# Impact of Cadmium On The Hypothalamus-Pituitary-Interrenal Axis Function In Rainbow Trout

by

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# AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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# Abstract

Cadmium (Cd) is a nonessential metal present in sublethal concentrations within the aquatic environment. Cd is an endocrine disruptor and high concentrations of this metal suppress stressor-induced cortisol production in fish. However, few studies have examined the effect of Cd at concentrations that are environmentally relevant on the functioning of the hypothalamus-pituitary-interrenal (HPI) axis. The HPI axis activity is essential in the stressor-induced cortisol production, a highly conserved adaptive response to stress in vertebrates. Elevation of plasma glucose in response to a rise in plasma cortisol is mediated through steroid activation of glucocorticoid receptors (GRs), but the mechanism of action of Cd in disrupting target tissue cortisol action is not known in fish.

The overall objective of this thesis was to examine the impact of sublethal and environmentally relevant levels of Cd on the stress response and target tissue metabolic capacities, and to investigate the mechanisms of action of this metal in disrupting cortisol production and target tissue cortisol action in rainbow trout (*Oncorhynchus mykiss*).

The impact of subchronic exposure to environmentally relevant levels of Cd on metabolic capacity and stress performance was identified through a 28 day (d) *in vivo* exposure of juvenile rainbow trout to either of two Cd concentrations (0.75 µg/L or 2.0 µg/L). During the exposure period, juvenile rainbow trout accumulated Cd within the liver, kidney and gills, but were able to adapt to exposure concentrations as no changes were observed in plasma cortisol, glucose and lactate levels. However, changes in abundance of mRNAs encoding proteins involved in corticosteroidogenesis, including melanocortin 2 receptor (MC2R), steroidogenic acute regulatory protein (StAR) and P450 side chain cleavage enzyme (P450scc), and liver GR protein expression suggesting endocrine disruption over the 28 d period. Also, target tissue metabolic capacities, including lower liver glycogen content and changes in intermediary metabolic enzyme activities in the liver and gill, were compromised by the 28 d exposure to Cd. The response to a secondary handling stressor at either 7 or 28 d exposure was attenuated suggesting that subchronic exposure to low levels of Cd disrupts the highly conserved adaptive stress response in rainbow trout.

Upon further investigation using *in vitro* head kidney slices exposed to 0, 10, 100 or 1000 nM of Cd and stimulated with adrenocorticotropic hormone (ACTH), a similar inhibition of cortisol production was observed, as demonstrated *in vivo*, suggesting that Cd disrupts interrenal corticosteroidogenesis in fish. The impact of Cd on ACTH-stimulated cortisol production involved

the suppression in the abundances of MC2R, StAR and P450scc transcripts. This response was also mimicked when head kidney slices from 7 d Cd exposed fish were incubated *ex vivo* with ACTH confirming that interrenal tissue is a key target for endocrine disruption by Cd. In both the *in vitro* and *ex vivo* incubations of head kidney slices 8-Bromo-cAMP (a cAMP analog) completely abolished the Cd-mediated cortisol inhibition demonstrating for the first time that Cd disruption of corticosteroidogenesis is occurring upstream of cAMP production.

Further investigation of Cd-mediated impact on MC2R showed alterations in MC2R mRNA transcripts during *in vivo* exposure after 7 days and an attenuation of MC2R mRNA levels after Cd-exposed fish were subjected to a handling stressor. Disruptions in the mRNA abundance of MC2R was associated with disruptions of melanocortin receptor accessory protein 1 (MRAP1), but not MRAP2; a phenomenon that was also observed in *ex vivo* head kidney slices. Cell transfection studies confirmed that rainbow trout MC2R/MRAP1 receptor complex displayed decreased activity in the presence of Cd. Taken together these results suggest that Cd directly targets the MC2R/MRAP1 complex to inhibit ACTH-stimulated cortisol production in juvenile rainbow trout. In addition to Cd inhibiting interrenal steroidogenesis, the results also suggest that Cd may impact the negative feedback regulation of cortisol through the suppression of brain mineralocorticoid receptor (MR), but this requires further investigation. At the target tissue level, Cd by itself did not affect liver metabolism, but inhibited the cortisol-induced glucose production in liver slices. This involved suppression of GR protein expression along with the suppression of GR-responsive genes, including phosphoenolpyruvate carboxykinase (PEPCK) and suppressor of cytokines signaling 1 (SOCS1) and changes in enzyme activities, including hexokinase, glucokinase, pyruvate kinase and PEPCK, pointing to a disruption in liver GR signaling by Cd.

Altogether, Cd exposure disrupts the organismal stress responses in juvenile rainbow trout. Furthermore, Cd impairs the ability of juvenile rainbow trout to respond to a secondary stressor, which is a vital adaptive process that is fundamental to successful stress performance. Most importantly, these studies highlight for the first time that disruption of the HPI axis to attenuate cortisol production occurs at the level of the MC2R/MRAP1 complex, suggesting that the mechanism of action for attenuation of cortisol occurs at the level of MC2R activation. Also, GR signaling is a key target for Cd and may be a mechanism leading to altered metabolic capacities in stressed fish from Cd-contaminated sites. Overall environmentally relevant levels of Cd disrupt cortisol production and target tissue action of this steroid in rainbow trout.

# Acknowledgements

# Dedication

I dedicate this thesis to my late grand mother, Tej Kaur Sandhu. Waheguru has you in His keeping; we have you in our hearts.

May you always live on forever.

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# Chapter 1
## Introduction

## 1.1 General Introduction

Many toxicants have been shown to disrupt cortisol production in teleosts including pharmaceuticals and consumer products (Benguira et al., 2002; Lacroix and Hontela, 2004; Gravel and Vijayan 2006, 2007; Hontela, 2006; Dann and Hontela, 2011), xenobiotics (Hontela et al., 1992; Pickering, 1993; Leblond et al., 2001), wastewater effluents (Miller et al., 2009; Ings et al., 2011a,b, 2012a,b; Miller and Hontela, 2011), and heavy metals (Brodeur et al., 1997; Playle 1998; Gravel et al., 2005; Gagnon et al, 2006), including Cd (Lacroix and Hontela, 2004;  Levesque et al., 2002, 2003; Hontela and Lacroix, 2006; Lacroix and Hontela, 2006; Gagnon et al., 2007; Sandhu and Vijayan, 2011 [Chapter 3]). However, the mechanism of action of these contaminants in impacting the stress and metabolic performance is poorly understood.

Cadmium (Cd) is a nonessential heavy metal found at trace levels within the aquatic environment and is a known endocrine disruptor in both mammals and teleosts (Ricard et al., 1998; Henson and Chedrese, 2004; reviewed by McGeer et al., 2012). Most studies have focused on the reproductive axis and only a few studies have examined its impact on the stress response (Hontela and Vijayan, 2008). In the presence of Cd, cortisol production in rainbow trout is attenuated (Lacroix and Hontela, 2006; Gagnon et al., 2007; Sandhu and Vijayan, 2011 [Chapter 3]); however the mechanism of action is unknown. Furthermore, current studies examining the impact of Cd on the stress response have looked at concentrations that are well above environmentally relevant levels. This chapter will briefly review the impact of sublethal Cd concentrations in the environment on fish physiology and the organismal and cellular stress responses.

## 1.2 Cadmium in the aquatic environment

Cd is considered as a toxic and nonessential heavy metals to animals. Due to its many industrial uses, Cd is greatly dispersed throughout ecosystems (TRI96, 1998; Pinot et al., 2000). Within the aquatic environment, Cd enters via natural (present in Earth's crust) and anthropogenic activities such as the smelting of iron lead and/or zinc, burning of fossil fuels such as coal and incineration of municipal waste (Thornton, 1983; WHO, 1992; Camusso et al., 1995; ASTDR, 1999; Frew et al., 1997; Okada et al., 1997; Dias and Edwards, 2003; Kumar et al., 2007; Vazques-Sauceda, 2011). Cd is also present in food grown in large amounts of phosphate, sewer sludge, cigarettes, batteries, paints, metal coatings and plastics (WHO, 1992; Okada et al., 1997; Roblenbeck et al., 1999; Olsson et al., 2002; Kumar et al., 2007). From these multiple sources, Cd enters the aquatic environment and gets biomagnified in the food chain (Harrison and Klaverkamp, 1989; Ayres, 1992; Cope et al., 1994; Burger et al., 2002, 2008; Seebaugh et al., 2005).

Cd is extremely toxic and lethal to aquatic animals at concentrations lower than for many other metals (Canadian Council of Ministers of the Environment (CCME), 1994, 1999). The toxic release inventory has reported that approximately 3 million kilograms of Cd are annually released in the environment due to mining and smelting (TRI96, 1998). The average Cd content in the world's oceans have variously been reported as low as 0.005 µg/L (WHO, 1992) and 0.005-0.02 µg/L (OECD, 1994; Jensen and Bro-Rasmussen, 1992), to as high as 0.5-1.5 µg/L (Pan et al., 2010) and > 8.9 µg/L (CRC, 1996; Singh, 2001; Kaushik et al., 2003). Within fresh waters across Canada, Cd concentrations range from < 0.1 to 122 µg/L (CCME, 1996). Exposure to toxicants such as cadmium in the aquatic environment can be defined as acute and chronic. Although the definitions can be loosely interpreted, acute exposure is generally accepted as a stressor that is applied to an organism and removed, allowing the organism to recover, whereas chronic exposure is a constant exposure. Currently, the freshwater quality criteria in water hardness ranging from 20-120 mg/L as $CaCO_3$ for aquatic life is 0.6 – 4.8 µg Cd/L for acute exposure and 0.3 – 1.3 µg Cd/L for chronic exposure (US EPA, 2001) in the United States and 0.017 µg Cd/L in Canada (CCME, 1999).

## 1.3 Cadmium bioavailability and water hardness

Cd within the aquatic environment is present in many forms, such as soluble fractions and bound to suspended particles or sediments. Cd is most readily absorbed in its free form Cd (II) (Calamari et al., 1980; Wright and Welbourn, 1994; AMAP, 1998; DiToro et al., 2001; May et al., 2001; Lyndersen et al., 2002; Okocha and Adedeji, 2011). Water chemistry influences Cd bioavailability: dissolved organic carbon and sulfur-containing compounds bind Cd, while calcium (Ca) (involved in water hardness) can compete with Cd uptake at the gills (Bucking and Wood, 2006).

Water hardness is considered to be the most important factor related to Cd sensitivity in fish (Playle et al., 1993b; Hollis et al., 2000) and Ca is considered to be the most important ion as it competes with Cd for uptake in fish at branchial uptake sites (Verbost et al., 1987, 1989; Playle et al., 1993a; Wood, 2001; Niyogi and Wood, 2003; Baldisserotto et al., 2005; Ng et al., 2009). In teleosts, toxicity of Cd has been demonstrated to increase in the absence of extracellular Ca *in vitro*, suggesting a reciprocal interaction between Ca and Cd (Verbost et al., 1989; Zohouri et al., 2001; Franklin et al., 2005; Galvez et al., 2006; Lacroix and Hontela, 2006). Ca is an important second messenger in steroid synthesis (Van Der Kraak, 1991; Yamazaki et al., 1998) and since Cd competes and/or interferes with Ca uptake through cationic channels within steroidogenic cells (Mathias et al., 1998; Lacroix and Hontela, 2006; Gagnon et al., 2007), it is possible that a link exists between Ca and Cd-mediated inhibition of steroidogenesis although this is yet to be verified.

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## 1.4 Cadmium route of entry and uptake in freshwater fish

Metal ions in fish are typically absorbed through passive diffusion or carrier-mediated transport across the gills, whereas metals that are associated with organic material are typically ingested and absorbed by endocytosis through the intestine (McDonald and Wood, 1993). The route of exposure is dependent upon the salinity of the environment (Klinck and Wood, 2013a) and since freshwater fish are actively taking up ions ($Na^+$, $K^+$, $Cl^-$ and $Ca^{2+}$) (Schoenmakers et al., 1993), uptake of Cd occurs primarily at the gills, although uptake also occurs through the gut and liver (Reid and McDonald, 1991; Pratap and Wendelaar Bonga, 1993; Franklin et al., 2005; Baldisserotto et al., 2006; Cooper et al., 2006; Wood et al., 2006; Ojo and Wood, 2008; Niyogi et al., 2008; Klinck et al., 2009; Kwong and Niyogi, 2009, 2012; Kwong et al., 2010, 2011; Klinck and Wood, 2011, 2012, 2013b; McGeer et al., 2012).

Binding of Cd to gills in freshwater fish occurs at either high affinity, low capacity sites or low affinity, high capacity sites (Playle et al., 1993a,b; Reid and McDonald, 1998). The high affinity, low capacity sites are considered "toxic sites" that are crucial in maintaining Ca homeostasis (Niyogi et al., 2008). At the gill, Ca influx occurs through active transport against a chemical gradient and is transcellular (Flik et al., 1985; Wood, 1991; Perry, 1997; Perry et al., 2003; Moyes and Schulte, 2008), whereas efflux occurs passively (Verbost et al., 1987). Ca transport at the gills to the blood occurs via $PNA^+$ MR cells (Galvez et al., 2006). Uptake of Cd occurs by competing with Ca at the apical Ca channel of chloride cells in gills via mechano-sensitive L-type Ca channels and to a lesser degree, via non-voltage gated Ca transporters (Goss et al., 2001; Perry et al., 2003; Galvez et al., 2006; Klinck and Wood, 2011; Klinck et al., 2012). Alongside competing for uptake through chloride cells, Cd also inhibits Ca uptake within ionocytes by inhibiting basolateral Ca-ATPase (Verbost et al., 1987, 1989; Schoenmakers et al., 1992; Kwong et al., 2011) leading to hypocalcaemia (Wong and Wong, 2000). Hypocalcaemia, which is a decrease of calcium in the blood, occurs by non-competitive inhibition by Cd, which irreversibly blocks the Ca-ATPase transport enzyme and inhibits Ca transport into the blood (Hollis et al., 2000; Szebedinsky et al., 2001). Overall, Cd appears to have multiple mechanisms of uptake into freshwater fish, however, the mechanism of basolateral extrusion they are still unclear.
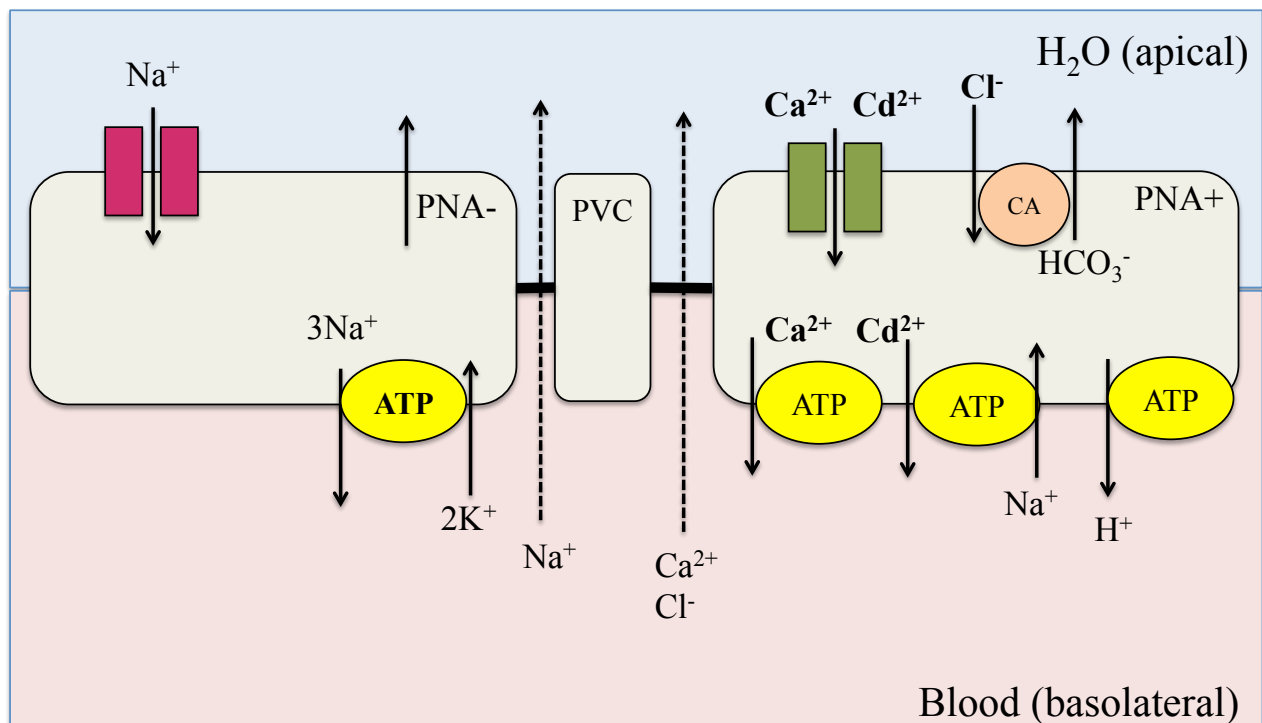
**Figure 1. Cadmium uptake in the gills of freshwater fish**. At the site of the gill, cadmium acts like a calcium analogue and uptake occurs through voltage independent epithelial calcium channels that are considered to be located on positive peanut lectin agglutinin (PNA+) cells. Once Cd crosses the gill epithelium, it is either sequestered at the gill or released into the blood. The basolateral extrusion of Cd occurs through Ca-ATPase channels or sodium/calcium (Na/Ca) exchange and is transferred by the blood to other internal tissues either bound to metallothioneins, plasma proteins, or in its conjugated form. (Abbreviations: negative peanut lectin agglutinin (PNA-), pavement cells (PVC), $Cd^{2+}$ (cadmium), $K^+$ (potassium), CA (carbonic anhydrase), adenosine triphosphate (ATP), $H^+$ (hydrogen), HCO3- (sodium bicarbonate), chloride (Cl-)).

## 1.5 Cadmium tissue accumulation

The initial site of accumulation and tissue damage occurs at the site of entry, which are the gills of freshwater fish (McDonald and Wood, 1993; Niyogi et al., 2008). After the gills, Cd is transferred through the bloodstream and distributed to internal tissues (McGeer et al., 2000b, 2007). The amount of metal in each of the internal tissues is dependent upon factors such as blood distribution, tissue specific uptake processes and detoxification mechanisms (McGeer et al., 2000b, 2007). Generally in freshwater fish, Cd accumulates predominately in the liver and kidney, after accumulation in gills (Norey et al., 1990; Chowdhury et al., 2004).

Physiological adjustments due to Cd exposure are typically accompanied by acclimation though enhanced tolerance of the metal (McDonald and Wood, 1993; Stubblefield et al., 1999; Hollis et al., 1999; McGeer et al., 2000a). This process, known as the damage-repair model, is characterized by three phases; the initial shock phase, the recovery phase and acclimation phase (McDonald and Wood, 1993). Briefly, upon exposure to Cd, there is an initial accumulation at the site of exposure, which is primarily the gills, resulting in homeostatic disruption (McDonald and Wood, 1993). Accumulation occurs predominantly in the gills before Cd begins to accumulate in other tissues, suggesting that there is a lag period and time is required to deliver Cd to internal target sites such as the liver (McGeer et al., 2000b; Hollis et al., 2001). Transfer of metals to target sites results in the recovery phase, which consists of reallocation of energy towards increasing metabolic activity and protein synthesis (McDonald and Wood, 1993). An important aspect of the recovery phase is the production of metal-binding proteins, metallothioneins (McDonald and Wood, 1993), which internalize metals in forms that are biologically unavailable to react with other target tissues (Wallace et al., 2003; Vijver et al., 2004; Rainbow, 2007). The high accumulation in some tissues, including the liver, is thought to be due to the presence metallothioneins that bind Cd and reduce the biologically active free pool (Olsson and Kille, 1997; Hollis et al., 1999; Kamunde, 2009). Metallothioneins will chelate and sequester Cd by binding of the metal to thiol groups, keeping Cd in the cytosol as and in soluble particulate structures (Lanno et al., 1987; Wallace et al., 2003). Although metallothioneins transcript levels are increased in the presence of Cd, the majority of Cd is sequestered in the liver and kidney (Flik et al., 1987), which is thought to be a result of the high affinity binding of Cd at Ca binding sites in the gill (Hollis et al., 1999). The reduction in internally available Cd by metallothioneins has been shown to protect organisms from chronic Cd exposure (Prakash and Rao, 1995; Kraemar et al., 2005). The metabolic cost associated with this protein synthesis may compromise tissue metabolic performance to chronic Cd exposure, but this remains to be tested. Finally, if Cd exposure is continuous (i.e. chronic) the

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final phase occurs, the acclimation phase, whereby the gills begin to acts as a barrier and reduce internal Cd loading (Hollis et al., 2000). Reduction in Cd accumulation results in the fish returning to pre-exposure conditions, or, a new functional state (McDonald and Wood, 1993).

## 1.6 Cadmium effects in fish

The sensitivity of aquatic organisms to Cd has been found to vary, and although the reason for this is unknown, it is thought to be due to variations in water chemistry, life stage, metal uptake, accumulation and sequestration (Spry and Wiener, 1991; Lacroix and Hontela, 2004; McGeer et al., 2000b; McGeer et al., 2012). In 2001, the United States Environment Protection Agency (US EPA) compiled a list of effects of Cd on an array of freshwater and saltwater organisms (US EPA, 2001). Amongst salmonids, rainbow trout are listed as the fourth most sensitive freshwater organism to Cd exposure (Sorensen et al., 1991; US EPA, 2001, Hansen et al., 2002) with a final acute value (value that is lower than LC50 and EC50 value for nearly all individuals in a genus) for total Cd at a hardness of 50 mg/L to be 2.108 μg/L (US EPA, 2001).

Accumulation of Cd in living organisms is a major ecological concern especially because of its long-half life and low excretion rate resulting in target tissue toxicity (ASTDR, 1999; Lippmann, 2000; Risso-de-faverney et al., 2001). Once Cd is absorbed and brought into the cells, it is accumulated and available to interact with cytoplasmic components which results in a variety of toxico-pathological responses (Bertin and Averback, 2006), including impaired tissue metabolism, anemia, osmo-ionic disturbances and hypocalcaemia along with changes to enzyme regulation (Pratap and Wendelaar Bonga, 1993; Vaglio and Landriscina, 1999; McGeer et al., 2000a, Wood, 2001; Almeida et al., 2002; Baldisserotto et al., 2004; Pratap and Wendelaar Bonga, 2007; Okocha and Adedeji, 2011). Furthermore, changes in behaviour (Scott et al., 2003; Sloman et al., 2003), decreased hatch success (Lazardo-Daudt and Kennedy, 2008) disturbances in respiration (Livingstone, 2001; Shaffi et al., 2001) and/or mitochondrial dysfunction (Bagchi et al., 1996; Fernandez et al., 2003; Kurochkin et al., 2011) along with impairment in blood parameters such as cortisol and glucose (Fu et al., 1990; Pratap and Wendelaar Bonga, 1990; Gill et al., 1993; Brodeur et al., 1998; Lacroix and Hontela, 2004) are observed, all of which can lead to mortality. Fish exposed to sublethal concentrations of Cd can also be visibly shorter and their reproductive competence, which includes the synthesis of sex steroids, can be reduced due to lack of energy allocated towards growth and reproduction (Versteeg and Giesv, 1986; Fu et al., 1990; Pratap and Wendelaar Bonga, 1993; Hontela, 1998; Ricard et al., 1998; Hollis et al., 1999; McGeer et al., 2000a; Jones et al., 2001; Hansen et al., 2002; Tilton et al., 2003).

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In the aquatic environment, organisms are typically not exposed to high, acutely toxic concentrations of Cd, unless they are restricted to that specific vicinity. Downstream of the initial site of exposure, Cd concentrations become diluted, which decreases the concentrations to lower levels that are defined as sublethal. At these sublethal concentration, Cd can cause changes in the tissue appearance at sites of Cd exposure and accumulation including the gastrointestinal tract, gills, kidney and liver at concentrations that are below levels resulting in cell death (Forlin et al., 1986; Lizardo-Daudt et al., 2007).

## 1.7 Cadmium as an endocrine disruptor

The World Health Organization defines an endocrine disruptor as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (WHO, 1992).

Cd was first identified as an endocrine disruptor in human breast cancer cells over a decade ago (Garcia-Morales et al., 1994; Stoica et al., 2000) and has been shown in great detail to impact both the male and female reproductive system in mammals (Henson and Anderson, 2000; Byrne et al., 2009; Iavicoli et al., 2009). Briefly, in females, Cd works similar to the sex hormone estradiol, and induces proliferation (Brama et al., 2007) in the transcription of genes regulated by both the progesterone and estrogen receptor (ER$\alpha$) (Stoica et al., 2000; Wilson et al., 2004; Martinez-Campa et al., 2006). Recent literature suggests that the mechanism of action by which Cd exerts toxicity on estrogenic genes is through both genomic binding of Cd to ER$\alpha$ and through nongenomic ER$\alpha$ pathways, specifically through the extracellular signal-regulated kinase (ERK1/2) and serine/threonine kinase (Akt) (Brama et al., 2007; Liu et al., 2008). In males, Cd is able to impact genes regulated by the androgen receptor (Martin et al., 2002), but it is not known whether Cd directly binds to the androgen receptor. Recent studies suggest that the mechanism of action by which Cd acts on androgen receptors should be similar to the direct binding observed in female estrogen receptors as the ligand binding domain which binds Cd in the estrogen receptor is highly conserved between steroid receptors, including the androgen receptor (Martin et al., 2002).

Although a majority of the work involving Cd as an endocrine disruptor has focused primarily in mammals, most of these studies have focused on the reproductive axis. In relation to the stress response, not many studies have focused on the mechanism of action of Cd, and even fewer on non-mammalian vertebrates. It is well known that Cd and other heavy metals impair ACTH-mediated cortisol production in both mammals and fish (reviewed by Hontela, and Vijayan, 2008). However, the mechanism of action is yet to be elucidated.

## 1.8 Cadmium as an endocrine disruptor in teleost reproductive axis

Studies have shown that Cd causes endocrine disruption of the reproductive axis in aquatic organisms through disruption of gonadal steroidogenesis (Mukherjee et al., 1994; Arcand-Hoy and Benson, 1998; Kime, 1999; Jalabert et al., 2000; Le Guével et al., 2000). Similar to mammals, most of the endocrine disrupting studies of Cd has been focused on the reproductive axis. Inhibition of gonadal steroidogenesis in fish can be due to many reasons such as morphological alterations in the gonads due to exposure (Mousa and Mousa, 1999; Sepulveda et al., 2002; Levesque et al., 2003), or as seen in mammals, through binding of Cd to sex steroid receptors.

17 β-estradiol ($E_2$) is the predominant estrogen hormone in reproductive oviparous vertebrates that acts within the liver to induce the expression of vitellogenin (egg yolk precursor), making both the hormone and its target (liver) an important site for examining the expression of estrogen-dependent genes. Cd has estrogenic effects through different molecular and cellular mechanisms (Safe, 2003). Mechanistically in teleosts, Cd has been shown to have a high affinity to the estrogen receptor and therefore competes with estrogen for binding sites. Once bound, Cd can reduce or inhibit the expression of genes that are regulated by $E_2$ (Le Guével et al., 2000)

11-Ketotestosterone (11-KT) is the major androgen in male teleosts that is required for gametogenesis and the development of male secondary sexual characteristics (Scott et al., 1980; Arcand-Hoy and Benson, 1998; Schulz et al., 2001). Within males, Cd has been shown to alter 11-KT levels in rainbow trout testes (Sangalang and O'Hallorn, 1973; Sangalang and Freeman 1974; Kime, 1984) *in vitro* and *in vivo*. Unlike females, the mechanism of action by which Cd inhibits testosterone production in males is not fully understood but it is thought to impact the hypothalamus-pituitary-gonadal (HPG) axis prior to cAMP formation (Lizardo-Daudt et al., 2008).

## 1.9 Cadmium as an endocrine disruptor in teleost stress axis

Apart from the reproductive axis, Cd also disrupts other systems (Taylor and Harrison, 1999; Hewitt and Servos, 2001; Vetillard and Bailhache, 2005) including the hypothalamus-pituitary-interrenal (HPI) axis (Hontela et al., 1992; Brodeur et al., 1997; Lacroix and Hontela, 2004). With respect to the stress response in teleosts, there is far less information on the interaction of Cd, or any endocrine disruptor, with the HPI axis, and the mechanism of action is not known. Furthermore, the current literature on the impact of Cd on cortisol production in teleosts is very inconsistent. While some papers report that Cd increases plasma cortisol (Tort et al., 1996), others suggest an inhibition (Lacroix and Hontela, 2004). The

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inconsistency is due to variations within the studies such as route of exposure, concentration, duration of exposure, the reproductive status of the fish (mature versus immature), and the type of fish species used (Harrison and Curtis, 1992; Szebedinszky et al., 2001; Niyogi and Wood, 2003; Chowdhury et al., 2004). Field studies of fish from metal contaminated sites have shown reduced plasma cortisol concentrations and a failure to respond to additional stressors (Hontela et al., 1992, 1997; Hontela et al., 1995; Brodeur et al., 1997; Girard et al., 1998; Norris et al., 1999, Kakuta, 2002; Levesque et al., 2003). Although the reason for the unresponsiveness of fish to a secondary stressor is unknown, Brodeur and colleagues (1998) suggested that a sustained stimulation of cortisol secretion and the high metabolic activity of the interrenal tissue could lead to exhaustion of steroidogenesis, and that this could account for the low levels of cortisol during metal exposure and impairment of the stress response to additional stressors. *In vitro*, Lacroix and Hontela (2004) showed that direct exposure of adrenal cells to various concentrations of Cd caused a dose-dependent inhibition in cortisol production after 60 min of exposure. Furthermore, this inhibition was abolished in Cd-treated cells stimulated with pregnenolone and 25-hydroxycholesterol (pure synthetic precursor for cortisol production) suggesting that Cd disrupts cortisol signaling upstream of adenylate cyclase activation (Leblond and Hontela, 1999; Tilton et al., 2003; Lizardo-Daudt et al., 2007).

