect differences in 11 β HSD2 transcript abundance in the head kidney tissue of dominant, subordinate and sham-treated trout did not, however, support this possibility. Nevertheless, given previous findings, it will be important to measure GR and 11 β HSD2 protein levels.

Dominant trout exhibited a trend for increased mRNA abundance of GR2 in the head kidney as well as a trend for higher db-cAMP-stimulated cortisol production. Dominant trout experience acute stress during hierarchy formation, as shown by their high cortisol and catecholamine levels (Øverli et al., 1999b; Thomas and Gilmour, 2006). The elevated mRNA levels reported for StAR and P450scc in the head kidney of dominant fish suggest that the acute

stress of hierarchy formation may prepare the stress axis for future responses (Jeffrey et al., 2012). In support of this idea, Geslin and Auperin (2004) found that mRNA levels for StAR and P450scc were elevated following an acute stressor comprising capture, confinement for 2 min, and anesthesia for 3 min. If GR2 are present in interrenal cells, the trend for increased mRNA abundance of GR2 suggests the possibility that this receptor might play a role in increasing the capacity for cortisol synthesis by increasing expression of StAR and P450scc; increased expression of these key proteins in cortisol synthesis would, in turn, account for greater cortisol production in response to db-cAMP. Clearly, however, further research is needed to investigate this possibility in dominant fish.

3.4.2 Effects of cortisol on cortisol production: evidence for an ultra-short-loop negative feedback mechanism

Regulation of plasma cortisol levels by negative feedback is well-established in fish, although the cellular mechanisms through which cortisol acts at each level of the HPI axis have yet to be fully described (Fryer and Peter, 1977; Fryer et al., 1984; Bradford et al., 1992; Alderman et al., 2012). The elevated plasma cortisol concentrations experienced by subordinate trout could act upon cortisol production in the head kidney in an ultra-short negative feedback loop (Bradford et al., 1992), thereby accounting for the attenuated cortisol production of head kidney preparations from subordinate trout. The present study showed that incubation of head kidney preparations with 100 ng mL⁻¹ of cortisol, a level typical of that exhibited by subordinate trout (Jeffrey et al., 2012), for 2-8 h resulted in a lower increase in cortisol production compared to controls when the preparations were stimulated *in vitro* with ACTH. Moreover, the effects of 7 h of incubation with cortisol could be reversed by removing cortisol for the last hour when the

head kidney was stimulated with ACTH. These findings argue for the presence in rainbow trout of an ultra-short-loop negative feedback mechanism, as has been previously shown in coho salmon (Bradford et al., 1992). Bradford et al. (1992) suggested that cortisol could potentially affect ACTH-induced cortisol production at many levels, including modulation of MC2R, regulation of the signalling-cascade activated by MC2R, or regulation of proteins involved in cortisol synthesis (e.g. P450_{scc}, StAR). These possibilities have yet to be investigated in fish head kidney preparations. However, using bovine adrenocortical cells, Hornsby (1980) found that incubation with cortisol reduced cortisol production because the steroid end products (i.e. cortisol) acted as a pseudo-substrate, resulting in a lipid peroxidation reaction that inhibited 11 β -hyd activity, the enzyme that produces cortisol. Similar studies are needed for fish head kidney preparations. Unexpectedly, cortisol production by *in vitro* head kidney preparations from trout treated with cortisol *in vivo* exhibited no difference from control fish. Numerous factors differ between *in vitro* and *in vivo* treatment with cortisol that could potentially account for the different results, but it is worth noting that preparations from trout that were treated with cortisol were not incubated with cortisol *in vitro*, possibly allowing for effects of negative feedback to be relieved.

Collectively, the findings of the present study suggest that an ultra-short-loop negative feedback mechanism contributes to the attenuation of cortisol production in the head kidney of subordinate rainbow trout. The cellular mechanisms responsible for this effect require more investigation. The trend for higher db-cAMP-stimulated cortisol production in head kidney preparations from dominant trout also requires more investigation, particularly to establish whether it is robust and whether it is linked to the acute stress experienced by these fish during hierarchy establishment. As a whole, this study has broadened our knowledge of how negative

feedback acts within the HPI axis of rainbow trout, particularly in the context of social interactions.