Apart from its impact on cortisol production, not much is known about the metabolic impact of Cd or its mechanism of action in teleosts (Arcand-Hoy and Benson 1998; Hontela, 1998; Kime, 1999).

## 1.10 General introduction to stress and stress response

Stress occurs when a perceived stressor threatens to create a homeostatic imbalance within the organism (Barton, 1997; Wendelaar Bonga, 1997; Chrousos, 1998; Norris, 2000; Flik and Wendelaar Bonga, 2001, Barton, 2002; Barton et al., 2002; Iwama et al., 2006; Chrousos, 2009; Vijayan et al., 2010). Briefly, stressors consist of intrinsic and extrinsic stimuli as well as abiotic and biotic factors, including physical (e.g. temperature and pH), chemical (e.g. exposure to pollutants), physiological (e.g. disease and starvation) and psychological stressors (e.g. predation threat and crowding) (Barton and Iwama, 1991; Iwama et al., 1992, 1997; Iwama et al., 2006). In order to re-establish or create a new homeostatic set point, organisms elicit a complex suite of compensatory and/or adaptive behavioural and physiological responses broadly characterized as the primary, secondary and tertiary response (Selye, 1950, 1973; Barton and Iwama, 1991; Pickering and Pottinger, 1995; Wendelaar Bonga, 1997; Barton, 1997; Chrousos, 1998; Iwama et al., 1998; Schreck, 2010).

Physiological responses to stress invoke the generalized primary and secondary stress responses that have been previously reviewed in detail (Barton and Iwama, 1991; Gamperl et al., 1994; Wendelaar Bonga, 1997; Iwama et al., 1998, 2006; Barton et al., 2002; Iwama et al., 2006; Vijayan et al., 2010). Although the pathways involved in either adapting or maladapting the animal to stress are not well established, the primary stress response involving the role of the hypothalamic-sympathetic-chromaffin (HSC) axis and the hypothalamus-pituitary-interrenal (HPI) axis (homologous to mammalian hypothalamus-pituitary-adrenal [HPA] axis), leading to the rapid release of catecholamines from chromaffin tissue (Randall and Perry, 1992; Reid et al., 1998; Iwama et al., 1999) and the more delayed production of cortisol from the interrenal tissue is a well documented area of stress adaptation (Figure 1) (Wendelaar Bonga, 1997; Mommsen et al., 1999). Unlike mammals, fish do not possess a discrete adrenal gland (Mommsen et al., 1999). Instead, catecholamines are stored in chromaffin cells (adrenal medulla homologue) and cortisol is produced from interrenal cells (adrenal cortex homologue) (Wendelaar Bonga, 1998; Barton, 2002). Both chromaffin cells and interrenal cells along with immune cells are intermingled in clusters and found around the walls of the post cardinal vein located within the anterior kidney in teleosts (Hart et al., 1989; Randall and Perry, 1992; Reid et al., 1998; Hontela, 2005).

The perception of a stressor by the central nervous system (CNS) immediately activates the HSC axis and signals sympathetic nerve fibers to stimulate the release of catecholamines, via the cholinergic receptors on chromaffin cells, into the blood stream within seconds to minutes (Figure 1) (McDonald and Milligan, 1992; Randall and Perry, 1992; Reid et al., 1998; Barton et al., 2002; Vijayan et al., 2010). Catecholamines, including epinephrine and norepinephrine, are crucial in adaptation of the cardiovascular and respiratory systems, which are necessary for reallocation of energy substrates in order to meet the increased energy demand associated with stress (Hart et al., 1989, Randall and Perry, 1992; Wood and Munger, 1994; Wendelaar Bonga, 1997; Vijayan et al., 2010).

The secondary stress response related to the upregulation of catecholamines and cortisol involves physiological and cellular changes such as energy substrate mobilization and reallocation, increased cardiac output and oxygen uptake by the gills (Mommsen et al., 1999; Iwama et al., 2006; Vijayan et al., 2010). The stress response, in the short-term, is important for metabolic adjustments and for upregulation of energy substrate to cope with a stressor (Mommsen et al., 1999). The tertiary stress response occurs when the organism is unable to effectively adapt, leading to whole animal and population changes which can render the physiological stress response mechanisms maladaptive causing impaired immune function,

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growth and reproduction as a result of the diversion of energy resources from these processes (Barton et al., 2002; Hontela and Vijayan, 2009).

Most studies related to stress detection in teleosts examine the functioning of the HPI axis because, unlike catecholamines, it is possible to obtain resting levels of plasma cortisol (Wedemeyer et al., 1990; Barton and Iwama, 1991; Gamperl et al., 1994; Iwama et al., 2006).

## 1.11 The Hypothalamus-Pituitary-Interrenal (HPI) axis

Unlike the release of catecholamines, the release of cortisol is delayed because cortisol is not stored for rapid release. The release of cortisol, the principal corticosteroid in teleosts during stress, follows within minutes to hours after stressor exposure and as previously mentioned, involves the HPI axis functioning (Figure 2) (Mommsen et al., 1999; Aluru and Vijayan, 2009; Vijayan et al., 2010). The primary step involves the stressor-mediated stimulation of the hypothalamus to release corticotropin-releasing factor (CRF) (Figure 2) (Feist and Schreck, 2001; Alsop and Vijayan, 2008; Alderman and Bernier, 2009). The axons of nucleus preopticus (NPO) CRF cells projects directly onto the pituitary gland, specifically the cells of the rostral pas distalis, where CRF stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary (Figure 2) (Flik et al., 2006). Although other neurohormones and peptides can stimulate cortisol release from interrenal tissues, ACTH is the primary cortisol secretagogue (Balm et al., 1994; Wendelaar Bonga, 1997; Barton et al., 2002; Iwama et al., 2006). Once ACTH is produced, it travels through the blood stream to the interrenal cells within the head kidney and binds to the melanocortin 2 receptor (MC2R), leading to the activation of the steroid biosynthetic pathway, and the production of cortisol (Figure 2) (Aluru and Vijayan 2008, 2009).

**Figure 2. The hypothalamic-sympathetic chromaffin cell (HSC) axis and hypothalamus-pituitary-interrenal (HPI) axis**. Schematic diagram of the HSC and HPI axes function preceding stressor exposure, adapted from Wendelaar Bonga (1997). Following stressor perception, the HSC axis is activated and sympathetic nerves from the hypothalamus stimulate the chromaffin cells in the head kidney to release catecholamines (epinephrine and nor-epinephrine) into the blood stream to stimulate physiological changes required for an alarm, or fight-or-flight response. During HPI axis activation, corticotrophin-releasing factor (CRF) released from the hypothalamus stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), the primary cortisol secretagogue, into the blood stream, which increases cortisol synthesis and release from the interrenal cells in the head kidney, which promote physiological changes required for regaining and maintaining homeostasis. The physiological changes involve either an increase (blue), decrease (red) or could result in both an increase and decrease (brown) depending on the duration of stressor. Once cortisol reaches a threshold level, production is inhibited by cortisol acting on glucocorticoid (GR) receptors in the hypothalamus and pituitary to suppress CRF and ACTH production, respectively.

Stress

**Hypothalamus**

CRF

**Pituitary**

ACTH

Sympathetic nerves

**Head Kidney**

| Chromaffin Cells | Interrenal Cells |

**Catecholamines Epinephrine and Nor-epinephrine**

**Cortisol**

Hydromineral balance
Liver glycogen
Plasma glucose
Cardiac output
Gill blood flow
Oxygen uptake and transfer
Immune functions
Plasma FFA

Muscle proteins
Immune functions
Growth
Reproduction
Hydromineral balance
Liver glycogen
Plasma FFA

## 1.12 Melanocortin 2 receptor and melanocortin 2 receptor accessory proteins

The melanocortin receptor (MCR) family consists of five seven-transmembrane G-protein coupled receptors (GPCRs) known as MC1R, MC2R, MC3R, MC4R and MC5R (Gantz and Fong, 2003; Cooray and Clark, 2011) in mammals. These subtypes have also been identified in teleosts (Ringholm et al., 2002; Logan et al., 2003; Haitina et al., 2004; Klovins et al., 2004a,b; Metz et al., 2006; Aluru and Vijayan, 2008) and show a high degree of homology to mammalian MCRs (Schiöth et al., 2005). Ligands that bind to MCRs include α-, β-, and λ-MSH and ACTH-peptide hormones that originate from the proopiomelanocortin precursor peptide (Mountjoy et al., 1992; Cone et al., 1993). All five MCRs act via the stimulatory G protein (Gs), which in turn activates adenylate cyclase to produce cAMP (Metz et al., 2006; Cerdá-Reverter et al., 2011; Veo et al., 2011).

Of the five MCRs, MC2R is the only one that is predominately found in the interrenal cells and is solely activated by ACTH, which leads to the production of cortisol (Gantz and Fong, 2003). Current mammalian literature demonstrates the presence of a trafficking protein that aids in folding, processing and translocation of MC2R from the endoplasmic reticulum to the cell membrane, enabling MC2R to bind to ACTH (Cooray and Clark, 2011). MC2R was long suspected to be functional through an accessory protein because unlike other MCRs, heterologous expression of MC2R does not occur in nonadrenocortical cell lines (Weber et al., 1993; Naville et al., 1996; Yang et al., 1997; Elias et al., 1999; Metherell et al., 2005; Roy et al., 2007; Sebag and Hinkle, 2007; Cooray et al., 2008), a phenomenon that is similar to many GPCRs (Cooray et al., 2008). However, accessory proteins have been demonstrated to modulate expression of all melanocortin receptors (Sebag and Hinkle 2009a,b; Chan et al., 2009). Within mammals, 25% of familial glucocorticoid deficiency (FGD) cases, which result in glucocorticoid deficiency due to unresponsiveness to ACTH occur through mutations in MC2R, whereas in the other 75%, the receptor is normal but mutations are found in a single transmembrane protein, which was later classified as the melanocortin receptor accessory protein (MRAP) (Xu et al., 2002; Clark et al., 2005, 2009; Metherell et al., 2005; Rumié et al., 2007).

The MRAP protein is a single transmembrane protein that consists of six exons (Cooray and Clark, 2011) and is found in the adrenal gland of mammals. Although MRAP has been identified in several species, it is not highly conserved (Webb and Clark, 2010). In humans, MRAP has been identified as two isoforms called MRAP1 and MRAP2 (Roy et al., 2007; Chan et al., 2009; Webb and Clark, 2010). Trafficking of MC2R is not fully known, but it is thought that a 15-residue conserved region in the N-terminus of MRAP1 interacts with MC2R and is required for trafficking, and the C terminus of MRAP1,

which is not well conserved amongst species, is involved in MC2R cell surface expression (Sebag and Hinkle, 2009a; Webb et al., 2009). MRAP1 and MRAP2 share a 39% amino acid identity in the N-terminus and transmembrane domain (Chan et al., 2009) and *in vitro* MRAP2 has functional similarities to that of MRAP1 (Chan et al., 2009). *In vivo*, MRAP2 is not able to produce a functional MC2R but increases MC2R expression in the presence of MRAP1, suggesting that MRAP2 is not crucial for MC2R trafficking and functionalization (Gorrigan et al., 2011). Experiments in Y1 and CHO cells suggest that MRAP2 plays an inhibitory role where it competes with MRAP1 to bind to MC2R, thus reducing cAMP production in response to ACTH stimulation (Sebag and Hinkle, 2010). Other studies suggest that MRAP2 has additive effects on MRAP/MC2R cAMP response to ACTH (Agulleiro et al., 2010); however, the exact role of MRAP2 is yet to be elucidated.

In mouse, it was recently proposed that MRAP forms a homodimer with another MRAP within the endoplasmic reticulum via its transmembrane domain (Figure 3) (Sebag and Hinkle, 2007). The MRAP forms a homodimer with another MRAP within the endoplasmic reticulum via its transmembrane domain (Figure 3) (Sebag and Hinkle, 2007). The importance of MRAPs in teleosts is a recent avenue of research and thus not a lot is known about MRAPs in fish. MRAPs have recently been identified in some nonmammalian vertebrates (Liang et al., 2011) including rainbow trout (MRAP1 and MRAP2) and zebrafish. Unlike trout, zebrafish possess three MRAPs known as MRAP1, MRAP2a and MRAP2b (Agulleiro et al., 2010). Recently Liang and colleagues (2011) demonstrated that functioning of rainbow trout MC2R requires MRAP1, as observed in mammals and this functional MC2R is due to interaction of MC2R with amino acids located at positions 30-33 in MRAP1, which are not located in MRAP2, suggesting that like mammals, MRAP1 but not MRAP2 is crucial in MC2R trafficking and signaling. Since mutations in MRAP1 have been demonstrated to cause familial glucocorticoid deficiency (FGD) in mammalian vertebrates (Clark et al., 2005, 2009), it is possible that inhibition of ACTH-mediated cortisol by other factors such as Cd could involve disruption of MRAP1; however, this is yet to be determined.
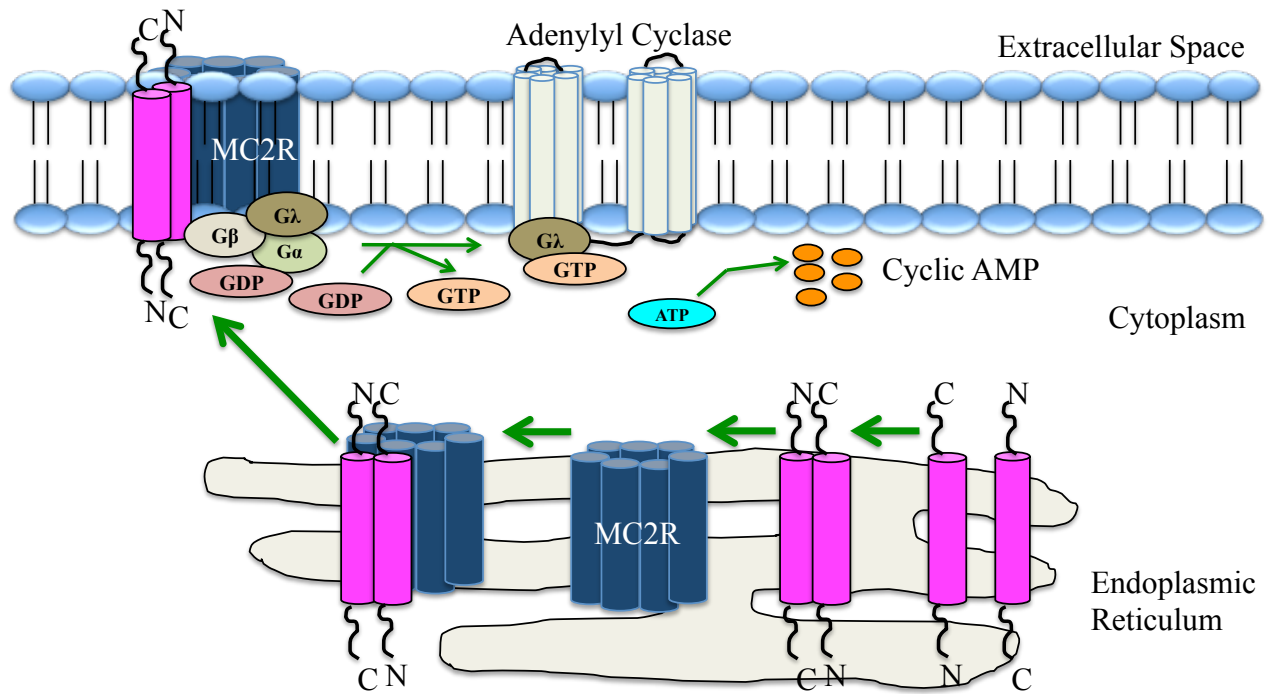
**Figure 3. Possible melanocortin 2 receptor (MC2R)/ MC2R accessory protein1 (MRAP1) interaction.**
Schematic diagram of the possible role of MRAP1 in proper functioning of MC2R in teleosts, adapted from
Clark et al., 2009. Upon production and release of adrenocorticotropic hormone (ACTH), MRAP (pink)
forms a homodimer that binds to MC2R (blue) and traffics the structure from the endoplasmic reticulum to
the plasma membrane. Upon arrival MRAP is thought to remain bound for proper interaction and binding
of ACTH or for production of an intracellular signal.

## 1.13 Cortisol biosynthesis

Under non-stressed conditions, cortisol is released in teleosts in a circadian manner to maintain regular physiological functions (Wendelaar Bonga, 1997). Stimulation of target tissue by cortisol has been implicated in a wide array of biochemical and physiological responses that are thought to be adaptive, including osmotic- and ionic-regulation, growth and metabolism, immune responses and reproduction (Laurent and Perry, 1990; Van der Boon et al., 1991; Wendelaar Bonga, 1997; Mommsen et al., 1999; Vijayan et al., 2005). In teleosts, cortisol is the primary circulating glucocorticoid and is released from the interrenal tissues (Mommsen et al., 1999). It is well established that a rapid increase in plasma cortisol occurs following an acute stress exposure and this transient increase in plasma cortisol concentrations returns to basal (resting) levels within a 24 h period (Mommsen et al., 1999; Vijayan et al., 2010). Since plasma cortisol levels rise dramatically during stress, an increase in plasma cortisol is a widely accepted indicator of stress in fish, since basal (resting) cortisol levels can be measured and are generally very low to stress-induced levels (Barton et al., 2002). Characteristically, cortisol elevations of fishes in response to acute stressors are typically in the range of 20 to 300 ng/ml, however, values can vary depending on the species of fish, hierarchy status (dominate versus subordinate) and integrity of the stress response (Wendemeyer et al., 1990; Barton and Iwama, 1991).

As previously mentioned, the secretion of cortisol in teleosts is under the control of the HPI axis (Wendelaar Bonga, 1997, Mommsen et al., 1999). Once ACTH binds to MC2R leading to a G-protein response (Midzak et al., 2011), cholesterol (the precursor of cortisol) is shuttled from the outer mitochondrial membrane to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR) (Figure 4) (Papadopoulos, 2004; Gravel and Vijayan, 2006). Once cholesterol is transported to the inner mitochondrial membrane, it is converted to pregnenolone by cytochrome P450 side chain cleavage enzyme (P450scc), specifically CYP11A1 (Figure 4) (Geslin and Auperin, 2004; Payne and Hales, 2004 Midzak et al., 2011). After pregnenolone is produced within the mitochondrion, it enters the endoplasmic reticulum and undergoes a string of hydroxylations and isomerizations to produce cortisol (Figure 4) (Mommsen et al., 1999). The order is as follows: 1) 17α-hydroxylase (17-OHase) to produce 17-α-hydroxypregnenolone; 2) 3β-hydroxysteroid dehdryogenase (3β-HSD) to produce 17-α-hydroxyprogesterone; 3) 21-hydroxylase (21-OHase) to produce11-deoxycortisol; and finally, 4) 11β-hydroxylase (11-β-OHase) to produce cortisol (Figure 4) (Mommsen et al., 1999; Geslin and Auperin, 2004; Papadopoulos, 2004).
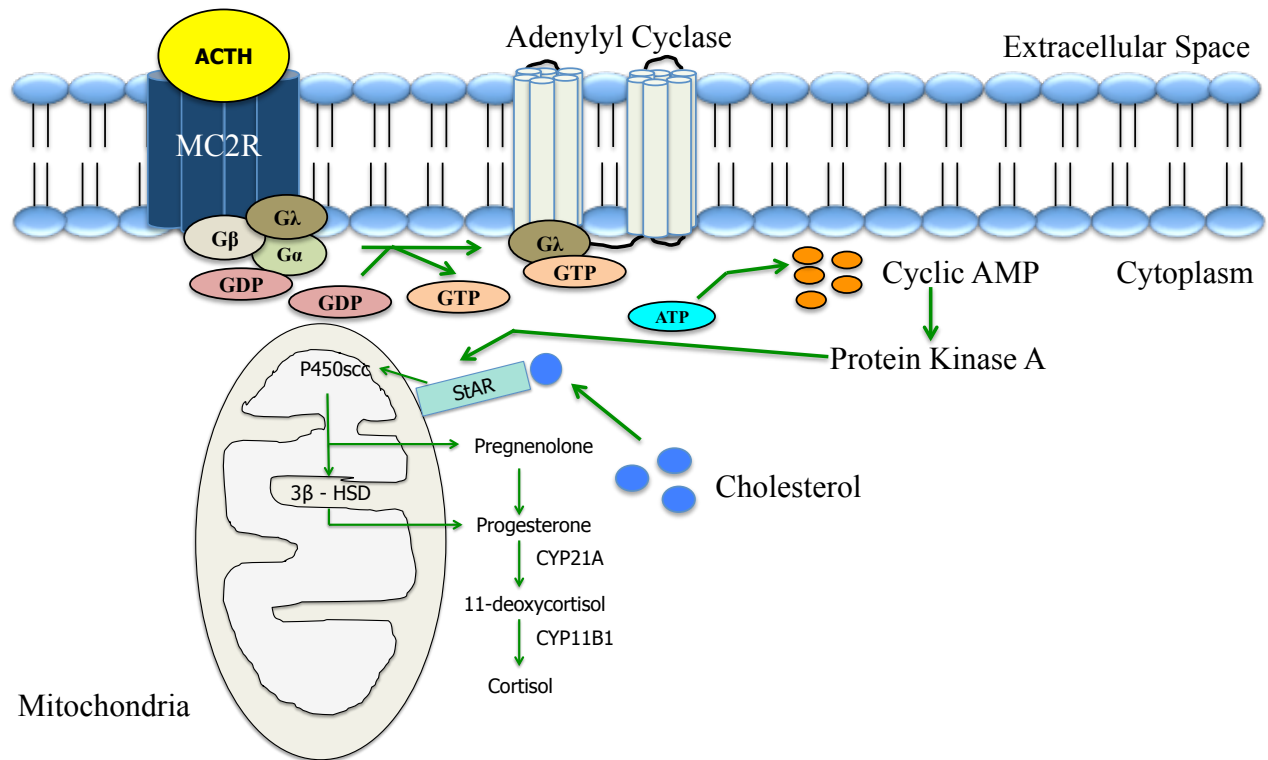
**Figure 4. ACTH-mediated corticosteroidogenesis.** Schematic diagram of G-protein coupled melanocortin 2 receptor (MC2R) signaling leading to cortisol biosynthesis. Binding of ACTH to MC2R on plasma membrane leads to increase in cyclic adenosine monophosphate (cAMP) from the activation of adenylyl cyclase (which converts ATP to cAMP). Elevation of cAMP leads to activation of protein kinase A, which phosphorylates steroidogenic acute regulatory protein (StAR). StAR then transports cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where it hydroxylated and cleaved by cytochrome P450 side chain cleavage enzyme (P450scc) to pregnenolone. Eventually through further hydroxylations and cleavages pregnenolone is converted to cortisol.

## 1.14 Cortisol release, corticosteroid receptors and negative feedback pathway

Once cortisol is produced in the interrenal cells it is circulated through the plasma and acts on target tissues (primarily the brain, liver, gill, and heart) where it provides an adaptive role in osmotic- and ionic-regulation, immune response, reproduction, growth and metabolism (Bamberger et al., 1996; Mommsen et al., 1999; Vijayan et al., 2003; Kudielka et al., 2006). In vertebrates it is well known that the transport of steroid hormones, including cortisol, in the blood in is mediated by specific protein systems (Hammond, 1995). In mammals and birds, the majority of cortisol (70-90%) is bound to cortisol binding globulin (CBG), while some cortisol (7-20%) is found bound to albumin (Gayrad et al., 1996; Aaron et al., 2004). In fish, there is no known cortisol binding protein

The mechanism(s) of action of cortisol on target tissues begin with the binding of cortisol to corticosteroid receptors, a steroid/thyroid/retinoid receptor superfamily of ligand-bound transcription factors (Bamberger et al., 1996; Mommsen et al., 1999; Aluru and Vijayan 2009). Within teleosts, corticosteroid receptors involved in cortisol signaling consist of multiple glucocorticoid receptors (GRs) and one mineralocorticoid (MR) receptor (Vijayan et al., 2005; Prunet et al., 2006; Alsop and Vijayan, 2008). Distribution of GR and MR varies in teleosts where an overall greater amount of MR is observed in the brain and a greater amount of GR is found in the kidney, gill, spleen, heart, and liver (Greenwood et al., 2003; Sturm et al., 2005; Stolte et al., 2008; Arterbery et al., 2010; Takahashi and Sakamoto, 2013).

Unlike mammals that possess a known ligand to bind to MR (aldosterone), no ligand has been found to bind specifically to MR in fish, although 11-deoxycorticosterone is a circulating corticosteroid in teleosts that can bind to MR *in vitro* (Sturm et al., 2005; Milla et al., 2006, 2008; Bury and Sturm, 2007; Kiilerich et al., 2007, 2011; Stolte et al., 2008; McCormick et al., 2008; Sakamoto et al., 2011). Furthermore, a role for the multiple GR isoforms has also not been established (Bury et al., 2003; Vijayan et al., 2005). In teleosts, cortisol binds to both GR and MR implicating a role of cortisol in hydromineral balance as aldosterone does in mammals (McCormick, 2001; McCormick and Bradshaw, 2006). Although cortisol binds to both receptors, MR typically has a 10-fold greater affinity for cortisol (Greenwood et al., 2003). The exact role of cortisol/MR in the stress response is not well known, thus most studies focus on cortisol/GR signaling (Aluru and Vijayan, 2009).

Once cortisol binds to GR the molecular chaperones inactivating GR dissociate and a homodimer forms (Figure 5). The cortisol-GR heterocomplex translocates from the cytoplasm to the nucleus where it then binds to a specific DNA region in the promoter of target genes known as glucocorticoid response elements (GREs) resulting in the transactivation or repression of glucocorticoid responsive genes (Evans,

1988, Pearce, 1994; Pickering and Pottinger, 1995; Farman and Rafestin-Oblin, 2001; Vijayan et al., 2005; Bury and Sturm, 2007; Prunet et al., 2006) (Figure 5). Two key aspects of glucocorticoid receptors are the activation of gluconeogenic genes in the liver to aid in the increased energy demand and suppression of corticotropin releasing factor (CRF) and adrenocorticotropin hormone (ACTH) from the hypothalamus and anterior pituitary, respectively, to suppress cortisol production (Figure 5) (Mommsen et al., 1999; Young, 2004; Vijayan et al., 2005). Suppression of cortisol is crucial by a negative feedback regulation as prolonged elevation of glucocorticoids in fish can result in suppression of reproduction, immune response and decrease in body weight (Wendelaar Bonga, 1997; Weyts et al., 1999; Norris, 2000).

**Figure 5. Genomic cortisol signaling.** Schematic diagram of the genomic cortisol response through glucocorticoid receptor activation. After cortisol is released into the circulation from the interrenal tissue, it crosses cell membranes and binds to glucocorticoid receptors (GR) in the cytosol leading to dissociation of molecular chaperones and dimerization of the activated receptor. The cortisol/GR heterotimeric complex then translocates to the nucleus and binds to glucocorticoid response elements (GREs) on target genes to initiate transcription.

Cortisol

Cortisol

Cortisol

GR

Molecular
Chaperones

Molecular
Chaperones

Cortisol

GR

Cortisol

GR

Cortisol

GR

Cortisol

GR

Nucleus

GRE

Genomic biological responses

23
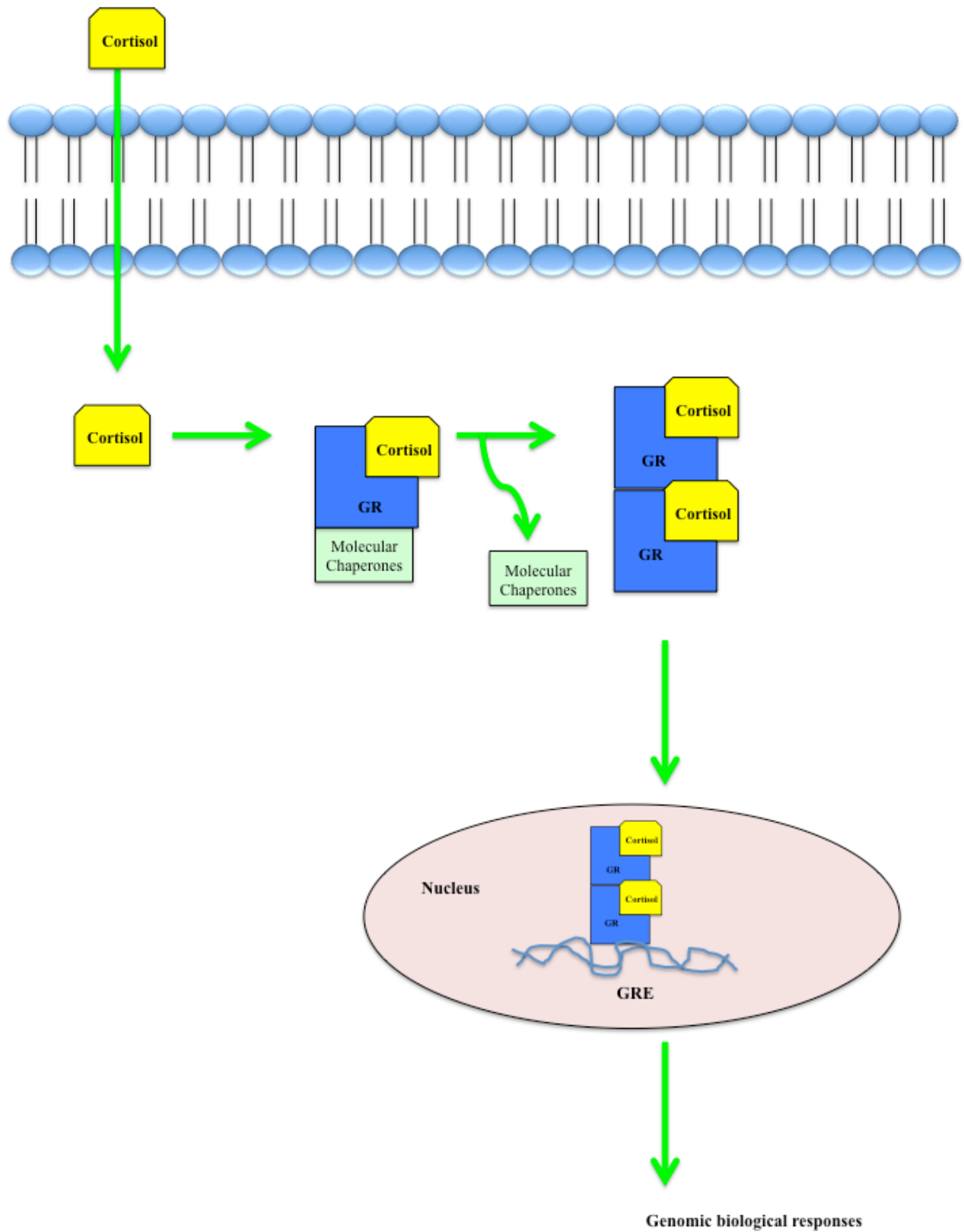
## 1.15 Metabolism

During stressor exposure, tissues including liver, brain, gills and heart show an increased energy demand (Mommsen et al., 1999). Cortisol plays a key role in stress adaptation by mobilizing energy substrates to fuel increased energy demand that is associated with stressor exposure (Vijayan et al., 2010). In teleosts, a clear link has been established between increased plasma cortisol levels and the corresponding increase in plasma glucose levels (Mommsen et al., 1999; Aluru and Vijayan, 2009; Vijayan et al., 2010; Ings et al., 2011a,b, 2012a,b). The initial stressor-induced release of glucose is mediated by catecholamine-induced glycogenolysis and the maintenance of plasma glucose levels (e.g. depletion of liver glycogen) is through cortisol-induced hepatic gluconeogenesis (Mommsen et al., 1999). In fish, cortisol has been shown to affect carbohydrate, protein and lipid metabolism, enabling the organism to increase plasma glucose to fuel homeostatic recovery following stressor exposure (Wendelaar Bonga, 1997; Mommsen et al., 1999; Vijayan et al., 2010). Plasma glucose and other substrates used for energy such as lactate have been used to assess the effects of stressors on metabolism (Vijayan et al., 1994, 2003, 2010).

A key target tissue for metabolic effects of cortisol is the liver, which increases glucose production primarily through gluconeogenesis, decreasing liver glycogen content (Figure 6) (Goldstein et al., 1992, 1993; Jones et al., 1993), and also through other mechanisms such as glycogenolysis, proteolysis and lipolysis (Mommsen et al., 1999). Previous studies have shown that both cortisol and stress increase the activity, and/or the transcript levels of key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase which is a rate-limiting step in gluconeogenesis that converts oxaloacetate into phosphoenolpyruvate and carbon dioxide as well as enzymes involved in amino acid metabolism such as alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) (Vijayan et al., 1994; Sathiyaa and Vijayan, 2003; Vijayan et al., 2003; Gravel and Vijayan, 2007; Wiseman et al., 2007) and the C3 substrates are channeled for gluconeogenesis. Glycolytic enzymes including hexokinase and pyruvate kinase, that are rate-limiting, along with lactate dehydrogenase are also upregulated in response to cortisol and/or stressor exposure (Vijayan et al., 1994; Gravel and Vijayan, 2007). The upregulation of these enzymes has also been shown to be associated with an increase in their transcript levels in the liver when there is an increase in glucose production (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003; Aluru and Vijayan, 2007; Momoda et al., 2007; Wiseman et al., 2007). Although a clear link has been shown between increased cortisol and increased plasma glucose and lactate levels, which can provide insight into energy repartioning in response to stress (Mommsen et al., 1999; Vijayan et al., 2010), very few studies have looked at target tissue metabolic adjustments, particularly in response to contaminant exposure, including

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Cd (Sangiao-Alvarellos et al., 2005, Tintos et al., 2006, 2007, Vijayan et al., 2006; Gravel and Vijayan, 2007). As the activation of HPI axis functioning is a highly conserved response in vertebrates and critical for stress adaptation, the disruption of the axis by Cd can lead to altered fish performances and reduced fitness (Hontela et al., 1992, Hontela, 1997; Vijayan et al., 1997a,b; Hontela and Vijayan, 2008, Ings et al., 2011a,b; Sandhu and Vijayan, 2011 [Chapter 3]), but the mechanisms are far from clear.
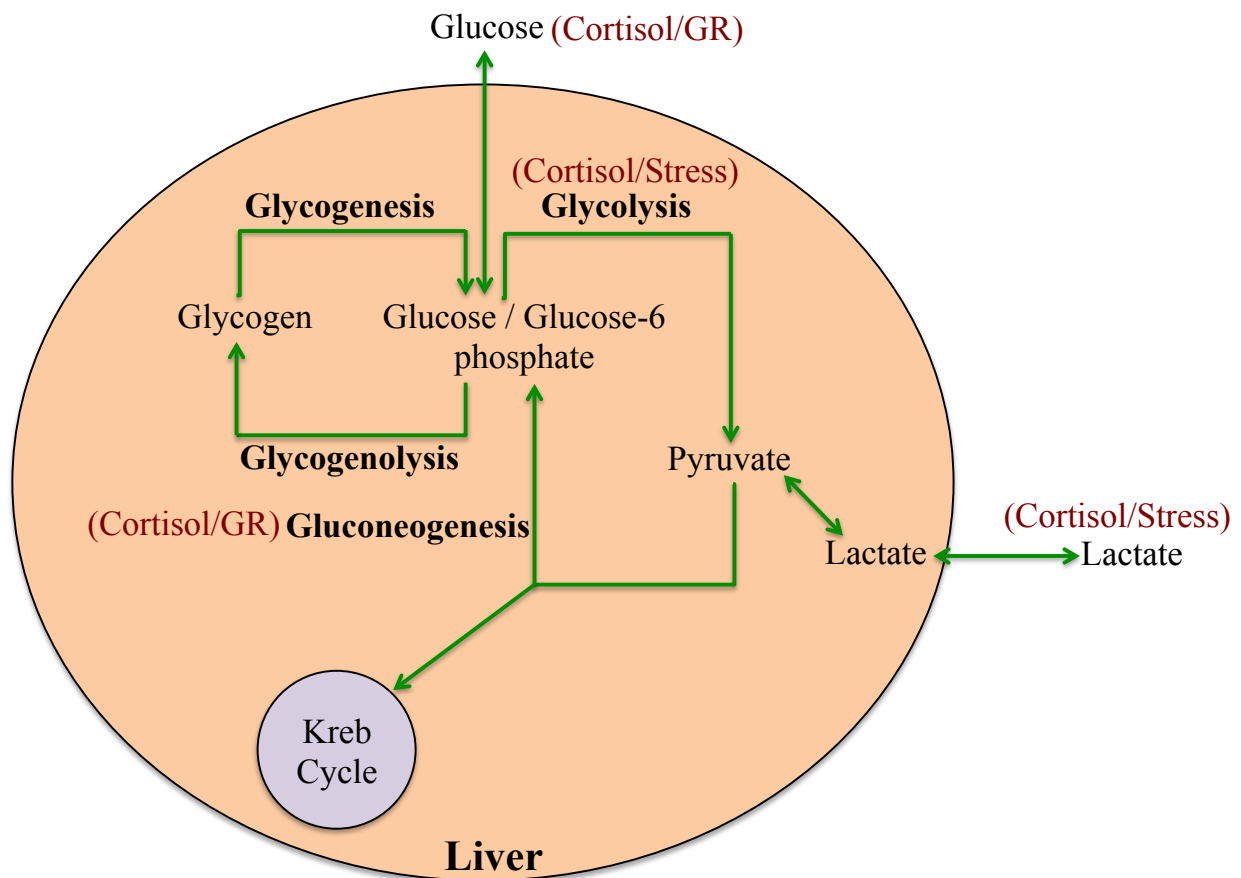
**Figure 6. Liver metabolism.** Glucose production in the liver of freshwater fish. Increase of glucose production in the liver that is associated with greater plasma cortisol levels occurs through genomic signaling to upregulate gluconeogenesis and to a lesser extent, glycogenolysis. Other pathways such as glycolysis have been previously shown to be upregulated in response to a stressor or greater plasma cortisol levels. Lactate, a byproduct of muscle proteolysis can also act as a substrate for glucose production. Once glucose is produced, it can either be used endogenously in the liver or is transported to other tissues that use glucose such as the brain and gill (Abbreviations: glucocorticoid receptor (GR)).

## 1.16 Thesis objectives

The overall objective of this thesis was to examine the impact of environmentally relevant sublethal Cd concentrations on HPI axis functioning in juvenile rainbow trout (*Oncorhynchus mykiss*). The overall objective was met by conducting a series of controlled lab studies either *in vivo* or *in vitro* using head kidney preparations to determine whole animal and metabolic responses and to investigate the mode of action of this metal in disrupting target tissue responses.

Specific objectives include:

1. Examine the impact of Cd during exposure and in response to a secondary stressor (Chapter 2);

2. Determine the impact of Cd on ACTH-mediated cortisol production within the hypothalamus-pituitary-interrenal (HPI) axis (Chapter 3);

3. Determine the impact of cadmium on the negative feedback pathway and at the level of the MC2R/MRAP1 complex (Chapter 4);

4. Determine the impact of cadmium on metabolic capacity of the liver (Chapter 5).


Specific hypotheses include:

1. Subchronic Cd exposure affects hormonal and metabolic levels during exposure and in response to a secondary stressor in rainbow trout *in vivo* (Chapter 2);

2. Cd exposure disrupts ACTH-mediated cortisol production by disrupting the HPI axis in rainbow trout *in vitro* (Chapter 3);

3. Subchronic Cd exposure impacts cortisol production through disruption at the hypothalamus and/or at the level of the melanocortin 2 receptor and its accessory proteins in rainbow trout *in vivo* and *ex vivo* (Chapter 4);

4. Exposure to Cd impacts liver target tissue response to cortisol, which is important in energy production during stress response *in vitro* (Chapter 5).

# Chapter 2

**Exposure to environmental levels of waterborne cadmium impacts corticosteroidogenic and metabolic capacities, and compromises secondary stressor performance in rainbow trout**

## 2.1 Overview

The physiological responses to waterborne cadmium exposure have been well documented; however, few studies have examined animal performances at low exposure concentrations of this metal. We tested the hypothesis that longer-term exposure to low levels of cadmium will compromise the steroidogenic and metabolic capacities, and reduce the cortisol response to a secondary stressor in rainbow trout (*Oncorhynchus mykiss*). To test this, juvenile rainbow trout were exposed to 0 (control), 0.75 or 2.0 µg/L waterborne Cd in a flow-through system and were sampled at 1, 7 and 28 d of exposure. There were only very slight disturbances in plasma cortisol, lactate or glucose levels in response to cadmium exposure over the 28 d period. Chronic cadmium exposure significantly affected key genes involved in corticosteroidogenesis, including melanocortin 2 receptor (MC2R), steroidogenic acute regulatory protein (StAR) and cytochrome P450 side chain cleavage enzyme (P450scc). At 28 d, the high cadmium exposure group showed a significant drop in the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) protein expressions in the liver and brain, respectively. There were also perturbations in the metabolic capacities in the liver and gill of cadmium-exposed trout. Subjecting these fish to a secondary handling disturbance led to a significant attenuation of the stressor-induced plasma cortisol, glucose and lactate levels in the cadmium groups. Collectively, although trout appears to adjust to subchronic exposure to low levels of cadmium, there may be a cost associated with impaired interrenal steroidogenic and tissue-specific metabolic capacities, that may play a role in the attenuated stress performance in rainbow trout.

## 2.2 Introduction

Fish encounter elevated cadmium (Cd) concentrations in their aquatic environment due to both natural and anthropogenic sources, which usually results in elevated Cd bioaccumulation in tissues (Hollis et al., 1999, 2001; reviewed by McGeer et al 2011). In vertebrates, Cd has no known physiological function but can exert toxicity at sublethal concentrations (McGeer et al 2011). The primary route of entry of Cd in fish is through their gills and/or GI tract and accumulates predominately in the kidney, gills and liver, and to a lesser extent in the brain (McGeer et al., 2007; Kamunde, 2009; McGeer et al 2011). The effects of chronic exposure to waterborne Cd at sublethal concentrations include disturbances in whole-body or plasma ion homeostasis, modifications in tissue-specific enzyme activities and metabolic capacity, as well as endocrine disruption (see McGeer et al 2011).

A well-known endocrine disrupting effect of sublethal Cd exposure involves the impairment of cortisol stress axis functioning in fish (Brodeur et al., 1997, 1998; Ricard et al., 1998; Lacroix and Hontela, 2004; Raynal et al., 2005; Lacroix and Hontela, 2006; Lizardo-daudt et al., 2007; Hontela and Vijayan, 2008;

Sandhu and Vijayan, 2011). The corticosteroid stress response in teleosts involves the activation of the hypothalamus-pituitary-interrenal (HPI) axis culminating in the release of cortisol into circulation (Aluru and Vijayan, 2009). The primary hormonal step in HPI activation involves the secretion of corticotropin-releasing factor (CRF) that stimulates the pituitary to produce adrenocorticotrophic hormone (ACTH), a proopiomelanocortin (POMC)-derived hormone (Aluru and Vijayan, 2009). ACTH activates melanocortin 2 receptor (MC2R), a G-protein coupled receptor leading to corticosteroid biosynthesis (Metz et al., 2006; Aluru and Vijayan, 2008a). The key steps in cortisol biosynthesis is thought to include the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR), as well as the conversion of cholesterol to pregnenolone catalyzed by the cytochrome P450 side chain cleavage enzyme (P450scc) (Payne and Hales, 2004; Stocco et al., 2005; Aluru and Vijayan, 2006). The terminal step in corticosteroid biosynthesis involves the conversion of deoxycortisol to cortisol by 11-β hydroxylase (Payne and Hales, 2004; Aluru and Vijayan, 2006).

The target tissue cortisol action is mediated by activation of either the glucocorticoid receptor (GR) and/or the mineralocorticoid receptor (MR) (Bury et al., 2003; Greenwood et al., 2003; Sturm et al., 2005; Prunet et al., 2006; Aluru and Vijayan, 2009; Takahashi and Sakamoto, 2013). In teleosts, unlike mammals, there are multiple copies of GRs but a functional role for these isoforms has not been established (Bury et al., 2003; Prunet et al., 2006; Aluru and Vijayan, 2009). The cortisol response during stress allows for physiological adjustments that are essential to restore homeostasis (Mommsen et al., 1999; Aluru and Vijayan, 2009). A primary role for stressor-induced cortisol elevation is to allow for metabolic adjustments, including stimulation of gluconeogenesis in the liver to restore homeostasis (Mommsen et al., 1999). While Cd has been shown to target the HPI axis and disrupt cortisol production (Hontela and Vijayan, 2008; Sandhu and Vijayan, 2011), the effect of subchronic exposure to environmentally relevant levels of Cd on target tissues involved and the mechanisms of action are far from clear.

We tested the hypothesis that subchronic exposure to Cd disrupts the metabolic capacity and compromises the cortisol stress performance to a secondary stressor in juvenile rainbow trout (*Oncorhynchus mykiss*). This was tested using a water-borne exposure in a flow-through system as described before (McGeer et al., 2000 a, b) and further detailed in Milne et al. (2013). Trout were exposed to either 0 (control), 0.75 (low exposure) or 2.0 μg/L (high exposure) Cd for 28 d and then subjected to a 5 min handling disturbances as described before (Ings et al., 2012). We examined plasma cortisol, glucose and lactate levels as markers of stress response, while transcript abundances of MC2R, StAR and P450scc in the head kidney tissue were

used as markers of steroidogenic capacity. Liver and gill metabolic capacities were determined by measuring the activities of glycolytic (hexokinase, glucokinase, pyruvate kinase and lactate dehydrogenase), gluconeogenic (phosphoenolpyruvate carboxykinase) and oxidative (citrate synthase) enzymes, while GR and MR protein expressions in the liver and brain were used to assess target tissue cortisol responsiveness.

### 2.2.1 Chemicals

Tricaine methanesulfonate (TMS) and sodium bicarbonate were purchased from Syndel Laboratories Ltd., (Vancouver, BC, CAN). Borosilicate and scintillation tubes, monobasic and dibasic sodium phosphate, potassium bicarbonate, perchloric acid, potassium chloride and sodium bicarbonate were purchased from Fisher Scientific (Fairlawn, NJ, USA). Scintillation cocktail and cortisol antibody were purchased from MP Biomedicals (Solon, OH, USA). [1,2,6,7-3H] cortisol tracer and ECl plus western detection system were purchased from GE Healthcare (Upsala, Sweden). D-glucose, Tween 20, Cadmium chloride and 96-well plates were purchased from Bioshop (Burlington, ON, CAN). Thimerasol, activated charcoal, dextran (from *Leuconostoc mesenteroides*) amyloglucosidase, imidazole, beta-nicotinamide adenine dinucleotide disodium salt (β-NADH), phosphoenol pyruvate (PEP), lactate dehydrogenase (LDH), pyruvate kinase, adenosine diphosphate (ADP), adenosine triphosphate (ATP), deoxyguanosinediphosphate (2-DGDP), magnesium chloride and sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Manganese chloride was purchased from Roche (Mannheim, Germany). Protein ladder and Sybr green for qPCR were purchased from Bio-Rad (Hercules, CA, USA).

### 2.3.2 Experimental animals and Cd exposure

Juvenile rainbow trout (27 ± 8 g) were obtained from Rainbow Springs Hatchery (Thamesford, ON, CAN) and held at Wilfrid Laurier University using previously described methods (McGeer et al., 2000a,b) and described in detail by Milne (2010). Initially, 420 fish were randomly divided among two 220 L polyethylene tanks (RTS Plastics, New Hamburg, ON, CAN) and acclimated for two weeks to flowing water at 140 mg/L $CaCO_3$ with Ca, Mg and Na concentrations of 868 ± 28, 480 ± 16 and 338 ± 16 uM (mean ± SEM), pH 7.2 and 11ºC) that was a mixture of local well water and reverse osmosis product water. Fish were fed 2% of their body weight daily as a single meal (Bio Oregon Protein Inc., Warrenton, OR, USA) and photoperiod (16 h: 8 h; light:dark) was maintained throughout the experiment.

Following the acclimation period, trout were nonselectively distributed to six 220 L polyethylene tanks (70 per tank) supplied with flowing (700 ml/min) water. Continuously flowing well and

reverse osmosis water to achieve the desired chemistry (see previous paragraph) were mixed in a 60 L polyethylene head tank and then delivered to three smaller 10 L polypropylene exposure head tanks before distribution to fish tanks. Concentrated stock solutions of Cd (as $CdCl_2$) were metered (QG6 pump, Fluid Metering Inc, New York) into two of the exposure head tanks to achieve the desired waterborne Cd concentration (either 0.75 or 2.0 µg Cd/L). Test solution (or unmodified (control) water) delivery from the exposure head tanks was split to replicate fish tanks (n=2 tanks of 70 fish each for control, 0.75 or 2.0 µg Cd/L). Measured total Cd concentrations were determined using graphite furnace atomic absorption spectrometry (see Milne et al 2013) and were $0.03 \pm 0.0002$ µg Cd/L, $0.71 \pm 0.101$ µg Cd/L and $1.85 \pm 0.119$ µg Cd/L for control, low and high exposures, respectively. Exposures were initiated by spiking tanks with appropriate volumes of concentrated stock solution to achieve the target exposure concentrations and throughout the exposure all header and fish tanks were aerated. The concentrated Cd stock solutions were renewed weekly and water pH , conductivity and temperature ) were also measured daily weekly using a pH meter (Seven Go, Mettler Toledo, Fisher Scientific, Mississauga ON) and a conductivity meter (YSI 30, Yellow Springs Instruments, Yellow Springs, OH). Feeding was stopped 24 h prior to sampling.

### *2.3.3* Sampling

The Cd exposure study was conducted for 28 d and fish were sampled at 1, 7 and 28 d after the start of Cd exposure. At each sampling time point 8 fish were sampled, except on day 28 for the 2.0 µg Cd/L Group, where only 6 fish (3 from each tank) were samples, from each treatment group (4 fish per replicate exposure tank) and euthanized quickly with an overdose of TMS buffered with an equal weight of sodium bicarbonate. On the last sampling day (28 d), only 6 fish (3 from each replicate tank) were sampled from the 2.0 µg Cd/L group.

Fish were bled by severing the caudal peduncle. The blood was collected in heparinized tubes and centrifuged at 10, 000 x g for 2 min to separate the plasma and stored at -80 °C until cortisol, glucose and lactate analysis. Plasma samples were used to measure cortisol, glucose and lactate levels in both primary (Cd) and secondary (chasing and netting) fish. Tissues (brain, liver and head kidney) were samples on 1, 7 and 28 d post-Cd exposure to assess enzyme activities, metabolite level, protein expression and mRNA abundances.

### 2.2.4 Acute stressor exposure

After 28 d exposure, fish in each group were subjected to a handling disturbance for 5 min as described previously (Ings et al., 2011). Fish were sampled at 1 and 24 h post-handling stressor. 0 h time point constitutes the pre-stress samples at 28 d

### 2.2.5 Plasma cortisol

Plasma cortisol was measured using a radioimmunoassay (RIA) as previously described (McMaster et al., 1995; Alsop and Vijayan, 2008).

### 2.2.6 Plasma glucose and lactate analysis

Plasma glucose and lactate were measured using commercially available colormetric kits (Raichem, CA, USA and Trinity Biotech, St Louis, MO, USA, respectively) as described in (Ings et al., 2011b).

### 2.2.7 Tissue preparation

Tissue preparation for protein and enzyme analyses followed the protocols described previously (Dindia et al., 2012; Ings et al., 2012). Briefly, 50 mg of tissue was added to 200 µL of 50 mM Tris and 1X proteinase inhibitor (PI). Samples for immunodetection were then diluted to a concentration of 2 mg/ml in Laemmli's buffer (Laemmli, 1970), and an aliquot was taken for enzyme determinationand added to 50% glycerol buffer (50% glycerol, 21 mM $Na_2HPO_4$, 0.5 mM EDTA-Na, 0.2% BSA, 5 mM β-mercaptoethanol, pH 7.5) as described before (Ings et al., 2012).

### 2.2.8 Liver glycogen analysis

Glycogen content (28 d samples only) was determined as glucose before and after hydrolysis to glucose with amyloglucosidase according to Ings et al., 2012. Briefly, samples were mixed with 35% perchloric acid (PCA) and endogenous glucose was measured colormetrically using glucose colour reagent as previously mentioned above. Samples were then mixed with $KHCO_3$ and amyloglucosidase (1 mg/ml in sodium acetate buffer) and incubated at 40 °C for 2 h, and the reaction stopped by the addition of 35% PCA and glucose measured.

### 2.2.9 Enzyme activity

All reagents were prepared using 50 mM imidazole buffer (pH 7.5) and liver and gill enzyme activities were measured kinetically at 340 nm at room temperature using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA) as described before (Vijayan et al., 2006). The enzymes

measured included glucokinase (GK), hexokinase (HK), lactate dehydrogenase (LDH), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK) and citrate synthase (CS). The following assay conditions were used:

- **HK (EC 2.7.1.1):** 1 mM glucose, 5 mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM PEP, 5 U/ml LDH, and 2.5 U/ml PK; reaction started with 1 mM ATP.

- **GK (EC 2.7.1.2):** 15 mM glucose, 5 mM $MgCl_2$, 10 mM KCl, 0.25 mM Nicotinamide adenine dinucleotide (NADH), 2.5 mM phosphoenolpyruvate (PEP), and 5 U/ml LDH, 2.5 U/ml PK; reaction started with 1 mM adenosine triphosphate (ATP).

- **PK (EC 2.7.1.40):** 10 mM $MgCl_2$, 3 mM KCl, 0.12 mM NADH, 20 U/ml LDH, 0.05mM PEP, and 2.5 mM ADP; reaction started with 2.5 mM PEP.

- **LDH (EC 1.1.1.27):** 0.12 mM NADH; reaction started with 1mM pyruvic acid

- **PEPCK (EC 4.1.1.32):** 20mM $NaHCO_3$, 0.12 mM NADH, 0.5 mM PEP, and 1 mM $MnCl_2$; reaction started with 5mM deoxyguanosine diphosphate (DGDP).

- **CS (E.C. 2.3.3.1):** 12 mM 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB), and 4 mM Acetylcoenzyme A; reaction started with 2.4 mM OXA. Soup was prepared in Tris-HCl buffer at pH 8.0 and samples were measured at 405nm.

The enzyme activity is expressed as micromole of substrate consumed or product liberated per minute (U) per gram protein.

## 2.2.10 GR and MR protein expression

Protein concentration was measured using the bicinchoninic acid (BCA) method with bovine serum albumin as a standard. Sample preparation and immunodetection followed the protocols described previously (Sathiyaa and Vijayan, 2003; Dindia et al., 2012). Briefly brain and liver samples in Laemmli's buffer were loaded (40 µg protein/sample) onto 8% SDS-PAGE gel. Samples were run for 40 min at 200 V using 1 X TGS running buffer and the separated proteins were transferred onto a nitrocellulose membrane using a semidry transfer unit (Bio-Rad, Hercules, CA, USA) at 20 V for 25 min in a transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine and 10% (vol/vol) methanol). The membranes were blocked for 1 h with 5%

skim milk in 1 X TTBS buffer (20 mM Tris, 300 mM NaCl, 0.1% Tween 20, with 0.02% sodium azide pH 7.5).

The blots were incubated with either rabbit polyclonal anti-trout GR (1:1000; Sathiyaa & Vijayan, 2003) for 1 h at room temperature or rabbit polyclonal anti-trout MR (1:1000; Jeffrey et al., 2012) overnight for 18 h at 4 °C. Blots were subsequently washed in 1 X TTBS three times (10 min/wash) and followed with a 1 h incubation with either alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000; Bio-Rad, Hercules, CA, USA) for GR or HRP-conjugated goat anti-rabbit IgG (1:3000; Bio-Rad, Hercules, CA, USA) for MR. Blots were then washed again with 1 X TTBS (3x10 min/wash) followed by one final wash in 1 X TBS (20mM Tris, 300mM NaCl, pH 7.5) for 10 min. The protein bands were detected using either NBT (0.033% w/v) and BCIP (0.017% w/v) color substrates (Bio-Rad, Hercules, CA, USA) for GR or ECl plus detection system (GE Healthcare, Upsala, Sweden) for MR. The blots were scanned and the intensities were quantified using AlphaEase software (AlphaEase, Innotech, San Leandro, CA, USA). Equal loading of samples were confirmed by probing the blots with β-actin (Cy3-coupled monoclonal primary antibody produced in mouse at 1:1000; Sigma-Alrdich, St. Louis, MO, USA).

### 2.2.11 RNA isolation and cDNA synthesis

Total RNA (DNase treated) was isolated from head kidney tissue using the RNeasy Mini Kit protocol (Qiagen, Mississauga, ON, CAN), and the RNA was quantified using a NanoDrop$^{TM}$ spectrophotometer (Thermo Scientific, Nepean, ON, CAN) at 260/280 nm. First strand cDNA was synthesized from 1 µg total RNA using First strand cDNA synthesis kit (MBI Fermentas, Burlington, ON, CAN). Briefly, total RNA was heat denatured (70 °C) and cooled on ice, and the sample was used in a 20 µl reverse transcriptase reaction using 0.5 µg oligo (dT) primers and 1 mM each dNTP, 20 U ribonuclease inhibitors and 40 U M-MuLV reverse transcriptase. The reaction was started by incubating at 37 °C for 1 h and stopped by heating for 10 min at 70 °C.

### 2.2.12 Primers

The primers (Table 1) were designed using rainbow trout MC2R, StAR and P450scc sequences and zebrafish β-actin sequence as the housekeeping gene (Gen-Bank accession nos. EU119870, AB047032, S57305.1 and AF157514 respectively). The primer pairs amplified ~ 100 bp fragments for genes used in quantitative real-time PCR (qPCR) (iCycler, Bio-Rad, Hercules, CA, USA). Table 1 lists primer names, sequence, melting temperature and product size for all genes analyzed.

**Table 1. Primer details.** Oligonucleotide primers [forward (F) and reverse (R)] for melanocortin 2 receptor (MC2R), steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (P450scc) and β-actin used in quantitative real-time PCR along with their melting temperature ($T_m$) and amplicon size.

| Gene | Primer | Sequence (5'-3') | $T_m$ | Product Size (bp) |
|------|--------|------------------|-------|-------------------|
| MC2R | F: | GAGAACCTGTTGGTGGTGGT | 63.9°C | 105 |
| | R: | GAGGGAGGAGATGGTGTTGA | 64.1°C | |
| StAR | F: | TGGGGAAGGTGTTTAAGCTG | 63.8°C | 101 |
| | R: | AGGGTTCCAGTCTCCCATCT | 63.8°C | |
| P450scc | F: | GCTTCATCCAGTTGCAGTCA | 64.1°C | 140 |
| | R | CAGGTCTGGGGAACACATCT | 63.1°C | |
| β -actin | F | TGTCCCTGTATGCCTCTGGT | 64.5°C | 121 |
| | R | AAGTCCAGACGGAGGATGG | 64.1°C | |

## 2.2.13 Real time quantitative PCR (qPCR)

Quantification of transcript levels was performed as previously described (Gravel and Vijayan, 2006) using qPCR (iCycler, Bio-Rad, Hercules, CA, USA). Briefly, cDNA samples were used as template for amplification of the housekeeping gene (β-actin) and target genes (MC2R, StAR and P450scc) using appropriate primers and their respective annealing temperatures (Table 1). A master mix containing Sybr green and RNase free water was prepared and the specific primers were added to 2 µL of sample. The samples were performed in triplicates on a 96-well plate and PCR was performed to amplify the predicted products using the following conditions: initial denaturation for 2 min at 94 °C, 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. PCR products were subjected to melt curve analysis to confirm presence of a single amplicon. Negative controls with no template were carried out for each gene analyzed. Transcript Ct values for β-actin between treatments and across time points were similar and, therefore, used as the housekeeping gene for normalization and the data are shown as percent control (0 µg Cd/L).