CHAPTER 4

GENERAL DISCUSSION

Collectively, the present thesis provides evidence that 5-HT and negative feedback play roles in the regulation of cortisol production at the head kidney level in rainbow trout. Chapter 2 focused on 5-HT as a regulator of cortisol production, testing the hypothesis that 5-HT acts as a paracrine regulator of cortisol production in the interrenal cells of rainbow trout via the 5-HT₄ receptor. A key finding of this chapter was that 5-HT₄ receptor mRNA was detected in interrenal cells. This result was supported by evidence from *in vitro* head kidney preparations, which showed that 5-HT-stimulated cortisol production was blocked by the 5-HT₄ receptor antagonist GR125487. The evidence for a paracrine role for 5-HT was less robust. Although 5-HT was detected by immunohistochemistry near interrenal cells, the cell type that stores 5-HT remains unknown and the relative scarcity of 5-HT-positive cells was not consistent with a paracrine regulatory role. Chapter 3 investigated potential factors underlying the attenuation of cortisol production in subordinate rainbow trout, which experience chronic social stress. The hypothesis that chronic social stress modulates the expression of key proteins involved in cortisol production as well as CR was tested, primarily using *in vitro* head kidney preparations as an experimental approach to assess interrenal cell function in dominant, subordinate and sham-treated trout. Based on measurements of ACTH-induced cAMP production, MC2R activity did not differ among dominant, subordinate and sham fish. Equally, no significant differences in CR or 11 β HSD2 transcript abundance were detected among dominant, subordinate and sham trout. However, measurement of db-cAMP-stimulated cortisol production, to assess the cortisol biosynthesis machinery downstream of MC2R, yielded a trend for higher cortisol production in dominant relative to sham and subordinate fish. This finding is consistent with previous work that has reported increased transcript abundance of P450_{scc} and StAR in the head kidney of dominant trout (Jeffrey et al., 2012) and raises the interesting possibility that acute stress

experienced by dominant fish during hierarchy formation primes the stress axis for subsequent responses to stressors. A second goal of Chapter 3 was to determine whether cortisol regulates its own production in the head kidney via an ultra-short negative feedback loop, as had been reported in coho salmon (Bradford et al., 1992). Again using *in vitro* head kidney preparations, a reduction in cortisol production was detected for tissue incubated with cortisol for as little as 2 h, as long as cortisol production was assessed in the presence of cortisol. If cortisol was not present when the head kidney was stimulated *in vitro* with ACTH, no reduction in cortisol production was detected even after 7 h of incubation in the presence of cortisol. These data argue for the existence of an ultra-short-loop negative feedback mechanism at the head kidney of rainbow trout.

Clearly, a common theme of the work described in the present thesis is the use of *in vitro* head kidney preparations to assess cortisol production by interrenal cells. Although a useful technique, head kidney preparations are not without drawbacks. First, interrenal cells comprise a small proportion of the total cell population of the head kidney, approximately 1 in 8,000 cells (Hontela et al., 2008). The interrenal cells, although relatively sparse, are collected into patches that surround the PCV (Chester et al, 1980). The patchy nature of interrenal cell distribution increases the difficulty of achieving an even distribution of interrenal cells across aliquots of head kidney tissue, and can lead to substantial disparities in cortisol production among replicates, a frequent problem in the present study. In addition, the potential for substantial differences between the internal environment normally experienced by interrenal cells and the *in vitro* environment in which cortisol production is assessed may also raise concerns. Despite these potential issues, the *in vitro* head kidney preparation remains a useful approach for assessing interrenal cell function.