## 2.2.14 Statistical analysis

All statistical testing was performed with Sigmaplot 11 (Systat Software Inc., San Jose, CA, USA) and data are shown as mean ± standard error of mean (SEM). Two-way analysis of variance (ANOVA) was used to determine changes in cortisol, glucose, lactate and transcript levels between treatments and sampling day.  Liver glycogen, liver and gill enzyme activities, and liver and brain protein expressions were compared using a one-way ANOVA. Significant differences between groups were compared using  the Holm-Sidak *post hoc* test or Student-Nuwman Keul's test for comparison of ranks. The data were transformed, wherever necessary, to meet assumptions of homogeneity of variance, although non-transformed data are shown in the table and figures. A probability level of P<0.05 was considered significant.

## 2.3 Results

### 2.3.1 Plasma analysis

Plasma cortisol levels were <3 ng/ml in all treatment groups over the 28 d period (Table 2). There was a significantly lower plasma cortisol levels in the Cd groups compared to the control group on day 1 and day 28 (only in the 2.0 µg Cd/L) but not on day 7 (Table 2). There was also a time effect and plasma cortisol levels were significantly lower at 7 and 28 d compared to day 1 (Table 2). There was no significant treatment effect on plasma glucose concentrations at 1 or 28 d post-exposure, but on day 7 glucose levels were significantly higher in the 2.0 µg Cd/L compared to the control group (Table 2). There was an overall time effect and plasma glucose levels were significantly higher at 7 and 28 d compared to day 1 (Table 2). There were no significant treatment effect on plasma lactate levels at 1 or 7 d, but on day 28 lactate levels were significantly higher in the cadmium groups compared tio the control group (Table 2).

### 2.3.2 MC2R, StAR and P450scc mRNA levels in the head kidney

MC2R mRNA levels were significantly higher in the 2.0 µg Cd/L group on all days compared to the 0.75 µg Cd/L s and control groups (Figure. 1A). MC2R mRNA levels were also significantly higher in the 0.75 µg Cd/L group compared to the control on days 1 and 28, but was significantly lower on day 7 (Figure. 1A). StAR mRNA levels were significantly higher in the 2.0 µg Cd /L group on all days compared to the control but not the 0.75 µg Cd / L group ( (Figure. 1B). StAR mRNA abundance was also significantly higher in the 0.75 µg Cd/L group compared to the control group at 28 d but not at 1 or 7 d of exposure (Figure. 1B). P450scc mRNA levels were significantly higher in the cadmium groups at 1 (only in the 2.0 µg Cd/L) and 28 d but not at 7 d compared to eh control groups ((Figure.1C).

**Table 2. Plasma analysis.** Plasma cortisol (ng/ml), glucose (mM) and lactate (mM) concentrations in juvenile rainbow trout sampled at 1, 7 and 28 d after exposure to 0 (control), 0.75 (low dose) or 2.0 (high dose) µg/L Cd. Values are expressed as mean ± SEM (n=8 for control and 0.75 µg/L; n=6 for 2.0 µg/L). Different letters indicate significant differences (P<0.05, two-way ANOVA) between time-points (time effect column). Asterisks indicate significant differences from control within a time-point (P<0.05, one-way ANOVA).

| Sampling Day | Stress Parameter | Control | 0.75 µg/L | 2.0 µg/L | Time Effect |
|---|---|---|---|---|---|
| 1 | | 2.95 ± 0.43 | 1.36 ± 0.40* | 1.36 ± 0.78* | A |
| 7 | Cortisol | 0.23 ± 0.17 | 1.30 ± 0.39 | 0.47 ± 0.45 | B |
| 28 | | 2.0 ± 0.58 | 0.84 ± 0.31 | 0.39 ± 0.30* | B |
| 1 | | 1.59 ± 0.07 | 1.52 ± 0.05 | 1.43 ± 0.04 | A |
| 7 | Glucose | 1.72 ± 0.07 | 1.83 ± 0.08 | 2.08 ± 0.17* | B |
| 28 | | 1.92 ± 0.08 | 1.86 ± 0.09 | 1.84 ± 0.04 | B |
| 1 | | 0.85 ± 0.07 | 0.81 ± 0.06 | 0.88 ± 0.05 | |
| 7 | Lactate | 0.79 ± 0.06 | 0.89 ± 0.09 | 0.89 ± 0.10 | |
| 28 | | 0.75 ± 0.08 | 0.99 ± 0.09* | 0.86 ± 0.07* | |

**Figure 1. Head kidney steroidogenic genes.** MC2R (A), StAR (B) and P450scc (C) mRNA levels in rainbow trout at 1, 7, and 28 d after exposure to 0, 0.75 or 2.0 µg/L Cd. Values are expressed as mean ± SEM (n=6); different letters within each sampling day are significantly different (one-way ANOVA; P<0.05).

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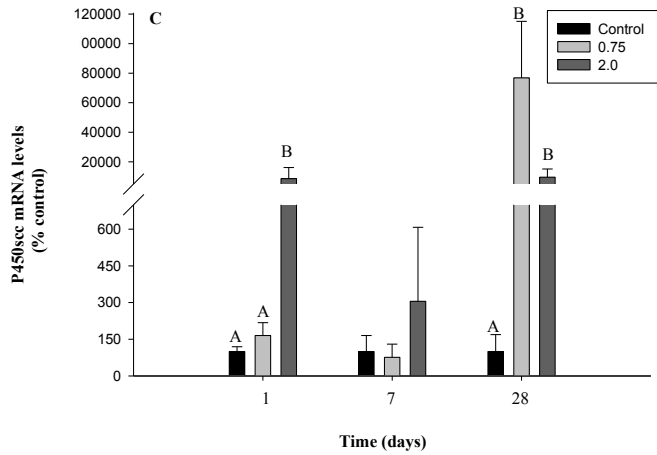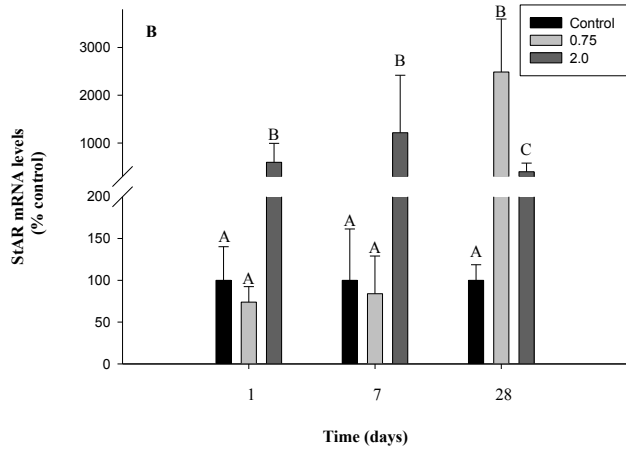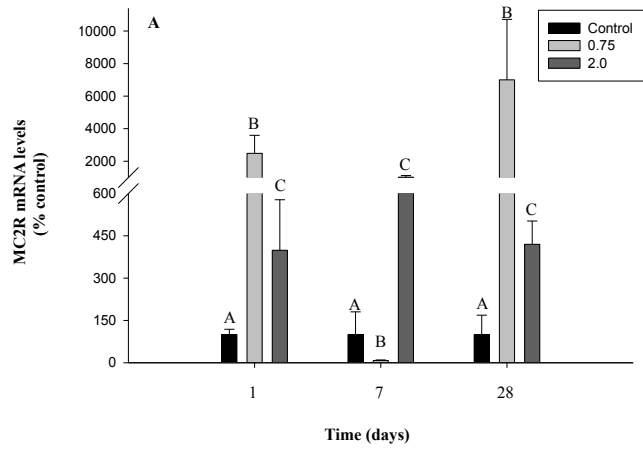
### 2.3.3 Liver glycogen content and enzyme capacity

Liver glycogen content in fish treated with 0.75 or 2.0 µg/L Cd were ~68% and ~52% significantly lower than control fish (Figure 2A). There was a significant increase of ~ 30% in liver HK (Figure 2B) and GK (Figure 2C) activities in both 0.75 and 2.0 µg/L Cd treated fish compared to the controls. No significant changes were seen in liver PK (Figure 2D), LDH (Figure 2E) or PEPCK (Figure 2F) activities between control and Cd treated groups.

### 2.3.4 Gill enzyme capacity

There was no significant difference in HK or PK activity between control and 0.75 µg/L Cd treated fish, whereas there was a ~20% significant increase in HK and PK activity in fish treated with 2.0 µg/L Cd compared to control fish (Figure 3A and 3B). No significant differences were seen in either LDH (Figure 3C) or CS (Figure 3D) activities between the control and Cd groups.

### 2.3.5 GR and MR protein expression in the liver and brain

At 28 d, GR protein expression in the liver was slightly lower (~60%) in fish treated with 2.0 µg Cd/L, but not 0.75 µg Cd/L, compared to the control group (Figure 4A), while there was no significant effect of Cd exposure on MR protein expression (Figure 4B). In the brain, there were no significant effect of Cd exposure in GR protein expression (Figure 4C), but MR protein expression was significantly lower (~50%) in fish exposed to 2.0 µg Cd/L, but not to 0.75 µg Cd/L, compared to control fish (Figure 4D)

**Figure 2. Liver metabolic capacity.** Liver glycogen (A) and activities of hexokinase (B), glucokinase (C), pyruvate kinase (D), lactate dehydrogenase (E), and phophoenolpyruvate carboxykinase (F) in rainbow trout after exposure to 0, 0.75, or 2.0 µg/L Cd for 28 d. Glycogen content is shown as micromoles glucosyl units per mg protein, while enzyme activity is expressed as micromoles of substrate consumed or product liberated per minute (U) per gram protein. Values are expressed as mean ± SEM (n=8 for control and 0.75 µg/L; n=6 for 2.0 µg/L). Bars with different letters are statistically significant (one-way ANOVA; $P<0.05$).

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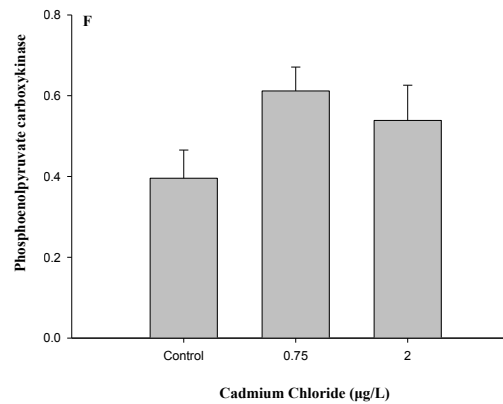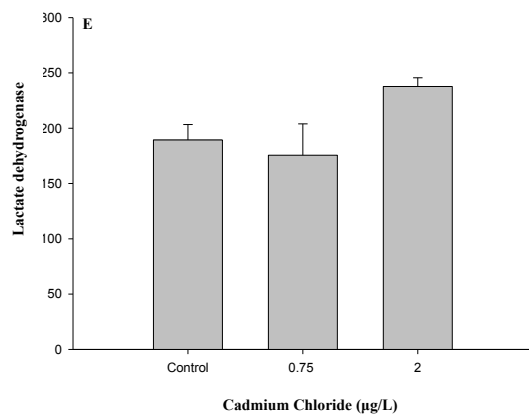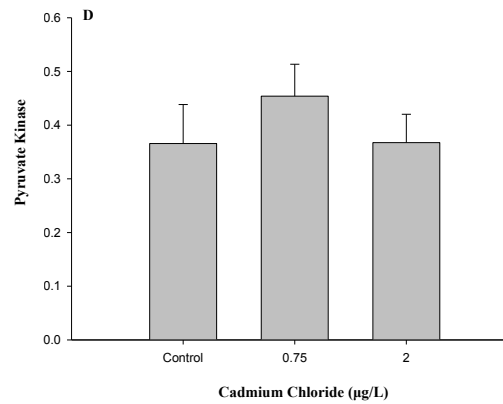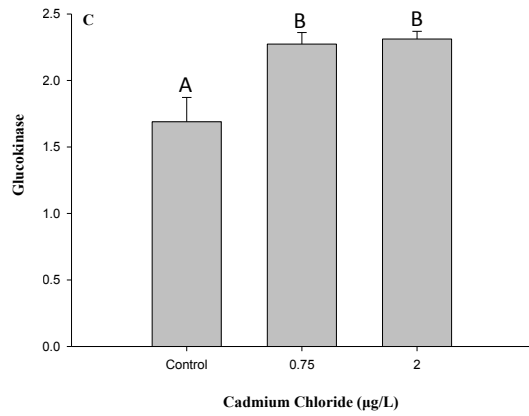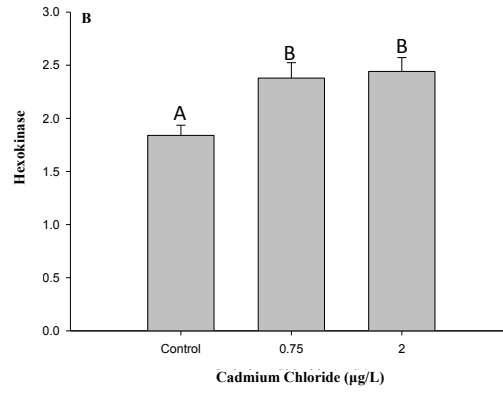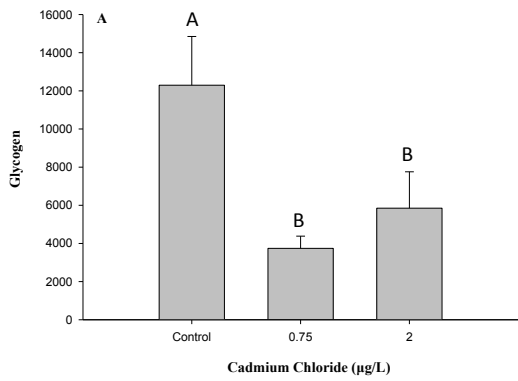
**Figure 3. Gill metabolic capacity.** Gill hexokinase (A), pyruvate kinase (B), lactate dehydrogenase (C), and citrate synthase (D) activities in rainbow trout after exposure to 0, 0.75, or 2.0 µg/L Cd for 28 d. Enzyme activity is expressed as micromoles of substrate consumed or product liberated per minute (U) per gram protein Values are expressed as mean ± SEM (n=8 for control and 0.75 µg/L; n=6 for 2.0 µg/L). Bars with different letters are statistically significant (one-way ANOVA; P<0.05).

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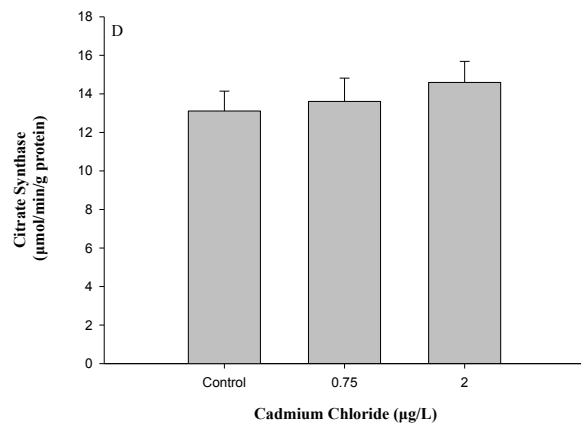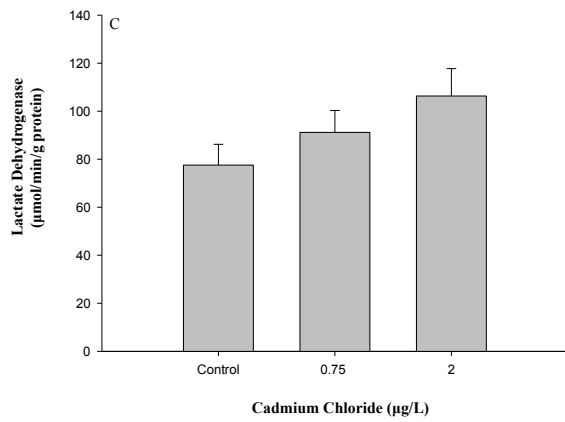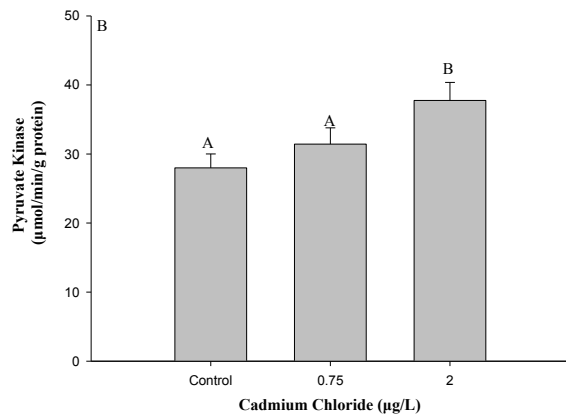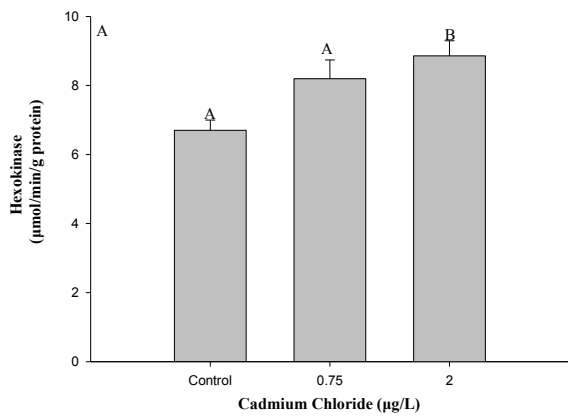
**Figure 4. Corticosteroid receptor protein expression.** Liver GR (A) and MR (B) and brain GR (C) and MR (D) protein expression in rainbow trout 28 d after exposure to 0, 0.75, or 2.0 µg/L Cd. The image above each histogram is a representative western. Values represent mean ± SEM (n=8 fish for control and 0.75 µg/L; n=6 for 2.0 µg/L). Bars with different letters are statistically significant (one-way ANOVA; $P<0.05$).

Control   0.75μg/L   2.0μg/L

90kDa

Control   0.75μg/L   2.0μg/L

96kDa

A

B

Control   0.75μg/L   2.0μg/L

Control   0.75μg/L   2.0μg/L

C

D

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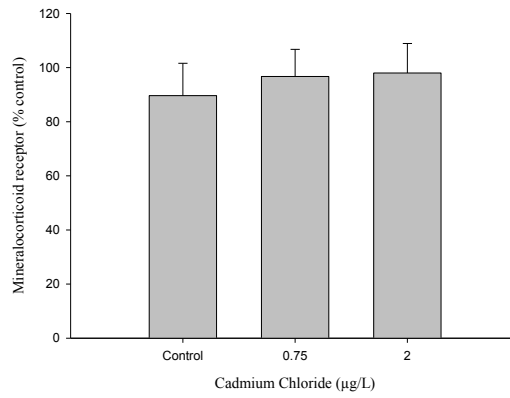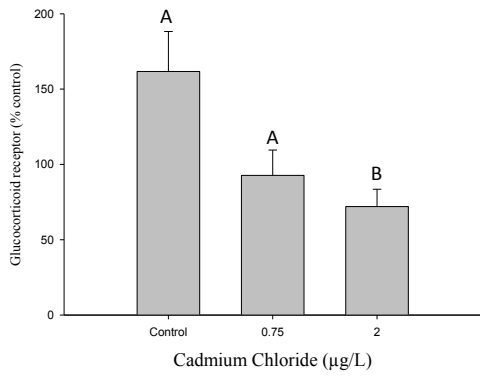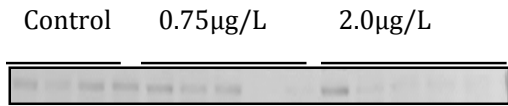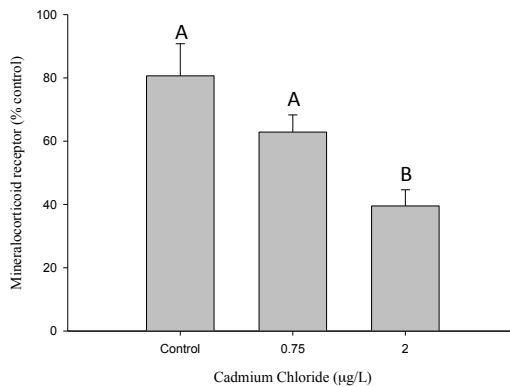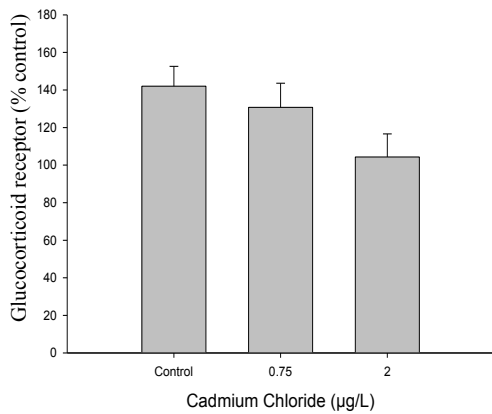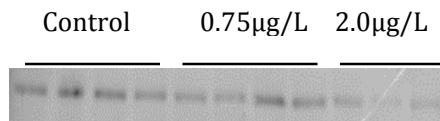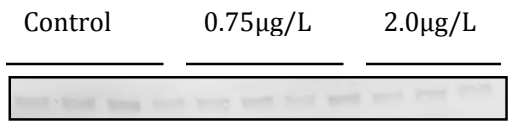
## 2.3.6 Secondary acute stress response

As expected, the stressor-induced cortisol levels showed a transient and significant increase 1 h after stressor exposure (time effect; two-way ANOVA) and dropped significantly below pre-stress levels at 24 h post-handling disturbance (Figure 5A). There was a was a significant reduction in plasma cortisol levels (treatment effect; 2-way ANOVA) in a dose-related fashion in the Cd groups compared to the control groups prior to and at 1 h but not at 24 h post-handling disturbance (Figure 5A). Plasma glucose levels were significantly elevated at 1 h after the handling disturbance, but returned to the pre-stress levels at 24 h post-disturbance (Figure 5B). Cadmium treatment significantly reduced the stressor-induced elevation of plasma glucose levels compared to the control group at 1 h post-handling disturbance, but not at other time points (Figure 5B).

There was a significant elevation in plasma lactate levels at 1 h but the levels were back to the pre-stress values at 24 h post-handling disturbance (Figure 5C).  Plasma lactate levels were significantly higher in the cadmium groups compared to the control group prior to stress and at 24 h post-handling disturbance (Figure 5C). However, the stressor-induced plasma lactate elevation seen in the control group at 1 h post-handling disturbance was significantly reduced in a dose-related fashion in response to cadmium exposure (Figure 5C).

**Figure 5. Acute stress performance.** Plasma cortisol (A), glucose (B) and lactate (C) levels in rainbow trout in response to an acute stressor challenge at 28 d after exposure to 0, 0.75, or 2.0 µg/L Cd. Values represent mean ± SEM (n=8 for control, 0.75 µg/L, and 2.0 µg/L at both 0h; 1h and 24 h; n=6 for 2.0 µg/L at 0 h). Lines above bars with different letters are statistically different from other sampling days (two-way ANOVA; $P<0.05$) and inset above graph represent statistical differences between overall treatment (two-way ANOVA, $P<0.05$). Different letters within each sampling days indicate significant difference between treatment groups within a time point (one-way ANOVA; $P<0.05$).
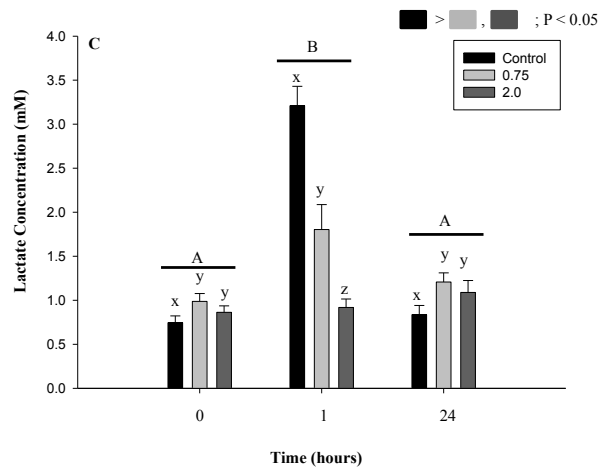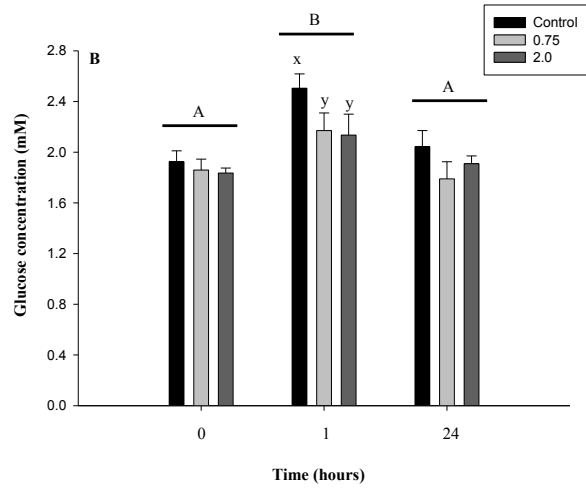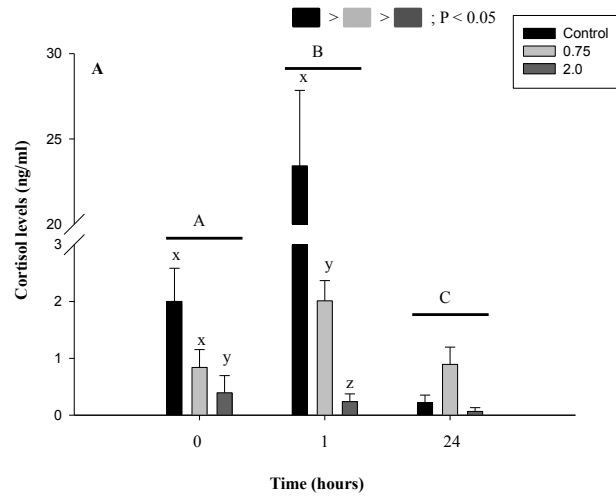
## 2.4 Discussion

The results demonstrate that subchronic exposure to low levels of Cd impacts longer-term tissue metabolic capacities and compromises the stress performance of rainbow trout. The Cd concentrations of 0.75 and 2.0 µg/L used in were within the concentrations measured in North American surface waters and within the US EPA freshwater quality criteria for aquatic life (US EPA, 2001; McGeer et al., 2011). While the fish did not show a chronic cortisol response to low level Cd exposure over a 28 d period, there were changes at the level of target tissue steroidogenic and metabolic capacities. Also, acute stressor-induced plasma cortisol and glucose responses were clearly compromised by low level Cd exposures in trout, implying perturbations in the highly conserved stress response that is essential to re-establish homeostasis.

The absence of a chronic plasma cortisol elevation in response to Cd exposures in this study is in agreement to a previous study that also showed a lack of cortisol response at lower Cd concentrations in juvenile trout (Ricard et al., 1998). However, the cortisol response to Cd exposure in fish is equivocal, and may be in part due to species differences, but also due to the generally unrealistic concentrations of Cd used in the majority of exposures (McGeer et al., 2011). Although plasma cortisol levels in all groups over the 28 d period of exposure were well below the resting levels reported for salmonids (Barton et al., 2002), there were treatment differences with levels significantly lower in the Cd groups. This supports studies that have reported impaired HPI axis activity in response to Cd exposure (Hontela and Vijayan, 2008)

Stressor-induced plasma cortisol elevation is achieved by the activation of the HPI axis (Mommsen et al., 1999; Aluru and Vijayan, 2006, 2009), and this involves the upregulation of key genes involved in steroid biosynthesis including MC2R, StAR and P450scc (Kusakabe et al., 2002, 2003, 2006; Geslin and Auperin, 2004; Hagen et al., 2006; Aluru and Vijayan, 2006; Gravel and Vijayan, 2007; Aluru and Vijayan 2008a, 2009). The stimulation of steroid biosynthetic pathway in the interrenal tissue involves the activation of MC2R, a G protein coupled receptor that increases adenylate cyclase activity, leading to elevated intracellular levels of cAMP and activation of protein kinase A (PKA) (Cooke, 1999; Gantz and Fong, 2003; Manna et al., 2006, 2009; Aluru and Vijayan, 2008a). PKA phosphorylates a suite of proteins, including StAR, making cAMP/PKA signaling cascade an important secondary messenger pathway for corticosteroidogenesis (Stocco, 2000). Cd has been shown to impair adrenocortical function in response to stressors (Hontela and Vijayan, 2008), leading to the proposal that the site of action of Cd-mediated cortisol disruption occurs within the intracellular signaling pathway (Leblond and Hontela, 1999; Lacroix and Hontela, 2004). Our previous study suggested that the inhibition of ACTH-mediated cortisol production

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might occur upstream of cAMP production, implicating MC2R as a possible target for endocrine disruption by Cd in trout (Sandhu and Vijayan, 2011).

In the present study although plasma cortisol levels were at resting levels (< 5 ng/ml) in the Cd groups, there was an upregulation of MC2R, StAR and P450scc mRNA abundances, especially at 28 d of exposure, suggesting disruptions in the molecular regulation of corticosteroidogenesis. These genes involved in steroidogenesis are upregulated in response to acute stress *in vivo* and or ACTH stimulation *in vitro* suggesting a key role in cortisol production (Kusakabe et al., 2002; Geslin and Auperin, 2004; Hagen et al., 2006; Aluru and Vijayan, 2006; Aluru and Vijayan 2008a). This was further confirmed by studies showing that treatments, including AhR agonist (Aluru and Vijayan, 2006) and Cd (Sandhu and Vijayan, 2011), that suppress ACTH-stimulated cortisol production also reduces the transcript levels of corticosteroidogenic genes. However, longer-term exposure to waterborne Cd in the present study led to higher mRNA abundances of steroidogenic genes, but this was not reflected in either elevated plasma basal cortisol levels or a secondary stressor-induced elevation in cortisol response, suggesting disruptions in interrenal steroidogenic capacity. The attenuated secondary stressor-induced plasma cortisol levels in the Cd group further confirms disruption in stimulated interrenal steroidogenesis by longer-term Cd exposure.

While altered steroidogenic capacity may be one factor leading to abnormal cortisol production, we cannot rule out other factors involved in HPI axis functioning as targets for Cd impact. For instance, a disturbance in negative feedback regulation may also lead to attenuated cortisol response to stressor exposure. To this end, brain GR, a key sensor thought to be involved in negative feedback regulation of cortisol (Aluru and Vijayan, 2008b), protein expression was not altered. However, brain MR protein expression was lower in the high Cd group and recently we proposed that brain MR signaling may be a key player in the suppression of stressor-induced cortisol elevation in fish (Alderman et al., 2012; Alderman and Vijayan, 2012). Although the mechanisms are unclear, subchronic low level exposure to Cd disrupts the stressor-induced cortisol response, a highly conserved stress response among vertebrates and essential for metabolic adjustments to re-establish homeostasis (Aluru and Vijayan, 2009), in rainbow trout. Also, the reduction in GR protein expression in the liver, a key target for metabolic regulation by cortisol (Mommsen et al., 1999; Aluru and Vijayan, 2009), by Cd in the present study suggests disruption also in target tissue cortisol signaling, while the mechanism is unknown. The reduced capacity to produce a cortisol response along with impaired steroid signaling may compromise the stress performance leading to reduced fitness in fish exposed to low levels of waterborne Cd. Given that short-term Cd exposure *in vitro* inhibits steroidogenic genes in trout (Sandhu and Vijayan, 2011), we hypothesize that the longer-term bioaccumulation of Cd

noted in the kidney (Milne et al., 2013) may be affecting mRNA stability and/or translation of genes encoding proteins involved in corticosteroidogenesis, but this remains to be tested.

A key role for stressor-induced cortisol elevation is the metabolic regulation that is essential to re-establish homeostasis (Mommsen et al., 1999; Momoda et al., 2007). The liver is an important target tissue for cortisol action (Vijayan et al., 1994, 2003, 2010) and stressor-induced cortisol elevation increases the metabolic capacity of this tissue (Mommsen et al., 1999; Aluru and Vijayan, 2009). A major role of cortisol in stress adaptation is to enhance glucose production by gluconeogenesis to meet the increased energy demand (Mommsen et al., 1999; Aluru and Vijayan, 2009). During the 28 d exposure period, there were no major changes in plasma glucose or lactate levels, suggesting either minor impact or rapid adjustment of plasma metabolite levels to low level Cd exposure. However, the metabolic capacity of the liver was clearly compromised after 28 d of Cd exposure. There was a significant accumulation of Cd in the liver in both treatment groups (Milne et al., 2013), similar to the concentration reported in trout from contaminated sites (McGeer et al., 2011). These results suggest that Cd accumulation increased the liver metabolic demand, and this was supported by the lower liver glycogen content at 28 d post-exposure. This along with the higher activities of hexokinase and glucokinase, key enzymes involved in glucose uptake, points to an increase in liver glycolytic capacity to meet the higher metabolic demand in response to Cd exposure in teleosts (Ricard et al., 1998; Levesque et al., 2002, 2003). The reduced stressor-induced plasma glucose levels seen in the Cd groups at 1 h after the handling disturbance may be related to the reduced liver glycogen content, as epinephrine-induced glycogenolysis is involved in the rapid elevation of plasma glucose levels after an acute stressor exposure (Reid et al., 1998; Mommsen et al., 1999; Vijayan et al., 2010).

A major target tissue that uses glucose as fuel is the gill and this tissue is also a key site for waterborne Cd uptake and accumulation. In the present study, Cd accumulated in the gills over a 28 d period (Milne et al., 2013), and our results suggest an enhanced metabolic capacity associated with this metal accumulation. The higher hexokinase and pyruvate kinase activities in the Cd groups confirm an increased glycolytic capacity to cope with the increased energy demand. The increased demand for glucose utilization by the gills coupled with a lower stressor-induced plasma glucose elevation may compromise gill function by reducing the fuel availability to cope with the stressor in the Cd exposed fish. The acute stressor-induced plasma lactate levels was also reduced in the Cd exposed fish suggesting disturbance to muscle metabolism. A recent study showed that cortisol elevation associated with exercise was involved in the muscle lactate production (Milligan, 2003). Consequently, the lower stressor-induced lactate levels in the present study in

the Cd groups may be due to their reduced cortisol levels and/or a reduction in target tissue GR signaling (Milligan, 2003), but this remains to be determined.

Altogether, 28 d subchronic exposure to low levels of waterborne Cd impacts liver and gill metabolic capacities and disrupts interrenal steroidogenic capacity in rainbow trout. Cd exposure also attenuates the secondary stressor-induced transient cortisol and glucose response that are essential to allow animals to cope with stress and regain homeostasis. Overall, subchronic exposure of trout to environmentally relevant levels of Cd impair the stress performance and may compromise their ability to respond to multiple stressors, leading to reduced fitness.

## 2.5 Acknowledgements

# Chapter 3

# Cadmium-mediated disruption of cortisol biosynthesis involves suppression of corticosteroidogenic genes in rainbow trout

## 3.1 Overview

Cadmium (Cd) is widely distributed in the aquatic environment and is toxic to fish even at sublethal concentrations. This metal is an endocrine disruptor, and one well established role in teleosts is the suppression of adrenocorticotrophic hormone (ACTH)-stimulated cortisol biosynthesis by the interrenal tissue. However the mechanism(s) leading to this steroid suppression is poorly understood. We tested the hypothesis that Cd targets genes encoding proteins critical for corticosteroid biosynthesis, including melanocortin 2 receptor (MC2R), steroidogenic acute regulatory protein (StAR) and cytochrome P450 side chain cleavage enzyme (P450scc), in rainbow trout (*Oncorhynchus mykiss*). To test this, head kidney slices (containing the interrenal tissues) were incubated *in vitro* with cadmium chloride (0, 10, 100 and 1000 nM) for 4 h either in the presence or absence of ACTH (0.5 IU/mL). In the unstimulated head kidney slices, Cd exposure did not affect basal cortisol secretion and the mRNA levels of MC2R and P450scc, while StAR gene expression was significantly reduced. Cd exposure significantly suppressed ACTH-stimulated cortisol production in a dose-related fashion. This Cd-mediated suppression in corticosteroidogenesis corresponded with a significant reduction in MC2R, StAR and P450scc mRNA levels in trout head kidney slices. The inhibition of ACTH-stimulated cortisol production and suppression of genes involved in corticosteroidogenesis by Cd were completely abolished in the presence of 8-Bromo-cAMP (a cAMP analog). Overall, Cd disrupts the expression of genes critical for corticosteroid biosynthesis in rainbow trout head kidney slices. However, the rescue of cortisol production as well as StAR and P450scc gene expression by cAMP analog suggests that Cd impact occurs upstream of cAMP production. We propose that MC2R signaling, the primary step in ACTH-induced corticosteroidogenesis is a key target for Cd-mediated disruption of cortisol production in trout.

## 3.2 Introduction

The elevation in plasma corticosteroid levels in response to stressor exposure is an evolutionary conserved response in vertebrates and is thought to be essential for re-establishing homeostasis (Sapolsky et al., 2000). Corticosteroid biosynthesis occurs in the adrenal cortex of mammals, while teleosts lack a discrete adrenal gland and the steroidogenic cells are predominately distributed in the anterior region of the kidney (interrenal tissue) (Wendelaar Bonga, 1997; Mommsen et al., 1999). Cortisol is the primary circulating corticosteroid in teleosts and its release involves the coordinated activation of the hypothalamus-pituitary-interrenal (HPI) axis. They key mediators include the release of corticotropin-releasing factor (CRF) from the hypothalamus, and stimulating the release of adrenocorticotropic hormone (ACTH) from the pituitary (Wendelaar Bonga, 1997). Circulating ACTH binds to melanocortin 2 receptor (MC2R) on the

steroidogenic cells within interrenal tissue and activates the signaling pathway leading to cortisol biosynthesis (Aluru and Vijayan, 2008).

MC2R is a G-protein coupled receptor and its activation by ACTH leads to enhanced adenylate cyclase activity, followed by elevated production of cyclic AMP (cAMP), a key second messenger triggering the onset of steroid biosynthesis (Mountjoy et al., 1994; Gantz and Fong, 2003). The two key steps in corticosteroid biosynthesis involve the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR), followed by the conversion of cholesterol to pregnenolone, the primary steroid intermediate, by cytochrome P450 side chain cleavage (P450scc) enzyme (Stocco, 2000; Payne and Hales, 2004). Consequently, either stressor-induced elevation of plasma cortisol *in vivo* or ACTH-induced production of cortisol *in vitro* using head kidney preparations corresponds with an upregulation of StAR and P450scc transcripts in rainbow trout (Geslin and Auperin, 2004; Hagen et al., 2006; Aluru and Vijayan, 2006; Gravel and Vijayan, 2006). Indeed, several biotic and abiotic stressors are known to modulate cortisol biosynthesis through alterations in the expression of genes encoding proteins involved in the functioning of the HPI axis (Hontela and Vijayan, 2008).

Cd is a metal that is widely distributed in the aquatic environment and is toxic to fish at sublethal concentrations (Pratap and Wendelaar Bonga, 1990; Hontela, 1997; Gillesby and Zacharewski, 1998; Lacroix and Hontela, 2006). Due to its long half-life and low excretion rate, Cd can also accumulate in various organs, primarily within the liver, kidney and reproductive and respiratory systems in fish (Norey et al., 1990; Sorensen et al., 1991; Hollis et al., 2000). This metal is known to disrupt head kidney corticosteroid production in fish (Hontela and Vijayan, 2008), while the mechanisms involved are far from clear. Recent studies suggest that the targets for Cd-mediated cortisol production are located upstream of pregnenolone synthesis (Lacroix and Hontela, 2004; Lizardo-Daudt et al., 2007), while the molecular mechanisms have received scant attention. We tested the hypothesis that Cd inhibits the expression of genes encoding proteins critical for cortisol biosynthesis in rainbow trout. To this end, we examined the expression of three key genes, including MC2R (ACTH signaling), StAR (cholesterol transport to the inner mitochondrial membrane) and P450scc (hydroxylation and cleavage of cholesterol to pregnenolone), essential for steroid biosynthesis in the interrenal tissue of fish (Aluru and Vijayan, 2006). This was tested using rainbow trout head kidney slices *in vitro*, a well established model system for studies pertaining to xenobiotics impact on corticosteroid biosynthesis (Hontela and Vijayan, 2008).

## 3.3 Materials and methods

### 3.3.1 Chemicals

ACTH (1-39), 2-Phenoxyethanol, gelatin, thimerasol, activated charcoal, dextran (from *Leuconostoc mesenteroides*) and 8-Bromo-cAMP were purchased from Sigma-Aldrich (St. Lous, MO, USA). $CdCl_2$ was purchased from BDH chemicals (Toronto, ON, CAN). D-Glucose was purchased from Bioshop (Burlington, ON, CAN), while monobasic and dibasic sodium phosphate, sodium bicarbonate and borosilicate tubes were purchased from Fisher Scientific (Ottawa, ON, CAN). Multiwell (24-well plate) tissue culture plates was from Sarstedt (Newton, NC, USA). 96-well and RNeasy mini kits were purchased from Qiagen (Mississauga, ON, CAN). First strand cDNA synthesis kit was from Fermentas (Burlington, ON, CAN). Scintillation cocktail and cortisol antibody were purchased from MP Biomedicals (Solon, OH, USA), while [1,2,6,7-3H] cortisol tracer was purchased from GE Healthcare (Upsala, Sweden).

### 3.3.2 Fish

Juvenile rainbow trout (average body mass 150 g) were obtained from Alma Aquaculture Research Station, Alma, Ontario, Canada, and maintained at the University of Waterloo Aquatic Facility. The experimental protocol was approved by the animal care committee at the University of Waterloo and is in accordance with the guidelines established by the Canadian Council for Animal Care. Fish were acclimated for 3 weeks in 2000-L tanks with continuous running water at 13 °C and 12-h light:12-h dark photoperiod before use in the *in vitro* studies. The fish were fed to satiety once daily 5 d a week with a commercial trout chow (Martin Feed Mills, Ellora, ON, CAN).

### 3.3.3 Cortisol production

Cortisol production *in vitro* was determined using head kidney tissue preparations as previously described (Aluru and Vijayan, 2006) with slight modifications. Briefly, trout were euthanized with an overdose of 2-phenoxyethanol (1:1000 dilution). The anterior region of the kidney (containing the interrenal tissues) from each fish was finely minced (approximately $1mm^3$ pieces) and rinsed with Hank's buffer (NaCl (136.9 mM), KCl (5.4 mM), $MgSO_4 \cdot 7H_20$ (0.8 mM), $Na_2HPO_4 \cdot 7H_20$ (0.33 mM), $KH_2HPO_4$ (0.44 mM), HEPES (5.0 mM), HEPES NA (5.0 mM), 5 mM $NaHCO_3$ and 5 mM glucose, pH adjusted to 7.63) to remove blood clots and the resulting mixture was distributed equally (500 μL buffer with approximately 50 mg of head kidney tissue in each well) into a 24-well tissue culture plate. The tissues were maintained for 2 h at 13 °C with gentle shaking. The buffer was replaced and the tissue was incubated for an additional hour after which the tissues were exposed to either fresh buffer (control) or fresh buffer

containing 0.5 IU/mL ACTH for 4 h. The ACTH concentration and the 4 h incubation period were chosen based on previous work from our laboratory (Aluru and Vijayan, 2006, 2008). At the end of the incubation period, samples were collected, quickly centrifuged down at 13,000 x g for 1 min and the supernatant and pellet were stored at -30 °C and -80 °C for later determination of cortisol concentration and gene expressions, respectively. Tissue viability was determined by measuring LDH leakage into the media according to established protocols (Boone et al., 2002).

### 3.3.4 Exposure to cadmium

Using the protocol described above, preliminary studies with head kidney slices involved exposing the head kidney preparation to a range of Cd concentrations (0, 0.01, 0.1, 1, 10 or 100 μM of cadmium chloride) and measuring either unstimulated or ACTH-stimulated cortisol production. Head kidney slices exposed to higher concentrations (10 or 100 μM) showed a high amount of cell lysis through lactate dehydrogenase leakage so lower concentrations were used (0, 10, 100 or 1000 nM cadmium chloride). The tissues were exposed to Cd 1 h prior to ACTH challenge. Cortisol released into the culture medium and tissue gene expressions were assessed either in the presence or absence of ACTH after 4 h incubation as mentioned above.

### 3.3.5 Exposure to 8-Bromo-cAMP

To examine if Cd effects may be due to impaired production of cAMP, a key second messenger for ACTH-induced cortisol production (Aluru and Vijayan, 2008), we exposed head kidney slices to 8-Bromo-cAMP (a cAMP analog) and measured cortisol production along with tissue transcript changes. For this experiment, head kidney slices were exposed to 0 or 100 nM (concentration that inhibited cortisol production by around 50% but did not affect cell viability) Cd exactly as mentioned above either in the presence or absence of 8-Bromo-cAMP (0.5 mM).

### 3.3.6 Cortisol concentration

Cortisol concentration in the media was determined by radioimmunoassay using the protocol described by Alsop and VIjayan (2008).

### 3.3.7 RNA isolation and first strand cDNA synthesis

Total RNA (DNase treated) from head kidney slices was extracted using RNeasy mini kit following the manufacturer's protocol (Qiagen, Mississauga, ON, CAN) and quantified by spectrophotometry at 260 nm using a nanodrop. First strand cDNA was synthesized from 1 μg of total RNA following the

manufacturers instructions (MBI Fermentas, Burlington, ON, CAN). Briefly, total RNA was denatured at 70 °C and cooled on ice, and the sample was used in a 20-μL reverse transcriptase reaction using 0.5 μg oligo dT primers and 1 mM each of dNTP, 40 U ribonuclease inhibitor, and 40 U M-MuLV reverse transcriptase. The reaction was started by incubating at 37 °C for 1 h and stopped by heating at 70 °C for 10 min.

### 3.3.8 Primers

The primers (Table 1) were designed using StAR, P450scc and MC2R cDNA sequences from rainbow trout and β-actin cDNA sequence from zebrafish (GenBank accession nos., AB047032, S57305.1, EU119870, and AF157514), respectively. The gene expression was analyzed using quantitative real-time PCR (iCycler, Bio-Rad, Hercules, CA, USA).

### 3.3.9 Quantitative real-time polymerase chain reaction (qPCR)

MC2R, StAR and P450scc mRNA levels were analyzed using qPCR and the values in the treatment groups are shown as relative to controls. Briefly, a master mix containing SYBR green, RNase free water and the specific primers were added to 2 μL of sample. The samples were in triplicates and PCR was performed to amplify the predicted products using the following conditions: initial denaturation for 2 min at 94 °C, 30 sec at 60 °C and 30 sec at 72 °C. β-actin was used as the housekeeping gene for data normalization. The threshold cycle for β-actin with the initial Cd study varied among samples and therefore, the genes of interest were quantified by equalizing the β-actin expression following the method described previously (Billiau and Vanderbroeck, 2001; Essex-Fraser et al., 2005; Ings and Van Der Kraak, 2006).

**Table 1. Primer details.** Oligonucleotide primers [forward (F) and reverse (R)] for steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (P450scc), melanocortin 2 receptor (MC2R) and β-actin used in semi-quantitative real-time PCR along with their melting temperature ($T_m$) and product size.

| Gene | Primers | $T_m$ | Product size (bp) |
|------|---------|-------|-------------------|
| StAR | F: 5'-TGGGGAAGGTGTTTAAGCTG-3' | 63.8 ºC | 101 |
|      | R: 5-AGGGTTCCAGTCTCCCATCT-3' | 63.8 ºC | 101 |
| P450SCC | F: 5'-GCTTCATCCAGTTGCAGTCA-3' | 64.1 ºC | 140 |
|         | R: 5'-CAGGTCTGGGGAACACATC-3' | 63.1 ºC | 140 |
| MC2R | F: 5'-GAGAACCTGTTGGTGGTGGT-3' | 63.9 ºC | 105 |
|      | R: 5'-GAGGGAGGAGATGGTGTTGA-3' | 64.1 ºC | 105 |
| β-actin | F: 5'-TGTCCCTGTATGCCTCTGGT-3' | 64.5 ºC | 121 |
|         | R: 5'- AAGTCCAGACGGAGGATGG -3' | 64.1 ºC | 121 |

### 3.3.10 Statistical analysis

All statistical analyses were performed on Sigmaplot 11 software (Systat Software Inc., San Jose, CA, USA) and data are shown as mean ± standard error of mean (SEM). However, for figures showing % change the SEM is only for reference and not a true reflection of the variance. Repeated measured one way analysis of variance (ANOVA) was used to determine changes in cortisol production and transcript levels in response to Cd and 8-Bromo-cAMP. Significant differences between treatment groups were compared using Holm-Sidak *post hoc* test or Student-Newman-Keul's test for rank comparisons. Paired t-test was performed to compare ACTH-mediated effect from control on cortisol production and gene expressions (see *inset* figures). The data were transformed wherever necessary to meet the assumption of homogeneity of variance, although non-transformed data are shown in the figures. A probability level of $P<0.05$ was considered statistically significant.

## 3.4 Results

### 3.4.1 Cortisol Production

ACTH-induced cortisol production was completely abolished at concentrations above 1 μM Cd (data not shown). As this also coincided with reduced cell viability all subsequent dose-response studies focused on Cd concentrations to a maximum of 1 μM (0, 10, 100 or 1000 nM). The cell viability was not affected at any of these concentrations as determined by lactate dehydrogenase leakage (~10% LDH leakage in all groups).

ACTH stimulation resulted in approximately a three-fold increase in cortisol production in trout head kidney slices (Figure 1A). Cd concentrations (10, 100 or 1000 nM) did not significantly affect unstimulated cortisol production compared to the control group (Figure 1B). There was a significant dose-related suppression of ACTH-mediated cortisol production by Cd in trout head kidney preparations (Figure 1C). The maximal suppression (> 50%) was seen at 100 and 1000 nM concentrations of Cd and this was significantly different from the control group.

**Figure 1. Cortisol levels.** (A) The basal (open bar) and ACTH-induced (closed bar) cortisol production in control tissues. (B) Effect of cadmium chloride exposure (0, 10, 100 or 1000 nM) on basal cortisol production. (C) Effect of cadmium chloride exposure (0, 10, 100 or 1000 nM) on ACTH (0.5 IU/ml) – stimulated cortisol production in rainbow trout head kidney tissue slices; values represent magnitude of change with ACTH stimulation compared to basal cortisol levels and expressed as % control (no Cd); data shown as mean ± SEM (n =5 fish); * significantly different from the no ACTH group (paired t-test, $P<0.05$); bars with different letters are statistically significant (one-way repeated measures ANOVA; $P<0.05$).

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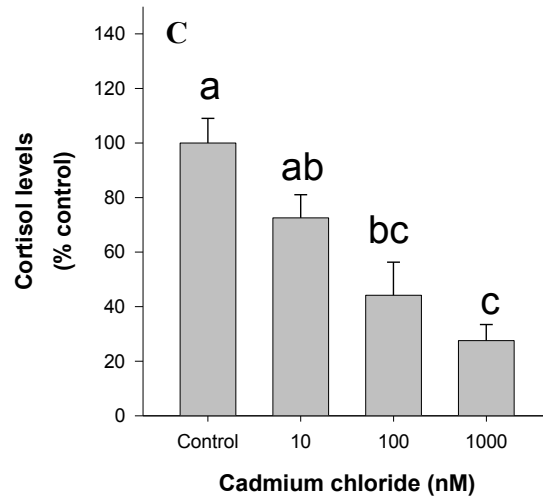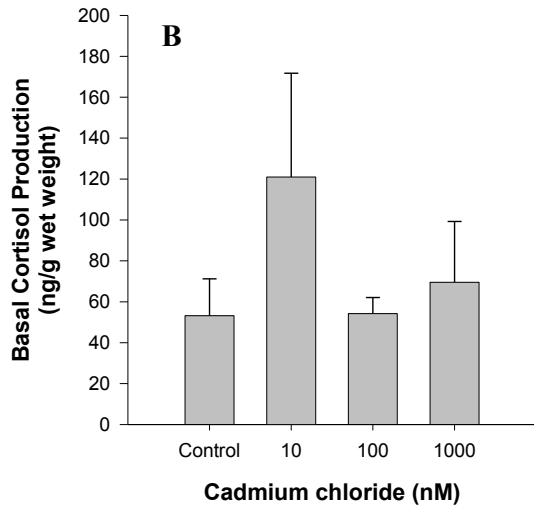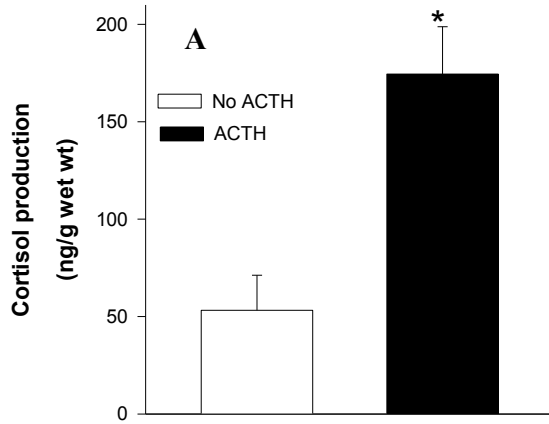
### 3.4.2 MC2R, StAR and P450scc gene expression

Expression of genes encoding proteins critical for cortisol biosynthesis, including ACTH signaling (MC2R), cholesterol transport to the inner mitochondrial membrane (StAR) and the rate-limiting primary enzymatic step (P450scc), were measured from head kidney slices (Figures 2-4). Cd did not significantly affect the basal (unstimulated) expressions of MC2R (Figure 2B) and P450scc (Figure 4B), whereas StAR expression was significantly reduced by around 60-90% in the Cd-treated groups (Figure 3B). ACTH exposure for 4 h elevated the mRNA levels of MC2R, StAR and P450scc by 5-, 3-, and 8-fold, respectively, in trout head kidney slices compared to the unstimulated slices (Figures 2A, 3A and 4A). The magnitude of change in gene expression seen with ACTH was significantly decreased by more than 60% at 100 and 1000 nM, but not at 10 nM Cd concentrations compared to the control group (Figures 2C and 3C). The mRNA levels of P450scc were significantly decreased by more than 50% at all concentrations of Cd tested compared to the control group (Figure 4C).

### 3.4.3 Effect of 8-Bromo-cAMP stimulation

Treatment of head kidney slices with the cAMP analog stimulated cortisol production and gene expressions and were not statistically different from ACTH-stimulated control groups (Figures 5A, 6A, 7A and 8A). The 100 nM Cd-mediated suppression of cortisol production and MC2R, StAR and P450scc gene expressions were completely abolished in the presence of 8-Bromo-cAMP exposure (Figures 5B, 6B, 7B and 8B).

**Figure 2. MC2R mRNA levels.** (A) The basal (open bar) and ACTH-induced (closed bar) MC2R mRNA levels in control tissues. (B). Effect of cadmium chloride exposure (0, 10, 100 or 1000 nM) on basal MC2R mRNA levels. (C) Effect of cadmium chloride exposure (0, 10, 100 or 1000 nM) on ACTH (0.5 IU/ml) – stimulated MC2R mRNA levels in rainbow trout head kidney tissue slices; values represent magnitude of change with ACTH stimulation compared to basal levels and expressed as % control (no Cd); data shown as mean ± SEM (n = 5 fish); *significantly different from the no ACTH group (paired t-test, $P<0.05$); bars with different letters are statistically significant (one-way repeated measures ANOVA; $P<0.05$).