The topics of the two studies in the present thesis, serotonergic regulation of cortisol production and cortisol production during chronic social stress, are linked by a common theme of chronic social stress. In particular, elevated brain serotonergic activity is a key characteristic of fish experiencing chronic social stress. For example, subordinate cichlid fish (*Astatotilapia burtoni*), Arctic charr and rainbow trout exhibit elevated 5-HIAA/5-HT ratios, an index of serotonergic activity, in several areas of the brain (Winberg and Nilsson, 1993; Winberg and Lepage, 1998; Höglund et al., 2000; Loveland et al., 2014). Cortisol, the product of the stress axis, may play a role in raising brain serotonergic activity in socially subordinate fish because treatment of unstressed rainbow trout with cortisol to raise circulating values to those typical of subordinate fish resulted in elevated brain 5-HIAA/5-HT ratios (DiBattista et al., 2005). In addition, in both rainbow trout and Arctic charr, 5-HIAA/5-HT ratios and circulating ACTH concentrations were correlated, as were 5-HIAA/5-HT ratios and plasma cortisol concentrations, suggesting an important role for 5-HT in the regulation of the HPI axis in chronically stressed fish (Winberg and Lepage, 1998; Øverli et al., 1999a; Höglund et al., 2000). To date, however, these studies have focused on the brain and there is a lack of data on serotonergic activity in the pituitary and head kidney of dominant, subordinate and sham fish. Moreover, potential sources of 5-HT at the head kidney should also be considered. The present study found that 5-HT is present in the head kidney but the cell type expressing 5-HT is still unknown. It is possible that 5-HT is locally produced within the head kidney, or it may be taken up from the blood passing through the head kidney via the PCV (Caamaño-Tubío et al., 2007). Immunohistochemistry for TPH, the rate-limiting enzyme in the synthesis of 5-HT, would be helpful in determining whether 5-HT is locally produced within the head kidney. Measurements of circulating 5-HIAA and 5-

HT levels (as in Caamaño-Tubío et al. 2007) in the blood of dominant, subordinate and sham fish also would be useful.

The present study focused on the head kidney, and evaluated the responses of cortisol production to 5-HT independently of the effects of cortisol on cortisol production. However, several studies have reported possible interactions between 5-HT and cortisol at the whole animal level, suggesting that it would be useful to evaluate the interactive effects of 5-HT and cortisol on cortisol production at the head kidney. For example, administration of the 5-HT_{1A} receptor agonist 8-OH-DPAT to Arctic charr stressed by handling dampened the cortisol response to the handling stressor, suggesting an inhibitory effect of the 5-HT_{1A} receptor in stressed fish (Höglund et al., 2002). Administration of 8-OH-DPAT to cortisol-treated Gulf toadfish failed to elicit an increase in plasma cortisol levels (Medeiros and McDonald, 2013). The lack of response may reflect an effect of high cortisol levels on brain 5-HT_{1A} receptor expression (Medeiros and McDonald, 2013), but could also reflect negative feedback effects of cortisol on cortisol production, as demonstrated by Medeiros and McDonald (2012) for Gulf toadfish and in the present study for rainbow trout. Similarly, *in vivo* administration of 5-HT resulted in a significant increase in plasma cortisol concentrations in dominant and sham trout but not in subordinate trout (M-È Bélair-Bambrick, BSc Honours thesis, uOttawa). The limitations on cortisol production in the head kidney of subordinate fish documented previously (Sloman et al., 2002; Jeffrey et al., 2014) and investigated in the present study likely accounted at least in part for the lack of response in subordinate fish, but it is also possible that high cortisol levels resulted in the downregulation of brain 5-HT receptors, as suggested by Medeiros and McDonald (2013). Whether cortisol treatment or social status affects 5-HT receptor expression in interrenal cells remains to be determined. Although head kidney tissue was found to express 5-

HT_{1A}, 5-HT₂ and 5-HT₄ receptors, cortisol production by *in vitro* head kidney preparations responded only to agonists selective for 5-HT₄ receptors. The present study strengthened the evidence in favour of the 5-HT₄ receptor playing a significant role in cortisol production at the head kidney level by localizing 5-HT₄ receptor mRNA to interrenal cells. Therefore, future experiments might focus on the 5-HT₄ receptor to determine whether this receptor is downregulated in the head kidney of subordinate trout.

In conclusion, it is clear that much remains to be learned about the interactions of 5-HT and cortisol in the regulation of the HPI axis both in general and specifically in the context of social stress. The present study has contributed to our understanding of these interactions by focusing on the roles of 5-HT and cortisol in regulating cortisol production at the head kidney. In particular, the present study localized the 5-HT₄ receptor to the interrenal cell and provided additional functional evidence that 5-HT regulates cortisol production via the 5-HT₄ receptor in the head kidney of rainbow trout. The source of the 5-HT that acts on this receptor, and whether 5-HT acts as a paracrine regulator of cortisol production, remain open questions. In addition, the present study provided evidence for the presence of an ultra-short-loop negative feedback mechanism in the head kidney of rainbow trout. This ultra-short-loop mechanism likely contributes to the inhibition of cortisol production that has been reported in subordinate rainbow trout. The cellular mechanisms through which cortisol inhibits cortisol biosynthesis require further investigation.

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