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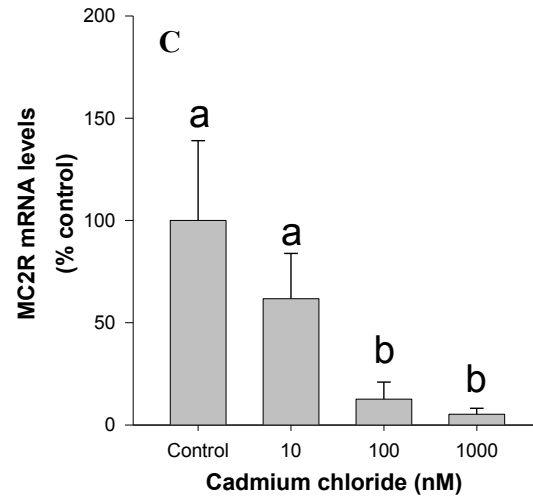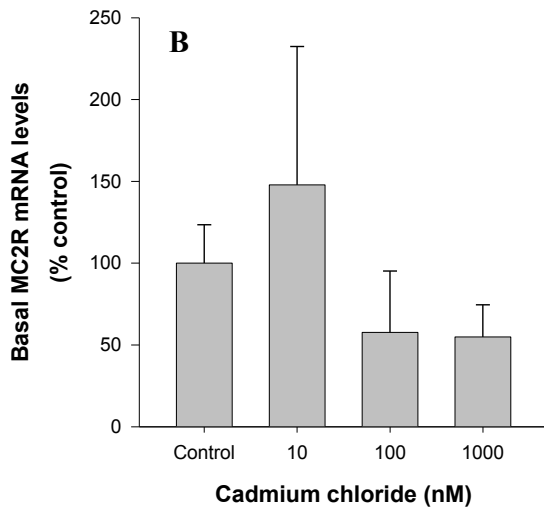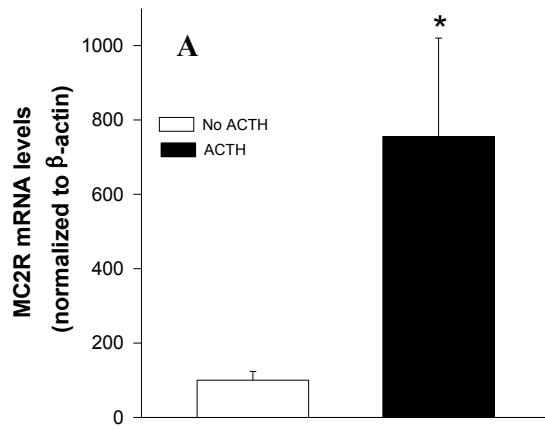
**Figure 3. StAR mRNA levels** The basal (open bar) and ACTH-induced (closed bar) StAR mRNA levels in control tissues. (B) Effect of cadmium chloride exposure (0, 10, 100 or 1000 nM) on basal StAR mRNA levels. (C) Effect of cadmium chloride exposure (0, 10, 100 or 1000 nM) on ACTH (0.5 IU/ml) – stimulated StAR mRNA levels in rainbow trout head kidney tissue slices; values represent magnitude of change with ACTH stimulation compared to basal levels and expressed as % control (no Cd); data shown as mean ± SEM (n = 5 fish); * significantly different from the no ACTH group (paired t-test, $P<0.05$); bars with different letters are statistically significant (one-way repeated measures ANOVA; $P<0.05$)

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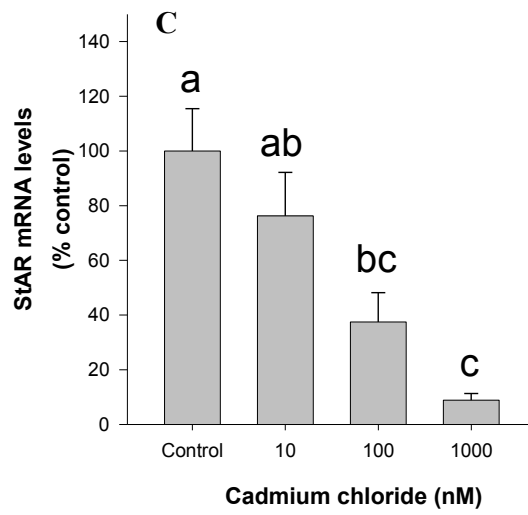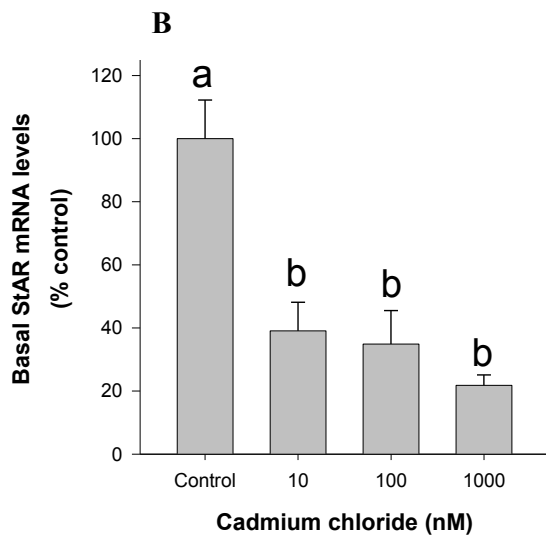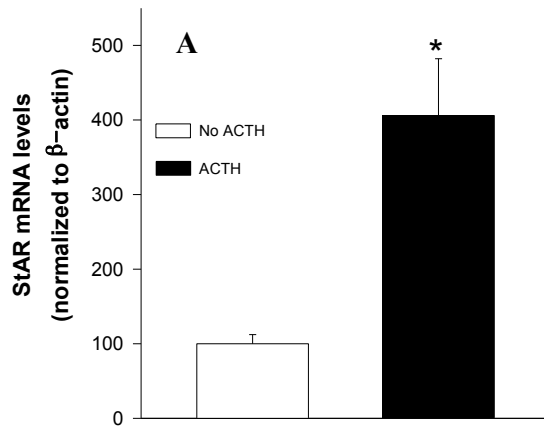
**Figure 4. P450scc mRNA levels.** (A) The basal (open bar) and ACTH-induced (closed bar) P450scc mRNA levels in control tissues. (B) Effect of cadmium chloride exposure (0, 10, 100 or 1000 nM) on basal P450scc mRNA levels. (C) Effect of cadmium chloride exposure (0, 10, 100 or 1000 nM) on ACTH (0.5 IU/ml) – stimulated P450scc mRNA levels in rainbow trout head kidney tissue slices; values represent magnitude of change with ACTH stimulation compared to basal levels and expressed as % control (no Cd); data shown as mean ± SEM (n = 5 fish); *significantly different from the no ACTH group (paired t-test, $P<0.05$); bars with different letters are statistically significant (one-way repeated measures ANOVA; $P<0.05$).
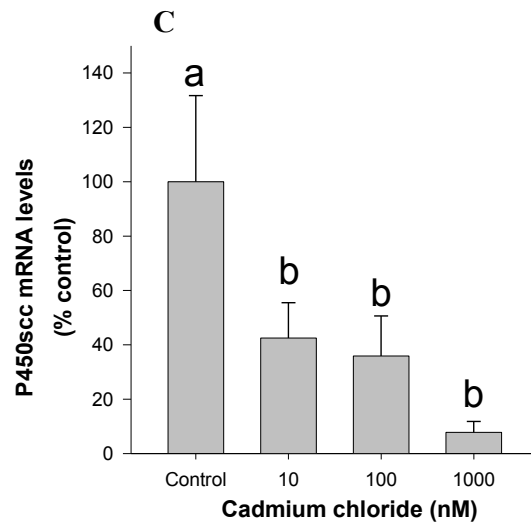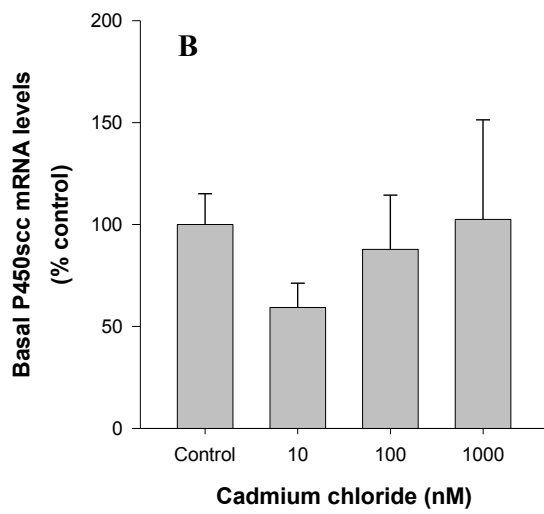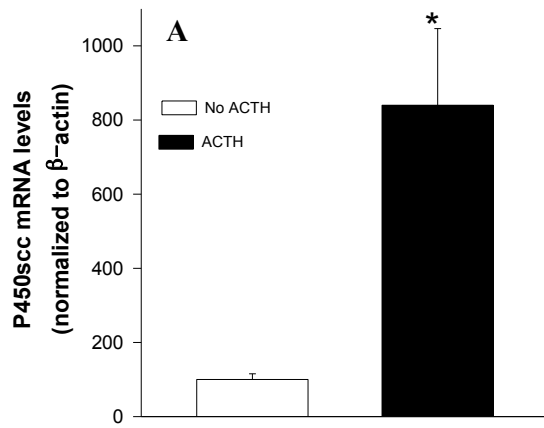
**Figure 5. Cortisol levels.** (A) Stimulation of cortisol secretion by ACTH or 8-Bromo-cAMP in head kidney slices of rainbow trout. (B) Effect of cadmium chloride (Cd) exposure (0 or 100 nM) on cortisol production in the presence of either ACTH or 8-Bromo-cAMP in the rainbow trout head kidney slices; values represent the magnitude of change with stimulation compared to the basal levels and expressed as % ACTH; data shown as mean ± SEM (n = 5 fish); bars with different letters are statistically significant (one-way repeated measures ANOVA; $P<0.05$).
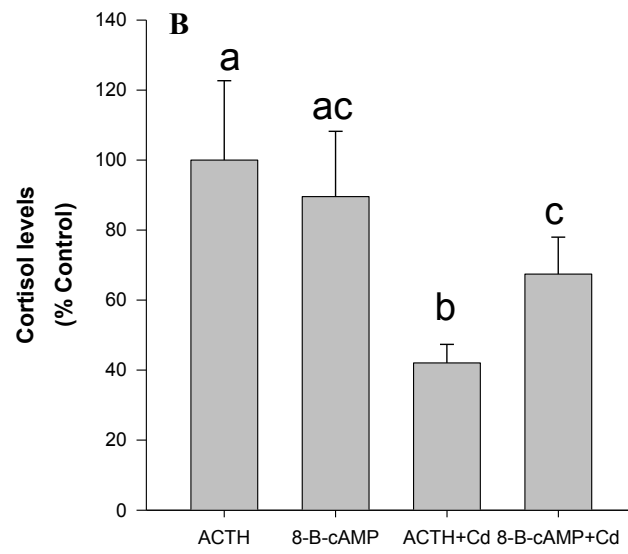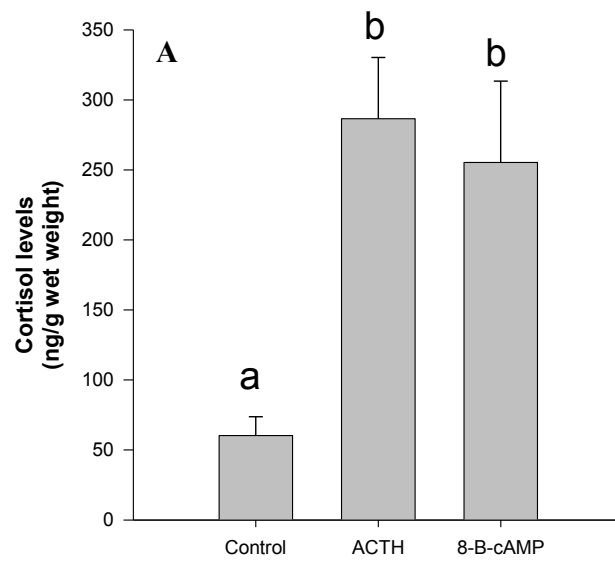
**Figure 6. MC2R mRNA levels.** (A) Stimulation of MC2R mRNA levels by ACTH or 8-Bromo-cAMP in head kidney slices of rainbow trout. (B) Effect of cadmium chloride (Cd) exposure (0 or 100 nM) on MC2R mRNA levels in the presence of either ACTH or 8-Bromo-cAMP in rainbow trout head kidney slices; values represent the magnitude of change with stimulation compared to the basal level and expressed as % ACTH; data shown as mean ± SEM (n = 5 fish); bars with different letters are statistically significant (one-way repeated measures ANOVA; P<0.05).
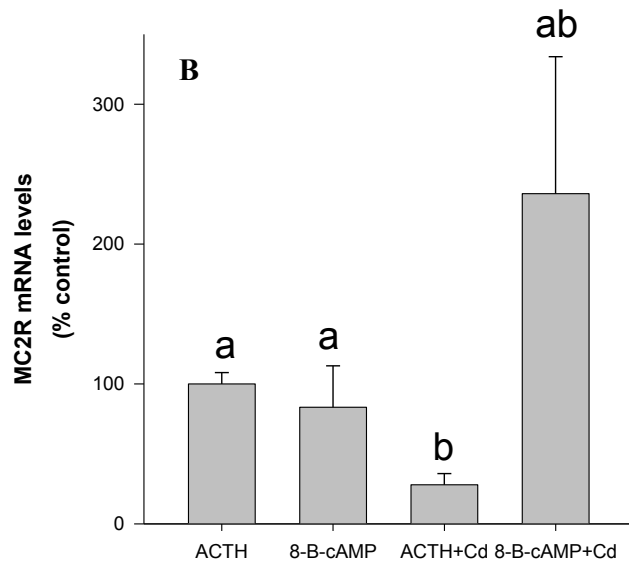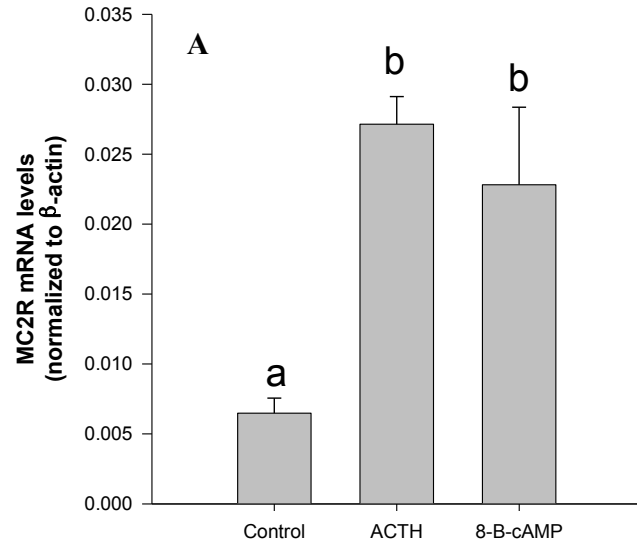
**Figure 7. StAR mRNA levels.** (A) Stimulation of StAR mRNA levels by ACTH or 8-Bromo-cAMP in head kidney slices of rainbow trout. (B) Effect of cadmium chloride (Cd) exposure (0 or 100 nM) on StAR mRNA levels in the presence of either ACTH or 8-Bromo-cAMP in rainbow trout head kidney slices; values represent the magnitude of change with stimulation compared to the basal levels and expressed as % ACTH; data shown as mean ± SEM (n = 5 fish); bars with different letters are statistically significant (one-way repeated measures ANOVA, $P<0.05$).
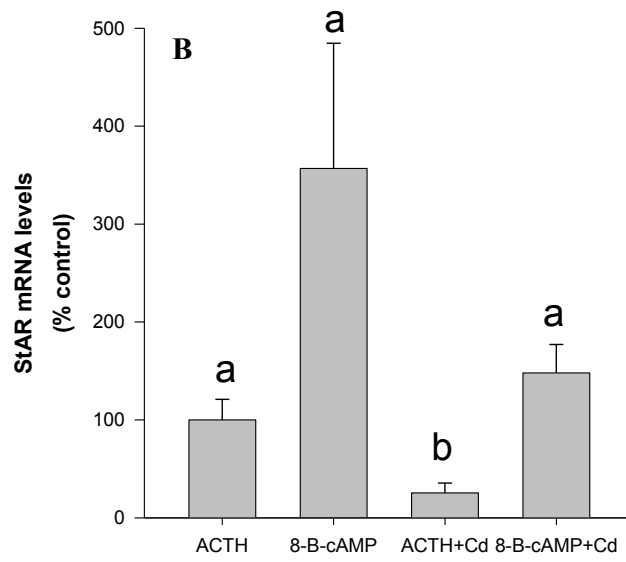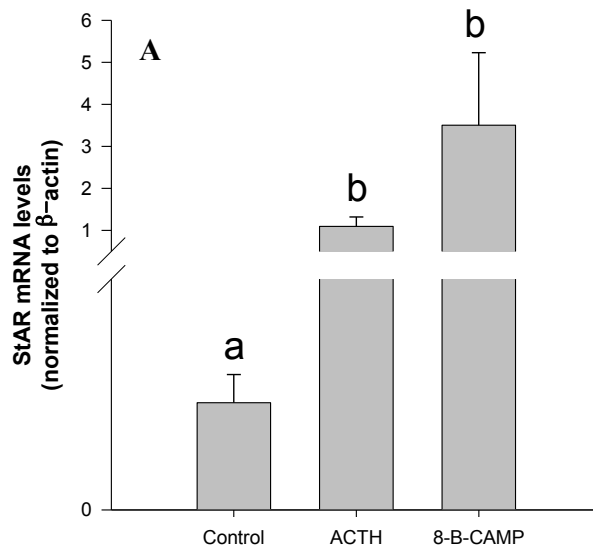
**Figure 8. P450scc mRNA levels.** (A) Stimulation of P450scc mRNA levels by ACTH or 8-Bromo-cAMP in head kidney slices of rainbow trout. (B) Effect of cadmium chloride (Cd) exposure (0 or 100 nM) on P450scc mRNA levels in the presence of either ACTH or 8-Bromo-cAMP in rainbow trout head kidney slices; values represent the magnitude of change with stimulation compared to the basal levels and expressed as % ACTH; data shown as mean ± SEM (n = 5 fish); bars with different letters are statistically significant (one-way repeated measures ANOVA; $P<0.05$).
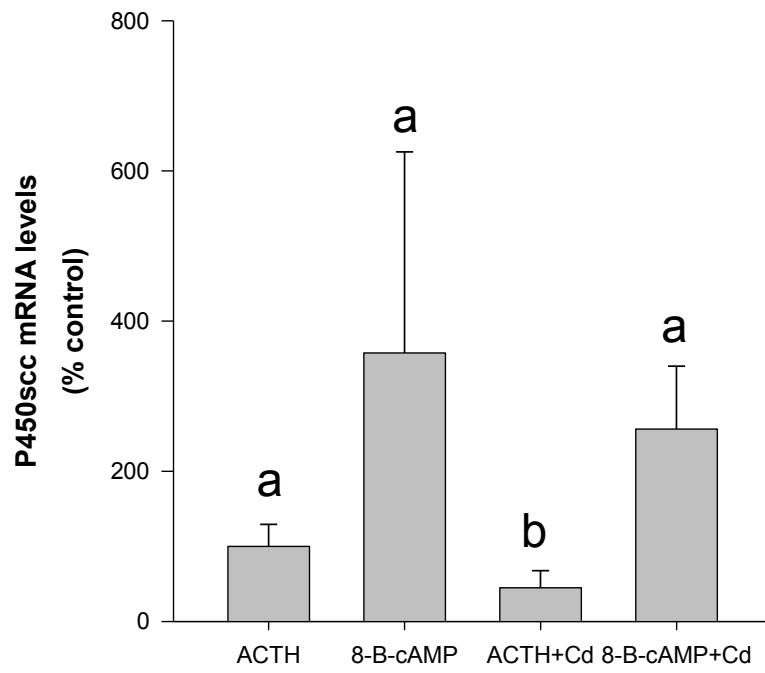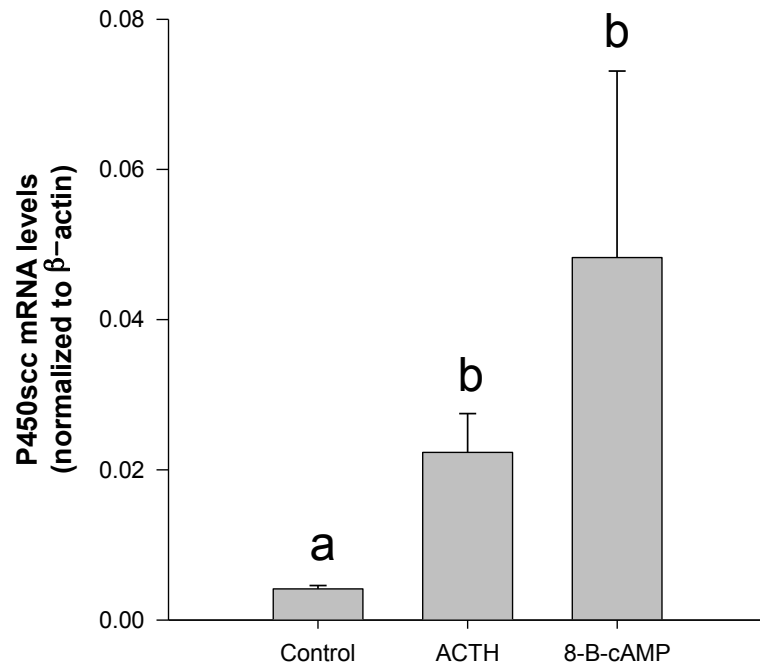
## 3.5 Discussion

We demonstrate for the first time that Cd impact on the interrenal (adrenal homolog of mammals) cortisol production involves suppression of genes encoding proteins critical for corticosteroid biosynthesis in rainbow trout. In teleosts, the corticosteroidogenic cells are distributed predominately in the anterior region of the kidney (Wendelaar Bonga, 1997), a key organ that accumulates and retains waterborne Cd during chronic sublethal exposures (Hollis et al., 2000; McGeer et al., 2000; Szebedinszky et al., 2000, 2001). Consequently, the interrenal tissues exposed to Cd during sublethal exposures *in vivo* leads to impaired plasma cortisol levels, especially in response to secondary stressors (Levesque et al., 2003). This was further confirmed *in vitro* using either dispersed interrenal cells or head kidney slices, underscoring the inhibition of ACTH-stimulated cortisol production by Cd (Ricard et al., 1998; Leblond and Hontela, 1999; Lacroix and Hontela, 2004; Raynal et al., 2005; Lizardo-Daudt et al., 2007).

Our results implicate Cd as a disruptor of corticosteroidogenic gene expression and this may play a role in the inhibition of ACTH-induced cortisol production in trout head kidney preparations *in vitro* (Lacroix and Hontela, 2004; Raynal et al., 2005; Lizardo-Daudt et al., 2007). The Cd-mediated transcript changes were seen mostly in the ACTH-stimulated tissue slices, except for StAR gene expression, and not under basal conditions suggesting that this metal impacts ACTH signaling and not the steroidogenic capacity of the tissue. Cd may be affecting StAR as their mRNA levels in the unstimulated tissue slices were suppressed. The absence of any changes in unstimulated basal cortisol output with Cd leads us to propose that this metal may be affecting the StAR mRNA stability and/or turnover. This is primarily based on the findings that higher StAR mRNA levels reflect a higher steroid production capacity *in vitro* and elevated plasma steroid levels *in vivo* (Aluru and Vijayan, 2008; Kocerha et al., 2010). However, we cannot rule out a direct effect of Cd on StAR regulation, but this will require characterization of the StaR promoter in trout.

In the present study, Cd exposures at very low concentrations, relative to other studies in trout (Lacroix and Hontela, 2004; Raynal et al., 2005; Lizardo-Daudt et al., 2007), suppressed the ACTH-stimulated cortisol production in a dose-related manner. The higher interrenal sensitivity to Cd in the present study may be due to the absence of calcium in the incubation medium. This is because calcium competes with Cd for cellular uptake and it has been shown that absence of extracellular calcium concentration increases Cd toxicity (Lacroix and Hontela, 2006). The absence of extracellular calcium led to a lower magnitude in cortisol response (3-fold decrease), compared to our other studies (Aluru and

Vijayan, 2006, 2008), but is in agreement with results obtained previously with trout adrenocortical cells *in vitro* (Lacroix and Hontela, 2006).

The rate-limiting steps in ACTH-stimulated steroid production involve the transport of cholesterol from the outer mitochondrial membrane by the steroidogenic acute regulatory protein (StAR), and the first enzymatic step, the cytochrome P450 side chain cleavage (P450scc) enzyme, which catalyzes the conversion of cholesterol to pregnenolone. In trout, the genes encoding StAR and P450scc are upregulated in response to ACTH stimulation in head kidney tissue slices *in vitro* and correspond with an increased cortisol output into the medium (Geslin and Auperin, 2004; Aluru et al., 2005, Aluru and Vijayan, 2006, 2008; Hagen et al., 2006; Gravel and Vijayan, 2007). This increase in transcript abundance of StAR and P450scc in the head kidney is also seen in response to an acute stressor-induced elevation in plasma cortisol levels *in vivo* (Aluru and Vijayan, 2008). This supports the overall enhancement of the steroidogenic capacity in response to ACTH stimulation.

While very few studies have actually examined ACTH signaling in trout, we have shown that MC2R is upregulated in response to ACTH stimulation *in vitro* or to an acute stressor *in vivo* (Aluru and Vijayan, 2008). The suppression of MC2R gene expression by Cd in the present study points to a disruption in ACTH signaling, leading to the inhibition of stimulated cortisol production. Indeed, StAR and P450scc mRNA levels were also suppressed by Cd suggesting other potential sites of impact in impairing corticosteroidogenesis. Although to out knowledge no other study has examined MC2R impact by contaminants, several studies have shown that StAR and P450scc are targets for xenobiotic impact, including AhR ligands, pharmaceuticals, tributyltin and nonylphenol (Aluru et al., 2005; Aluru and Vijayan, 2006; Gravel and Vijayan, 2007; Kortner et al., 2009; Pavlikova et al., 2010). While the mechanisms involved are far from clear, our results also include Cd to the list of contaminants targeting the steroidogenic genes leading to the disruption of steroid output in fish (Arukwe, 2008).

Interestingly, the suppression in cortisol production and expression of steroidogenic genes in response to Cd exposure is completely abolished in the presence of cAMP analog (8-Bromo-cAMP). A key signal for stressor-induced cortisol production is the binding of ACTH to the MC2R and activation of the signaling pathway leading to cAMP production, which is a key inducer of StaR gene expression in fish (Geslin and Auperin, 2004; Aluru and Vijayan, 2006; Hagen et al., 2006; Aluru and Vijayan, 2008). Although there are five known melanocortin receptors (MCRs) (MC1R-MC5R) in tetrapods, and there are multiple ligands for some of these receptors, only ACTH appears to bind to MC2R (Gantz and Fong, 2003; Klovins et al., 2004a,b; Metz et al., 2005; Aluru and Vijayan, 2008). Melanocortin receptors, including

MC2R, are G-protein coupled receptors containing seven-transmembrane domains with extracellular and intracellular amino- and carboxyl-terminals, respectively (Schiöth, 2001; Schiöth et al., 2005). Functionally, MC2R is coupled to adenylate cyclase and its effects are mediated through the cAMP-dependent signaling pathway (Schiöth, 2001). Specifically, upregulation of adeynylate cyclase increases production of cAMP which in turn activates protein kinase A leading to phosphorylation of StaR on the serine 194/195 residues and the subsequent activation of the steroid biosynthetic pathway (Schiöth, 2001; Lacroix and Hontela, 2001; Schiöth et al., 2005; Aluru and Vijayan, 2008).

The observation that 8-Bromo-cAMP rescues the Cd-mediated suppression of corticosteroidogenesis clearly implies that the mechanism leading to steroid disruption is upstream of StAR gene expression. This is in agreement with recent studies suggesting that the toxic action of Cd on cortisol production may be acting upstream of pregnenolone synthesis in trout (Lacroix and Hontela, 2004; Lizardo-Daudt et al., 2007). Similar to 8-Bromo-cAMP exposure, we also observed rescue of Cd-mediated cortisol production and steroidogenic genes with forskolin (an adenylyl cyclase activator; data not shown) leading us to hypothesize that Cd toxicity may involve disrupting MC2R signaling, leading to reduced cAMP production in the steroidogenic cells. The dose-related suppression of ACTH-stimulated MC2R gene expression by Cd supports the above contention. Whether this involves disruption of MC2R gene transcription, translation, and/or regulation by accessory proteins remains to be established. The rescue of MC2R gene expression by a cAMP analog in the present study is not surprising, especially given the recent finding that this gene is upregulated by its own ligand in rainbow trout (Aluru and Vijayan, 2008).

Taken together, disruption of ACTH signaling may be a mechanism leading to the impairment of cortisol production by Cd in fish. We propose that Cd disruption of MC2R activation, the key signaling step in ACTH-induced steroidogenesis, is involved in the inhibition of cortisol production, while the mechanisms involved remain to be established.

## 3.6 Acknowledgements

**Chapter 4**

**Sublethal cadmium concentrations impair the cortisol stress response by disrupting melanocortin 2 receptor (MC2R) and MC2R accessory protein function in juvenile rainbow trout**

## 4.1 Overview

We tested the hypothesis that juvenile rainbow trout continuously exposed to low levels of cadmium (Cd), commonly detected in the environment, will exhibit impaired functioning of the hypothalamus-pituitary-interrenal axis. Specifically, we tested the hypothesis that melanocortin 2 receptor disruption is a key mechanism leading to the inhibition of steroid biosynthesis in the interrenal tissue. Fish were exposed to either one of two sublethal concentrations of Cd (0.75 or 2.0 μg/L) for 7 days (d), and were then subjected to a handling disturbance for 5 min and the fish were allowed to recover over a 24 hour (h) period after which samples were collected at 1, 4 and 24 h after exposure to secondary stressor (0 h). Cd accumulation was greatest in the liver followed by the kidney and brain in both concentration groups. Cd exposure for 7 d did not affect plasma cortisol and ACTH concentrations or mRNA levels encoding corticotropin-releasing factor (CRF) and corticotropin-releasing factor binding protein (CRF-BP) in the preoptic area (POA) or proopiomelanocortin receptor (POMCA or POMCB) in the hypothalamus or melanocortin receptor accessory protein 1 and 2 (MRAP1 and MRAP2) in the head kidney. However there was a significant increase in head kidney melanocortin 2 receptor (MC2R) mRNA levels in both Cd groups. In the brain, a significant decrease was observed in mineralocorticoid receptor (MR) protein expression in the high Cd dose group, but no changes were seen in glucocorticoid receptor (GR) expression. Subjecting the fish to a secondary handling stressor led to a transient attenuation in the stressor-induced plasma cortisol and ACTH levels in both Cd groups 1 h post-stressor exposure. No significant changes were observed in CRF, CRF-BP or MRAP2 mRNA levels in Cd or control groups at 1, 4 or 24 h post-stressor exposure. However, there was a significant decrease in POMCB mRNA levels 1 h after exposure to secondary stressor. Furthermore there was a decrease in stressor-induced MC2R and MRAP2 mRNA levels at 1 h for both Cd groups. An *ex vivo* study using head kidney tissue from the Cd groups at 7 d confirmed attenuation of ACTH-stimulated cortisol production that was abolished by 8-Bromo-cAMP treatment, confirming for the first time that MC2R activation is the likely target for Cd-mediated cortisol disruption. Furthermore, MC2R and MRAP1, but not MRAP2, mRNA levels were impaired in both Cd groups. Reporter assays with CHO cells transiently transfected with rainbow trout MC2R (rtMC2R) and zebrafish MRAP1 (zfMRAP1) cDNAs showed a dose-related inhibition of ACTH stimulation with Cd (0, 0.1, 10, or 100 nM). Collectively, 7 d exposure to sublethal Cd concentrations compromises the stressor-induced cortisol response by disrupting multiple pathways involved in HPI axis functioning. Our results highlight for the first time that the MC2R/MRAP1 complex is a key target for Cd-mediated inhibition of interrenal steroidogenesis in rainbow trout.

## 4.2 Introduction

Accumulation of Cd and other metals in aquatic organisms is dependent upon the route of exposure, which are either waterborne or dietary pathways (Kamunde et al., 2002; Franklin et al., 2005; Kwong et al., 2011) and uptake occurs primarily through the gut and/or gills (Alazemi et al., 1996; Alquezar et al., 2008; Ojo and Wood; 2008; Klinck et al., 2009; Klinck and Wood, 2011) where toxicity is dependent upon calcium competing with Cd for uptake (Zohouri and Wood, 2001; Adiele et al., 2011). Within fish, Cd is known to accumulate predominantly in metabolically active tissues, including gills, liver, gastrointestinal tract, and to a lesser extent in the muscle and brain (Norey et al., 1990; Gill et al., 1991; Lemaire-Gony and Mayer-Gostan, 1994; Melgar et al., 1997; Camusso et al., 1999; Kamunde, 1999; McGeer et al., 2000b; Chowdhury et al., 2004; Franklin et al., 2005). Typically, subchronic exposure to elevated waterborne Cd is accompanied by physiological adjustments and enhanced tolerance (McDonald and Wood, 1993; McGeer et al., 2000a,b). However, at sublethal concentrations, such as those found in the environment (Jensen and Bro-Rasiriussen, 1992; CCME, 1994, 1999), Cd can act as an endocrine disruptor, by inhibiting cortisol biosynthesis in the interrenal tissues of teleosts (Hontela, 1997; Gillesby and Zacharewski; 1998; Burger et al., 2002; Lacroix and Hontela, 2004; Vetillard and Bailhache, 2005; Lacroix and Hontela, 2006; Hontela and Vijayan, 2008; Sandhu and Vijayan, 2011 [Chapter 3]).

The stress response is a highly conserved response and essential for re-establishing homeostasis after a stressor exposure (Wendelaar Bonga, 1997; Mommsen et al., 1999; Sapolsky et al., 2000; Barton, 2002; Bury and Sturm, 2007; Prunet et al., 2008; Alsop and Vijayan, 2008; Aluru and Vijayan, 2009; Pankhurst, 2011). Cortisol production in teleosts is regulated by the hypothalamus-pituitary-interrenal (HPI) axis function (Wendelaar Bonga, 1997; Mommsen et al., 1999; Sapolsky et al., 2000; Alsop and Vijayan, 2008; Aluru and Vijayan, 2009). Briefly, activation of the HPI axis occurs through stressor-induced stimulation of the hypothalamus, leading to the release of corticotropin-releasing factor (CRF), which in turn stimulates the anterior pituitary to release adrenocorticotrophic hormone (ACTH), a peptide derivative of pro-opiomelanocortin (POMC), the primary cortisol secretagogue (Baker et al., 1996; Wendelaar Bonga, 1997; Mommsen et al., 1999; Pepels et al., 2004; Flik et al., 2006; Alderman and Bernier, 2007, 2009; Dores and Baron, 2011; Fuzzen et al., 2011). Within teleosts, two versions of POMC (POMCA and POMCB) exist as a result of the salmonid genome duplication event (Gonzalez-Nunez et al., 2003; Karsi et al., 2004; Leder and Silverstein, 2006). ACTH binds to the melanocortin 2 receptor (MC2R), a G-protein coupled receptor, on the steroidogenic cells of the interrenal tissues and activates the signaling cascade leading to cortisol production (Mountjoy et al., 1994; Wendelaar Bonga, 1997; Schiöth, 2001;

Gantz and Fong, 2003; Charmandari et al., 2005; Aluru and Vijayan; 2008). The presence of MC2Rs on the plasma membrane for binding and activation by ACTH has recently been shown to require the presence of melanocortin receptor accessory proteins (MRAPs) (Rachel et al., 2005; Cooray et al., 2008, 2009; Hinkle and Sebag, 2009; Sebag and Hinkle, 2009a; Agulleiro et al., 2010; Webb and Clark 2010; Cooray and Clark, 2011; Veo et al., 2011; Liang et al., 2011, 2013a; Valsalan et al., 2013). In trout, two MRAPs have been isolated and sequenced (MRAP1 and MRAP2), but only MRAP1, and not MRAP2, is essential for MC2R activation (Sebag and Hinkle, 2009a; Liang et al., 2011, 2013b). However, very little is known about MRAP regulation *in vivo* in response to stressor exposure in animal models.

In addition to corticosteroid biosynthesis, a key aspect of the stress response is the action of cortisol on target tissues, which is mediated by activation of either glucocorticoid receptor (GR) and/or mineralocorticoid receptor (MR) (Lee et al., 1992; Mommsen et al., 1999; Bury and Sturm, 2007). In teleosts, there are multiple copies of GR and one MR with an extensive tissue distribution, including the brain (Bury and Strum, 2007; Alsop and Vijayan, 2008), although functionally distinct roles for GR isoforms have not yet been reported (Bury et al., 2003; Greenwood et al., 2003; Prunet et al., 2006; Stolte et al., 2008; Kim et al., 2011. Plasma cortisol levels after stressor exposure are returned to basal levels by negative feedback regulation that involves the binding of this steroid to GR and/or MR to inhibit CRF and/or ACTH secretion, respectively (Mommsen et al., 1999; Aluru and Vijayan, 2009; Alderman et al., 2012; Alderman and Vijayan, 2012). This integrated stress response is considered a potential target for endocrine disruption by contaminants (Hontela and Vijayan, 2008), but little is known about the impact of environmentally relevant levels of Cd on HPI regulation in teleosts.

The objective of this study was to determine the mode of action of sublethal Cd levels in impacting key aspects of the HPI axis functioning in juvenile rainbow trout (*Oncorhynchus mykiss*). Specifically, the hypothesis tested was that MC2R signaling is a key target for Cd-mediated endocrine disruption of the cortisol stress axis. For this, trout were exposed to Cd for 7 d and then subjected to a secondary chasing and netting stressor to assess the functionality of the stress performance. Plasma cortisol and ACTH levels were measured as indicators of stress response, while brain GR and MR protein expression were used to assess brain corticosteroid responsiveness. Transcript abundances of CRF and CRF-BP from the preoptic area (POA) and POMCA and POMCB from the hypothalamus were used to determine regulation of the stress axis at the hypothalamus. Transcript abundances of MC2R, MRAP1 and MRAP2 from the head kidney were analyzed to indicate responsiveness of interrenal tissue to ACTH, while ACTH-stimulated cortisol production in *ex vivo* head kidney tissues indicated steroid biosynthetic capacity. Also, a reporter assay

using CHO cells transfected with MC2R and MRAP1 was used to confirm a direct effect of Cd on ACTH-stimulated MC2R signaling.

## 4.3 Materials and methods

### 4.3.1 Chemicals

Tricaine methanesulfonate (TMS) and sodium bicarbonate were purchased from Syndel Laboratories Ltd., (Vancouver, BC, CAN). RNase free water was purchased from Qiagen (Toronto, ON, CAN). Nitric acid, borosilicate and scintillation tubes, monobasic and dibasic sodium phosphate, potassium bicarbonate, perchloric acid, potassium chloride and sodium bicarbonate were purchased from Fisher Scientific (Fairlawn, NJ, USA). Scintillation cocktail and cortisol antibody were purchased from MP Biomedicals (Solon, OH, USA). [1,2,6,7-3H] cortisol tracer and ECl plus Western Detection System were purchased from GE Healthcare (Upsala, Sweden). Cadmium chloride, D-glucose and Tween 20 were from Bioshop (Burlington, ON, CAN). 96-well plates were purchased from VWR (Mississauga, ON, CAN). Thimerasol, activated charcoal and dextran (from *Leuconostoc mesenteroides*) were purchased from Sigma –Aldrich (St. Louis, MO, USA). DNase and bicinchoninic reagent were from ThermoScientific (Nepean, ON, CAN). Protease inhibitor tablets were purchased from Roche (Mannheim, Germany). Protein ladder for western analysis and Sybr green for qPCR were purchased from Bio-Rad (Hercules, CA, USA).

### 4.3.2 Rainbow trout holding conditions

Experimental holding conditions for the 7 d *in vivo* study were similar to those conducted during 28 d *in vivo* study (Chapter 2) and by McGeer and colleagues (2000a,b). 7 d was used as a time point based on previous studies showing significant accumulation within day 7 (Milne, 2010). Briefly, juvenile rainbow trout (*Oncorhynchus mykiss*) (30.4 ± 1.1 g) were obtained from Rainbow Springs Hatchery (Thamesford, ON, CAN) and held at Wilfrid Laurier University using previously described methods (McGeer et al., 2000a,b) and described in detail by Milne (2010). Fish were initially held in 220 L tanks (2 tanks with 50 fish in each) (RTS Plastics, New Hamburg, ON, CAN) with water flowing though each tank at 700 ml/min. Water was a 1:1 mixture of well water and soft water produced by reverse osmosis (500 mg/L as $CaCO_3$, 650 μS/cm, pH 7.2, 15 °C). Fish were acclimated to moderately hard water by gradually decreasing the flow of the well water over a two-week period. After the two weeks, fish were randomly distributed among six 200 L polyethylene tanks (16-17 fish in each). 60 L polyethylene mixing head tank received 2.4 L/min of soft water plus 0.6 L/min of well water, for a total of 3 L/min to achieve the chemistry of moderately hard water used for experimental exposures (140 mg/L as $CaCO_3$, 786 ± 25 Ca, 440 ± 18 Mg, 383 ± 32 Na

(all in µM ± 1SD, n=63), with a conductivity (YSI 30, Yellow Springs Instruments, Yellow Springs, OH, USA), pH (Seven Go, Mettler, Toledo, Fisher Scientific, Mississauga, ON, CAN), and temperature of 255 µS/cm, pH 7.2, 15 °C respectively. The mixing head tank delivered water (2 L/min) to three smaller 11.2 L polyethylene head tanks that have equally split outflows of water delivered to the two fish tanks running in duplicate. In order to reach the desired concentration of Cd, the flow rate for 0.75 µg Cd/L was 1290 ml/min and 1210 ml/min for 2.0 µg Cd/L. The flow rate for the control tanks was 1250 ml/min. All water in head tanks and fish tanks were well aerated. Fish were acclimated to their respective tanks for two weeks before Cd exposure and fed at 2% of their body weight daily as a single meal (Bio Oregon Protein Inc., Warrenton, OR, USA).

### 4.3.3 Cadmium exposure

Two head tanks were used to receive desired concentrations of 0.75 and 2.0 µg Cd/L (as $CdCl_2$, VWR International Mississauga, ON, CAN) and the remaining head tank was used for control (0 µg Cd/L). Dissolved actual Cd concentrations measured in fish tanks were (means ± SEM): -0.2 ± 0 0.03 µg Cd/L (control), 0.73 ± 0.12 µg Cd/L, 2.38 ± 0.35 µg Cd/L. All three exposure conditions (control (0), 0.75 and 2.0 µg Cd/L) were done in duplicate, therefore n = 24 fish for control, 0.75 and 2.0 µg Cd/L. Initially, head tanks and fish tanks were spiked with appropriate volume from a master stock of 1.0 g/L of Cd to achieve exposure concentrations. Additionally, appropriate volumes from a master stock were added to two 10 L carboys, each delivering Cd solution to the head tanks via pumps (FIM lab pump, Fluid Metering Inc., Oyster Bay NY, USA; 1.2 ml/min) to maintain the desired Cd concentrations in the exposure tanks. Water pH meter (Mettler Toledo SevenGO™, Fisher Scientific, Fairlawn, NJ, USA) and conductivity and temperature were measured using a conductivity meter (YSI 30, Yellow Springs Instruments, Yellow Springs, Ohio, USA).

### 4.3.4 Sampling

Rainbow trout were exposed to 0 (control), 0.75 (low dose) or 2.0 (high dose) µg Cd/L for 7 days and plasma and tissue samples were collected (0 h time point). Following the 0 h sampling, the remaining fish were exposed to a secondary stressor consisting as a 5 min handling disturbance as previously described (Ings et al., 2011; Wiseman et al., 2011) and the fish allowed to recover. Samples were taken at 1, 4 and 24 h post-stressor exposure. Fish were euthanized with an overdose of 0.3 g/L MS-222 buffered with 0.6 g $NaHCO_3$/L. One ml of blood was collected from the caudal peduncle in 1.5 ml centrifuge tubes containing 5 mM EDTA as an anticoagulant. Blood samples were immediately centrifuged at 10,000 x g for

2 min. Plasma was separated and stored at -30 °C to measure cortisol and ACTH levels later. Head kidney and regionalized brain tissues were stored at -80 °C for transcript and protein expression analyses later. The brain was regionalized into the preoptic area to measure CRF and CRF-BP and the hypothalamus to measure POMCA and POMCB.

### 4.3.5 *Ex vivo* study

Juvenile rainbow trout were sampled at 7 d post-Cd exposure as previously described. Trout were euthanized with an overdose of TMS buffered with sodium bicarbonate and the anterior region of the kidney (containing interrenal tissues) from each fish was finely minced (approximately 1 mm$^3$ pieces) and rinsed with modified Hank's buffer (NaCl (136.9 mM), KCl (5.4 mM), MgSO$_4$•7H$_2$0 (0.8 mM), Na$_2$HPO$_4$•7H$_2$O (0.33 mM), KH$_2$PO$_4$ (0.44 mM), HEPES (5.0 mM), HEPES NA (5.0 mM), 5mM NaHCO$_3$ and 5mM glucose pH adjusted to 7.63) to remove blood clots. The resulting mixture was distributed equally (500 μL modified Hank's buffer with approximately 50 mg of head kidney tissue in each well) into a 24 well tissue culture plate (Sarstedt, Newton, NC, USA). The tissues were maintained for 2 h at 13 °C with gentle shaking. After 2 h, the buffer was replaced and the tissues were incubated for an additional 1 h after which the tissues were replaced with fresh buffer containing no ACTH (control), 0.5 IU/mL ACTH or 5 mM 8-Bromo-cAMP for 4 h. The ACTH and 8-Bromo-cAMP concentrations and the incubation period were chosen based on previous work from our laboratory (Aluru and Vijayan, 2006, 2008; Sandhu and Vijayan, 2011 [Chapter 3]). At the end of the incubation period, samples were collected, quickly centrifuged (13,000 x g for 1 min) and the supernatant and pellet were separated and stored frozen at -30 °C or -80 °C, respectively, for later determination of medium cortisol concentration and tissue transcript abundance.

### 4.3.6 MC2R/MRAP1 CHO cell expression

The functional expression of rainbow trout MC2R/MRAP was performed in CHO cells. CHO cells were grown and transfected as previously described (Liang et al., 2011). Briefly, 2 μg rtMC2R, zfMRAP1 and CRE-Luc reporter plasmid were transfected using a Cell Line Nucleofector Kit (Amaxa Inc., Gaithersburg, MD, USA) with solution T and program U-23, and seeded on a 96-well plate. Two days after transfection, cells were treated with 0, 0.1, 10, or 100 nM Cd and stimulated for 4 h at 37 °C in the absence or presence of ACTH (0.1 nM). At the end of the incubation, 100 μl of Bright-Glo luciferase assay reagent (Promega, Inc, San Luis Obispo, CA, USA) was applied to each well and incubated for 5 min at room temperature. Luminescence was measured using Bio-Tek Synergy HT plate reader to determine MC2R

activity (Winooski, VT, USA). The cell viability was not affected in response to Cd treatment (data not shown).

### 4.3.7 Tissue Cd accumulation

Cd concentrations were measured as previously described (Playle et al., 1993a, 1993b; Janes and Playle, 1995; McGeer et al., 2000b). Briefly brain and kidney samples (60-250 mg) were weighed, thawed and digested using approximately 5 volumes of 1N TraceMetal grade $HNO_3$ and then baked for 3 h at 80 °C. After digestion, tissues were vortexed for 5 seconds and then centrifuged for 2 min at 10,000 rpm (Spectrafuge 16M; Labnet International, Edison, NJ, USA) and left to settle. The supernatant was then diluted between 10-to 100-fold with 1% acidified ultrapure water. The resulting sample was then measured for Cd using graphite furnace atomic absorption spectrophotometer (GFAAS; SpectraAA 880 GTA 100 atomizer, Varian, Mississauga, ON, CAN). The concentration of Cd in the brain and head kidney is expressed as μg/g wet weight.

### 4.3.8 Plasma ACTH and cortisol

Plasma cortisol was measured using a radioimmunoassay (RIA as previously described (Sandhu and Vijayan, 2011 [Chapter 3]). Plasma ACTH was measured using a commercially available ImmuChem ^{125}I-labeled RIA kit (MP Biomedicals, Santa Ana, CA, USA). This assay has been previously validated for measuring plasma ACTH levels in trout plasma (Aluru and Vijayan, 2004; Doyon et al., 2006; Aluru and Vijayan, 2008).

### 4.3.9 Gene expression

RNA from head kidney, hypothalamus and POA were extracted using Ribozol RNA extraction reagent (Amresco, Solon, OH, USA). Briefly, 500 μl of Ribozol was added to 30-50 mg of tissue. Tissues were sonicated for homogenization and chloroform was added. The samples were then spun at 12,000 x g for 15 min and the aqueous phase (top layer) containing RNA was added to a new microcentrifuge tube. RNA was then precipitated using isopropanol and the dried pellet was redissolved in RNase free water. RNA was then DNase treated using manufacturer's instructions (Fermentas, Pittsburgh, PA, USA) and stored at -80 °C until analyzed. One microgram of total RNA was reverse-transcribed with high capacity cDNA reverse transcription kit (Applied Biosystems, Streetsville, ON, CAN).

Real-time quantitative PCR (qPCR) was used to measure transcript abundances for MRAP 1, MRAP 2 and MC2R within the head kidney; CRF and CRF-BP in the POA; and POMCA and POMCB in

the hypothalamus. Primer pair sequences, melting temperatures, and amplicon sizes are listed in Table 1. qPCR was performed using iCycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 2 min at 94 °C followed by 40 cycles of 15 sec at 94 °C, 30 sec at desired melting temperature (Table 1), and 30 sec at 72 °C. PCR products were subjected to melt curve analysis to confirm presence of a single amplicon. Negative controls with no template were carried out for each gene analyzed. Elongation factor 1 alpha (EF1α) was used as the housekeeping gene as no differences were observed between treatments or time-points.

### 4.3.10 GR and MR protein expression

Tissue homogenization and sample preparation for immunodetection was described previously (Sathiyaa and Vijayan, 2003; Dindia et al., 2012). Briefly, 50 mg of brain tissue was added to 200 μl of 50 mM Tris and 1X proteinase inhibitor (PI). Protein concentration was measured using the bicinchoninic acid *BCA) method with bovine serum albumin as a standard and samples for immunodetection were diluted to a concentration of 2 mg/ml in Laemmli's buffer (Laemmli, 1970). Samples for GR and MR protein detection were loaded (40 μg/g protein) onto 8% SDS-PAGE gel. Samples were run for 40 min at 200 V using 1 X TGS running buffer and the proteins were separated by mass. The separated proteins were transferred to a 0.45 μM nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) using the Transblot ® SD-semi-dry electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA) at 20 V for 25 min with a transfer buffer (25 mM Trish, pH 8.3, 192 mM glycine, and 10% methanol). The membrane was blocked with 5% non-fat dry milk with 0.02% sodium azide made in 1 X TTBS (2 mM Tris, 300 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h at room temperature. The membranes were probed with polyclonal rabbit antibodies to either trout GR (1:1000 Jeffrey et al., 2012) for 1 h at room temperature or polyclonal anti-trout MR (1:1000 Jeffrey et al., 2012) overnight for 18 h at 4 °C, followed by incubation with the secondary anti-rabbit horseradish peroxidase (HRP)-labeled secondary antibody (Bio-Rad, 1:3000 dilution in 5% skim milk). Protein bands were detected using ECl Plus[TM] chemiluminescence (GE Health Care, Baie, d'Urfe, QC, CAN) and imaged using Pharos FX molecular Imager (Bio-Rad, Hercules, CA, USA). Equal loading was confirmed through incubation of membranes with Cy3[TM] conjugated monoclonal mouse β-actin antibody (1:1000; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature.

### 4.3.11 Statistical analysis

All statistical analyses were performed using SigmaPlot 11 software (Systat Software Inc., San Jose, CA, USA) and data are presented as mean ± SEM. Data comparisons for plasma cortisol, ACTH and transcript

levels during 7 d study utilized two-way analysis of variance (ANOVA), and significant differences within each time point were assessed using one way-ANOVA. Significant differences among cortisol and transcript levels in the *ex vivo* study and CHO cell expression of MC2R/MRAP1 were confirmed using two-way repeated measures-ANOVA and a paired t-test to compare differences within treatments. Significant differences in brain GR and MR protein levels and tissue Cd accumulation were assessed using a one-way ANOVA. Significant differences between treatment groups for cortisol, ACTH and transcripts were compared using Holm-Sidak *post hoc* test or Student-Newman-Keul's test for comparison of ranks. The data were transformed wherever necessary to meet the assumption of homogeneity of variance, although non-transformed data are shown in the figures. A probability level of $P<0.05$ was considered to be significant.

**Table 1. Primer details** Oligonucleotide primers [forward (F) and reverse (R)] for corticotropin-releasing factor (CRF), corticotropin-releasing factor binding protein (CRF-BP), proopiomelanocortin (POMCA and POMCB), melanocortin 2 receptor (MC2R), melanocortin receptor accessory protein (MRAP1 and MRAP2) and elongation factor 1 alpha (EF1α) used in quantitative real-time PCR along with their melting temperature ($T_m$) and amplicon size and accession number.

| PRIMER | SEQUENCE | Amplicon Size (bp) | Accession # | Annealing $T_m$ (°C) |
|---|---|---|---|---|
| CRF | F: ACAACGACTCAACTGAAGATCTCG | 54 | NM_001124286.1 | 60 |
| | R: AGGAAATTGAGCTTCATGTCAGG | | | |
| CRF-BP | F: GGAGGAGACTTCATCAAGGTGTT | 51 | NM_001124631.1 | 60 |
| | R: CTTCTCTCCCTTCATCACCCAG | | | |
| POMCA | F: AGGGTTGAGGGAGGAAGAGA | 116 | NM_001124718.1 | 60 |
| | R: TGTCAGAGGACAGGGCTTTT | | | |
| POMCB | F: CCAGAACCCTCACTGTGACGG | 199 | NM_001124719.1 | 60 |
| | R: CCTGCTGCCCTCCTCTACTGC | | | |
| MC2R | F: GAGAACCTGTTGGTGGTGGT | 105 | EU119870 | 60 |
| | R: GAGGGAGGAGATGGTGTTGA | | | |
| MRAP1 | F: GACGAGCGCAAACTGAAA | 116 | NM_001246347.1 | 60 |
| | R: CTGACTGAACGGGACATGAA | | | |
| MRAP2 | F: CGGACCCGGACTACAAGTGGA | 111 | NM_001246353.1 | 60 |
| | R: GGCCCACCCAGAAGCCTATCA | | | |
| EF1α | F: CATTGACAAGAGAACCATTGA | 95 | NM_00124339.1 | 56 |
| | R: CCTTCAGCTTGTCCAGCAC | | | |

## 4.4 Results

### 4.4.1 Cadmium accumulation

Cd accumulation was measured in brain and head kidney for fish treated with control, 0.75 and 2.0 µg Cd/L (Figures 1A and B). There was no significant difference in Cd accumulation in the brain of Cd exposed fish relative to control fish (Figure 1A). Basal Cd levels for control samples in brain (0.14 µg/g) and head kidney (0.24 µg/g) were above background levels. In the kidney, there was only a significant increase in Cd accumulation in the fish treated with 2.0 µg Cd/L (0.32 µg /g) compared to control (Figure 1B).

### 4.4.2 Plasma cortisol and ACTH levels

No significant differences were measured in plasma cortisol levels between treatments during 7 d Cd exposure (0 h), but there was an overall treatment effect with plasma cortisol levels significantly lower in the Cd groups compared to the control group (Figure 2A). A time dependent effect was also observed whereby cortisol production at 1 h after application of a stresor was greater than cortisol production at 0, 4 and 24 h post-stressor exposure (Figure 2A). In the Cd groups, plasma cortisol levels were significantly reduced in a dose-related fashion compared to the controls at 1 h (~30% for 0.75 µg/L and ~64% for 2.0 µg/L Cd), but not at 4 and 24 h post-stressor exposure (Figure 2A).

No significant differences were observed between treatments in plasma ACTH concentrations during 7 d Cd exposure (0 h). There was an overall temporal effect on plasma ACTH levels during the post-stressor exposure period. ACTH concentrations were higher at 1 h than at those observed at 0, 4 and 24 h post-stressor exposure (Figure 2B). The stressor-induced elevation in plasma ACTH levels were significantly lower in the Cd groups at 1 h (~18% for 0.75 µg /L and ~38% for 2.0 µg/L Cd) but not at 0, 4 and 24 h post-stressor exposure (Figure 2B).

**Figure 1.** Cd accumulation in brain (A) and head kidney (B) of juvenile rainbow trout exposed to 0, 0.75 or 2.0 µg/L Cd for 7 days. Bars represent mean ± SEM  (n=6). Different letters above bars indicates significant differences among concentrations (P<0.05; one-way ANOVA)
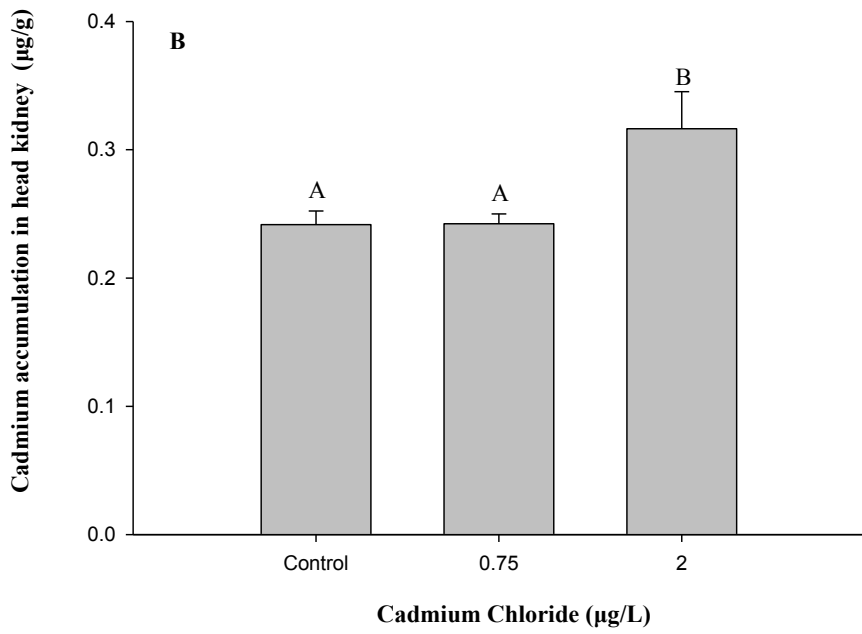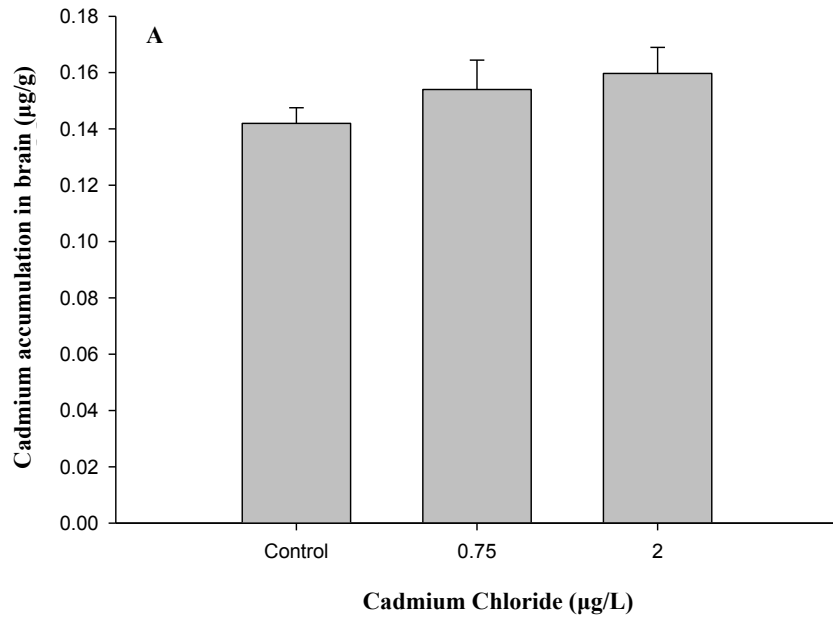
**Figure 2. Plasma analysis.** Plasma cortisol (A) and plasma ACTH (B) from juvenile rainbow trout exposed to 0, 0.75 or 2.0 μg/L Cd after 7 d exposure (0 h) and after 1,4 and 24 h after exposure to a secondary stressor. Bars represent mean ± SEM (n=6). Different letters above lines indicate significant differences between time-points (P<0.05; two-way ANOVA). Lower case letters indicate significant differences within the time-point (P<0.05; one-way ANOVA). Inset indicates significant differences between treatments (P<0.05; two-way ANOVA).
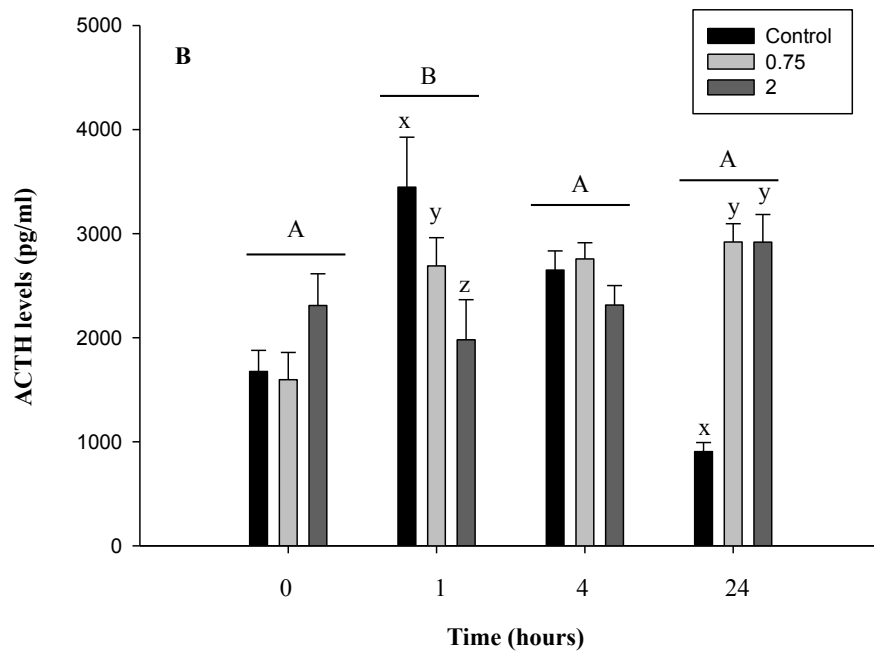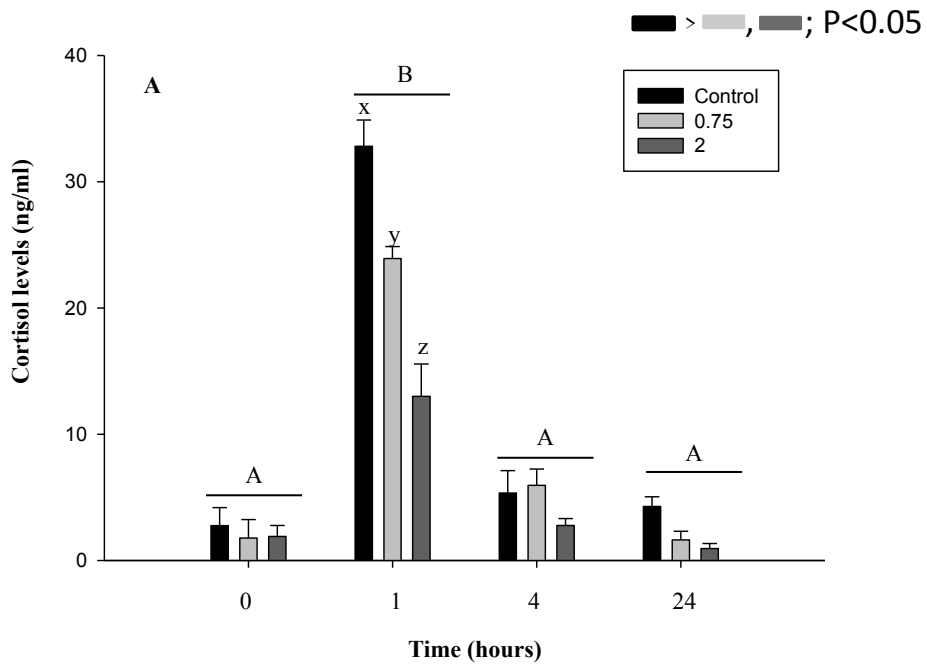
### 4.4.3 CRF and CRF-BP mRNA abundance in the preoptic area (POA)

No significant differences due to Cd exposure were observed in CRF or CRF-BP mRNA levels in the POA during 7 d exposure (0 h) (Figures 3A and B). There was a time-dependent increase in CRF mRNA levels after exposure to a secondary stressor over the 24 h period (Figure 3A). CRF-BP mRNA abundance also showed a time effect with levels significantly higher at 1 and 4 h compared to 0 and 24 h post-stressor exposure (Figure 3B). Cd exposure did not modify CRF-BP mRNA levels in the present study.

### 4.4.4 POMCA and POMCB mRNA abundance in the hypothalamus

POMCA mRNA levels were not statistically different between control and Cd-treated fish at any time point (Figure 4A). Although there was no treatment effect, a time-dependent effect was observed whereby POMCA mRNA levels were statistically different between 1 and 24 h post-stressed compared to 0 and 4 h (Figure 4A).

POMCB mRNA levels were not statistically different between control or Cd-treated fish at any time point except at 1 h post-stressed where POMCA mRNA levels were significantly decreased by ~72% and ~75% in fish exposed to 0.75 μg Cd/L or 2.0 μg Cd/L, respectively (Figure 4B). No other time or treatment effect was observed (Figure 4B).

### 4.4.5 Brain GR and MR protein expression

GR and MR protein expressions in the brain were measured after 7 d exposure to Cd. There was no significant effect of Cd exposure on GR protein expression (Figure 5A), whereas MR protein expression was significantly reduced by ~83% in the 2.0 μg Cd/L group compared to the control fish (Figure 5B).

**Figure 3. Preoptic area mRNA levels.** CRF (A) and CRF-BP (B) mRNA levels in preoptic area of juvenile rainbow trout exposed to 0, 0.75 or 2.0 μg/L Cd after 7 d exposure (0 h) and after 1,4 and 24 h after exposure to a secondary stressor on day 7. Bars represent mean ± SEM (n=6). Different letters above lines indicate significant differences between time-points (P<0.05; two-way ANOVA).
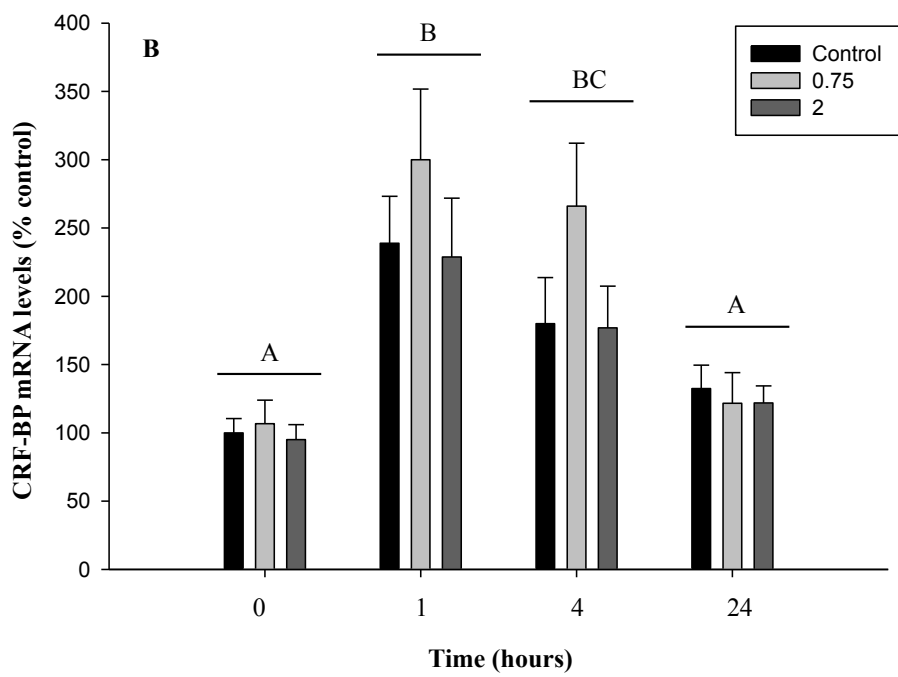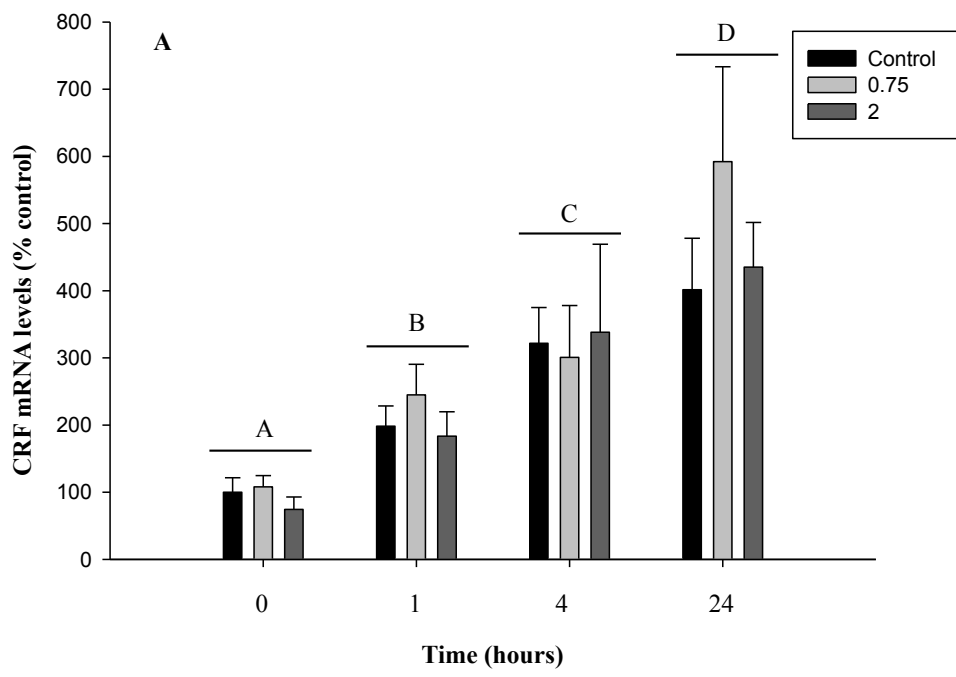
**Figure 4. Hypothalamus mRNA levels.** POMCA (A) and POMCB (B) mRNA levels in the hypothalamus of juvenile rainbow trout exposed to 0, 0.75 or 2.0 µg/L Cd after 7 d exposure (0 h) and after 1, 4 and 24 h after exposure to a secondary stressor on day 7. Bars represent mean ± SEM (n=6). Different letters above lines indicate significant differences between time points (P<0.05; two way ANOVA). Lower case letters indicate significant differences within the time-point (P<0.05; one way ANOVA).
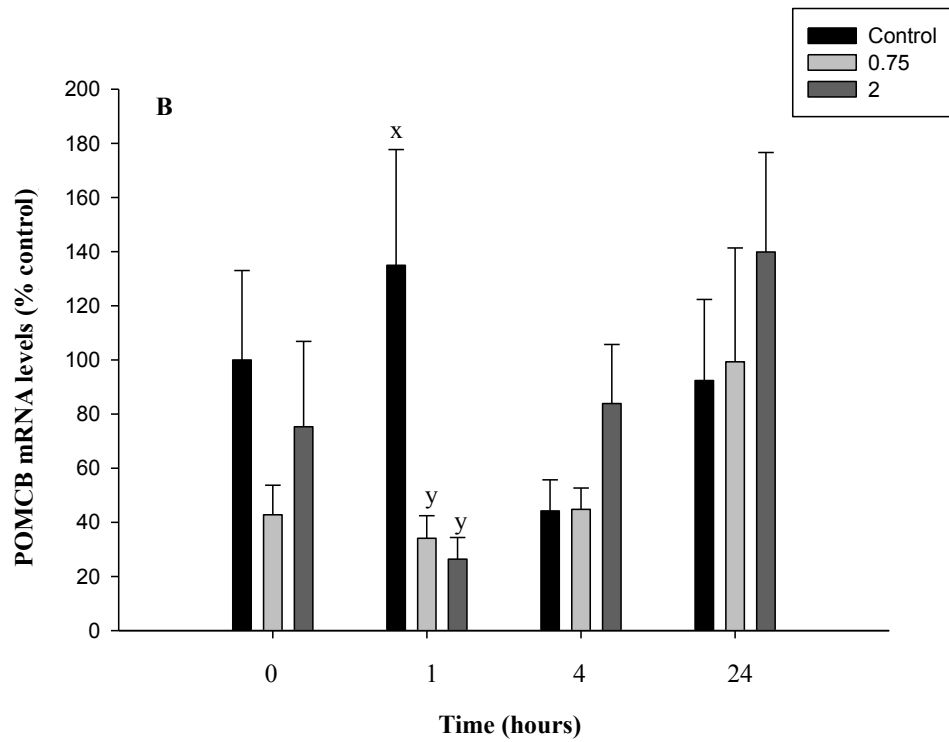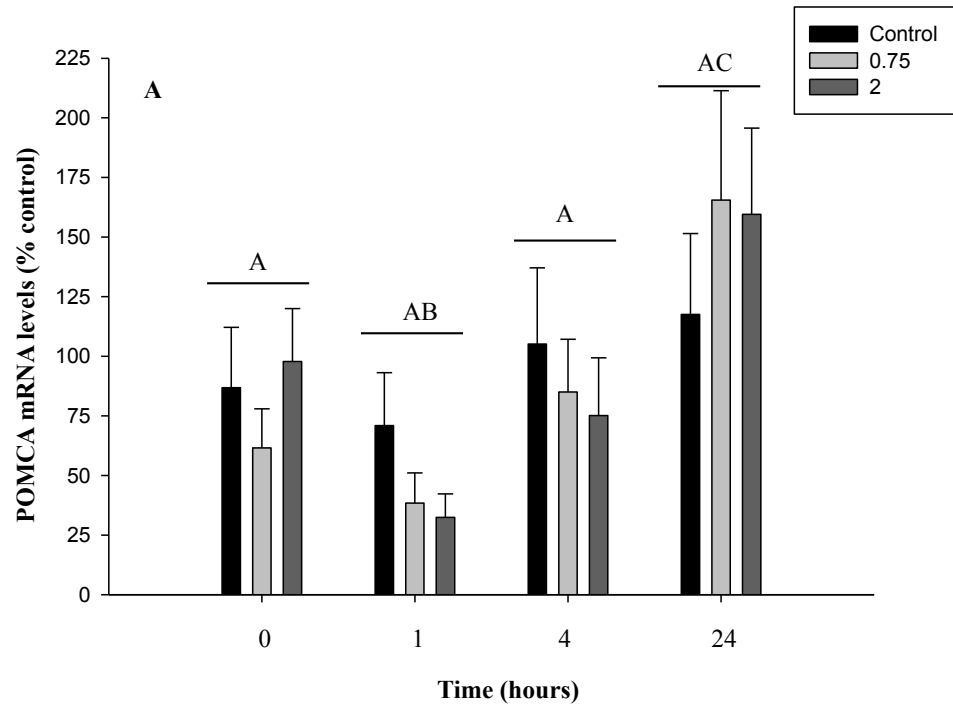
**Figure 5. Corticosteroidogenic protein expression.** GR (A) and MR (B) protein expression in whole brain of juvenile rainbow trout exposed to 0, 0.75 or 2.0 μg/L Cd for 7 days. Bars represent mean ± SEM (n=6). Different letters above bars indicates significant differences between concentrations ($P<0.05$; one-way ANOVA). A representative immunoblot for both GR and MR is shown. Equal loading was confirmed by β-actin (representative immunoblot image shown).
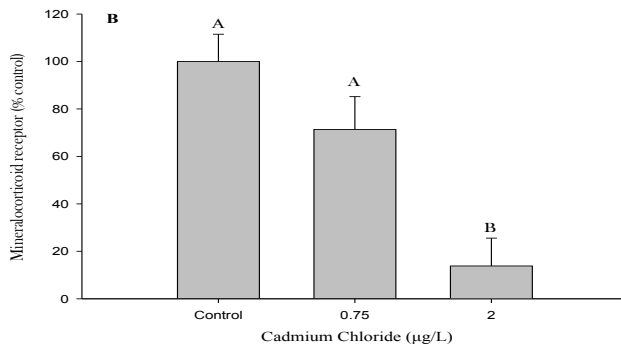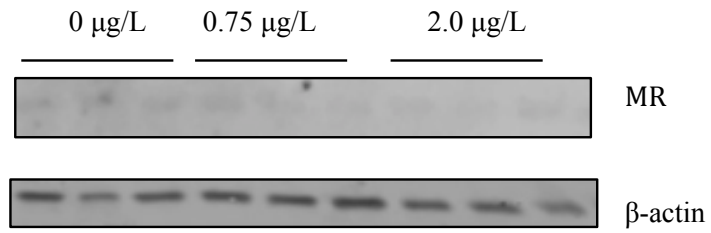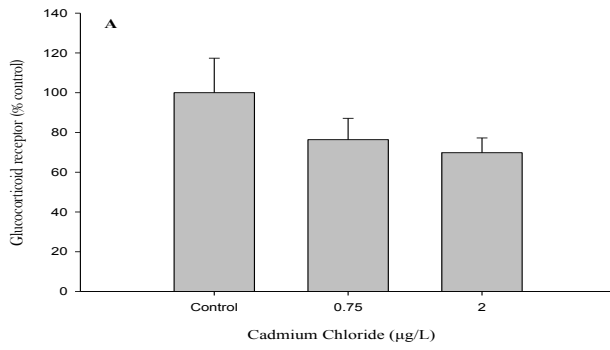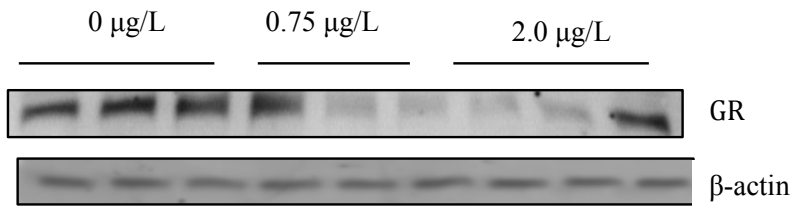
### 4.4.6 Stressor effect on MC2R, MRAP1 and MRAP2 mRNA levels in the head kidney

At 7 d exposure (0 h), MC2R mRNA levels were increased by ~50% and ~75% in in fish exposed to 0.75 μg Cd/L and 2.0 μg Cd/L, respectively, compared to control fish (Figure 6A). After 1 h post stressor exposure MC2R mRNA levels increased ~97% compared to control at 0 h (Figure 6A). In response to a secondary stressor, no significant changes were observed in MC2R mRNA levels at 4 or 24 h, but there was a ~97% decrease in MC2R levels in both Cd groups 1 h after exposure to a secondary stressor compared to the control group (Figure 6A). There was an overall treatment effect with the MC2R mRNA abundance significantly lower in the Cd groups compared to the control groups (Figure 6A). Furthermore, a time-dependent effect was observed whereby MC2R mRNA levels were statistically different at 1 and 4 h compared to 0 and 24 h post-stressor exposure (Figure 6A).

At 7 d exposure (0 h), no significant changes were observed in MRAP1 mRNA levels in any of the Cd groups with respect to control fish (Figure 6B). After 1 h post stressor exposure, MRAP1 mRNA levels increased ~91% compared to control at 0 h (Figure 6B). In response to a secondary stressor, no significant changes were observed in MRAP1 mRNA levels between treatments at 4 or 24 h, but there was a ~63% and ~91% decrease in MRAP1 mRNA abundance in fish exposed to 0.75 μg Cd/L and 2.0 μg Cd/L, respectively, at 1 h post-stressor exposure compared to the control group (Figure 6B). There was an overall treatment effect on MRAP1 mRNA levels showing greater values in control relative to fish treated with either Cd concentration. There was a time-dependent effect and MRAP1 mRNA levels were greater at 1 and 4 h, but not at 24 h after exposure to secondary stressor compared to 0 h samples (Figure 6B). No time-dependent effects were observed in time between 0 and 24 h (Figure 6B). There was also no significant effect of Cd exposure or time post-stressor exposure on MRAP2 mRNA abundance (Figure 6C).

**Figure 6. Interrenal mRNA levels.** MC2R (A), MRAP1 (B) and MRAP2 (C) mRNA levels from juvenile rainbow trout exposed to 0, 0.75 or 2.0 μg/L Cd after 7 d exposure (0 h) and after 1,4 and 24 h after exposure to a secondary stressor on day 7. Bars represent mean ± SEM (n=6). Different letters above lines indicate significant differences between time-points (P<0.05; two-way ANOVA). Lower case letters indicate significant differences within the time-point (P<0.05; one-way ANOVA). Inset indicates significant differences between treatments (P<0.05; two-way ANOVA).
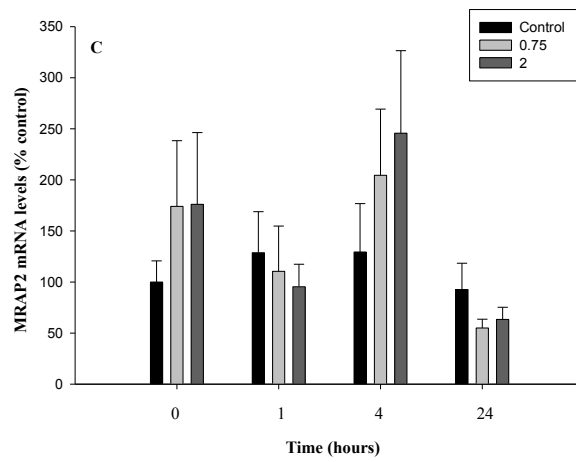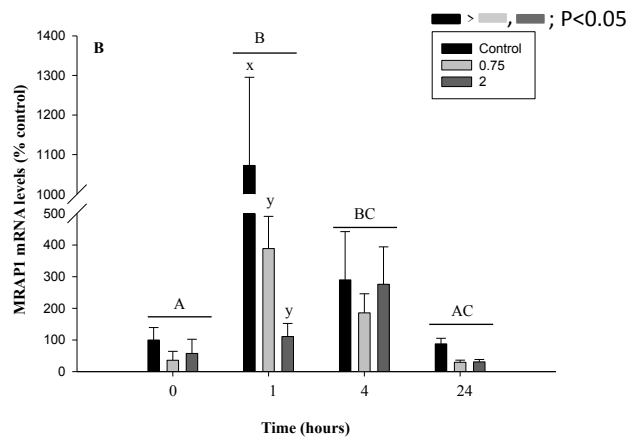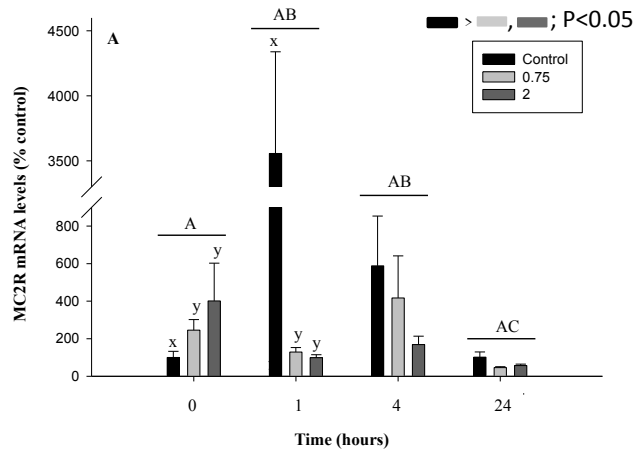
### 4.4.7 *Ex vivo* cortisol levels and MC2R, MRAP1 and MRAP2 mRNA levels in the head kidney

ACTH-stimulated cortisol production was significantly reduced by ~34% and ~68% in head kidney slices from fish exposed to 0.75 μg Cd/L and 2.0 μg Cd/L, respectively, compared to control (Figure 7A). No significant differences in cortisol production were observed between control and Cd exposed slices stimulated with 8-Bromo-cAMP (Figure 7A). However there was a ~39%, ~57% and ~78% increase in cortisol production in control, 0.75 μg Cd/L and 2.0 μg Cd/L head kidney slices stimulated with 8-Bromo-cAMP, respectively, relative to each treatment group stimulated with ACTH (Figure 7A). A stimulant effect was also observed whereby slices stimulated with 8-Bromo-cAMP showed greater cortisol levels than those stimulated with ACTH (Figure 7A).

Overall, MC2R levels were greater in the presence of ACTH relative to control (sham) (Figure 7B). In control (sham) slices stimulated with ACTH, MC2R levels significantly increased by ~96%, however no significant differences were observed in MC2R mRNA levels in control (sham) or ACTH-treated slices in either Cd group (Figure 7B). An overall treatment effect was observed whereby MC2R mRNA levels were greater in control fish relative to fish treated with 0.75 or 2.0 μg Cd/L (Figure 7B).

MRAP1 mRNA levels showed a stimulant effect where slices stimulated with ACTH had greater MRAP1 mRNA abundance than control (sham) (Figure 7C). MRAP1 mRNA levels significantly increased by ~96% in control slices stimulated with ACTH relative to control (sham), however no significant differences were observed in MRAP1 mRNA levels in control (sham) or ACTH-treated slices in either Cd groups (Figure 7C). MRAP1 had an overall treatment effect where MRAP1 mRNA abundance was greater in control fish relative to fish treated with 0.75 or 2.0 μg Cd/L (Figure 7C). No significant effect of Cd exposure or stimulant was observed on MRAP2 mRNA abundance (Figure 7D).

**Figure 7. Interrenal mRNA levels.** Magnitude of change in cortisol levels (A) and MC2R (B), MRAP1 (C) and MRAP 2 (D) mRNA levels in head kidney slices of juvenile rainbow trout exposed to 0, 0.75 or 2.0 μg/L Cd for 7 days *in vivo* and stimulated with ACTH or 8-Bromo-cAMP *in vitro*. Cortisol values show changes with respect to ACTH and 8-Bromo-cAMP stimulation whereas transcript values show changes with respect to ACTH. Values represent mean ± SEM (n=8). Lines with difference letters for cortisol data represent significant difference between control and Cd groups. Inset indicates significant difference between stimulants ($P<0.05$, two-way repeated measure ANOVA). Asterisks above bars represent significant differences within treatments ($P<0.05$; t-test).
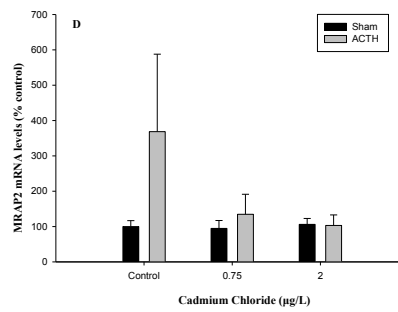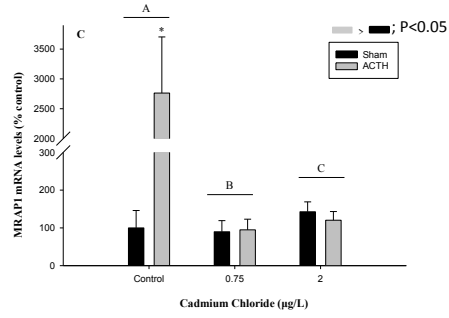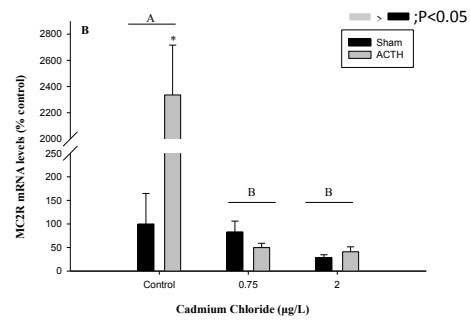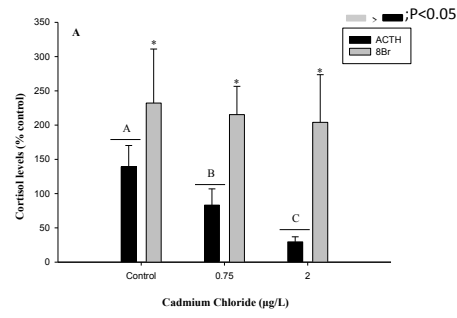
## 4.4.8 Functional activation of MC2R/MRAP1

MC2R/MRAP1 expression in CHO cells was not significantly different in control cells (background levels) exposed to 0, 0.1, 10 or 100 nM Cd (Figure 8). MC2R/MRAP1 expression in the presence of ACTH was ~90% greater in CHO cells exposed to 0 nM Cd relative to control cells (Figure 8). In the presence of Cd MC2R/MRAP1 expression was inhibited by ~42% (0.1 nM), ~41% (10 nM) and ~49% (100 nM) compared to expression in 0 nM CHO cells stimulated with ACTH (Figure 8).
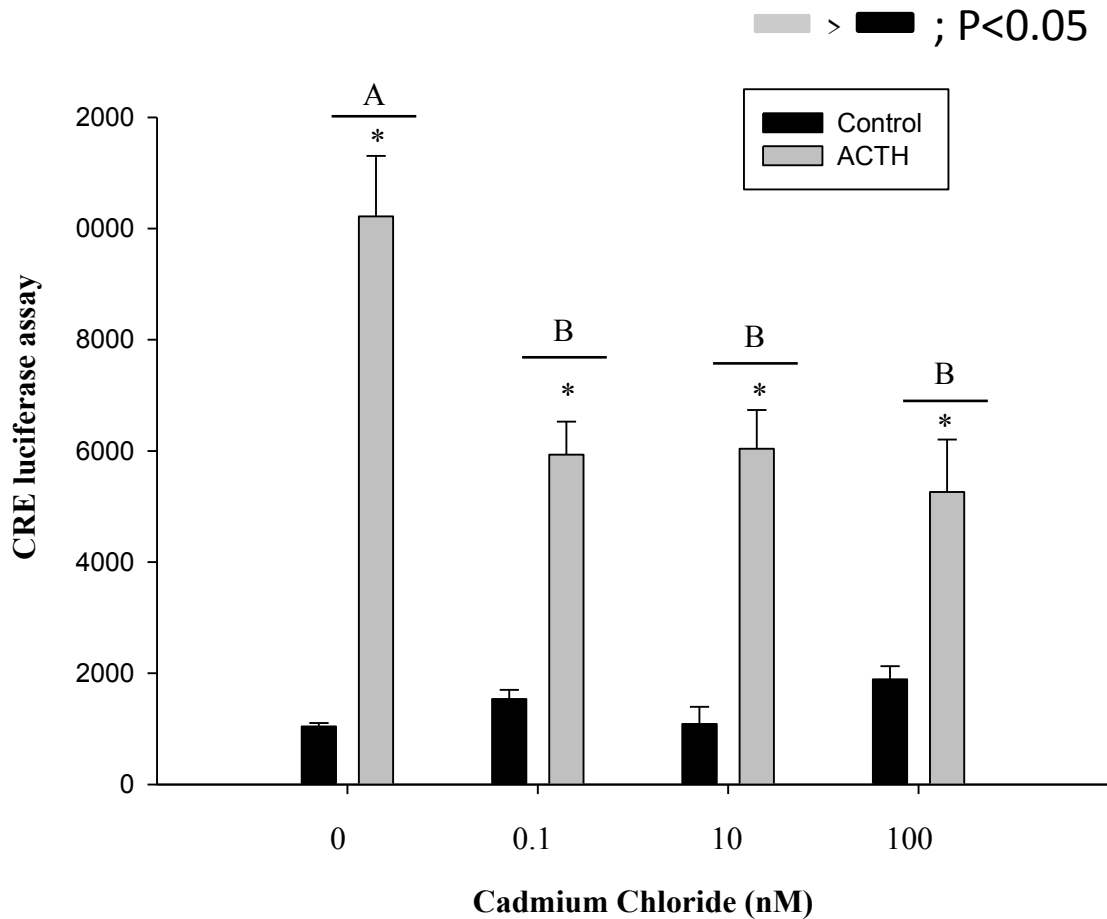
**Figure 8. CHO cell expression.** Maximal responses of rtMC2/zrMRAP1 receptor complex expressed in CHO cells exposed to 0, 0.1, 10 or 100 nM of Cd for 4 hour in the presence of absence of ACTH (0.1 nM). Values represent mean ± SEM (n=3). Inset indicates overall stimulant effect between control and ACTH (P<0.05, two-way repeated measures ANOVA). Lines with different letters above it represent significant differences in ACTH groups between control and Cd treatments (P<0.05; one-way ANOVA). Asterisks above bar represent significant differences in cell expression within treatments (P<0.05; t-test).

## 4.5 Discussion

Cd is an endocrine disruptor of the stress axis in teleosts. Our results suggest that Cd exerts its toxic effects at multiple sites, including the brain and interrenal tissue, in impairing the functioning of the cortisol stress axis in juvenile rainbow trout. This is the first study to demonstrate that the MC2R/MRAP1 complex, an essential component of ACTH signaling, is a target for Cd-mediated inhibition of interrenal steroidogenesis in rainbow trout.

### 4.5.1 Stress response disruption

Exposure to environmentally realistic Cd concentrations led to significant accumulation in the head kidney, but not the brain, which is in agreement with previously reported studies in fish (Norey et al., 1990; Gill et al., 1991; Lemaire-Gony and Mayer-Gostan, 1994; Kamunde, 1999; McGeer et al., 2000b; Franklin et al., 2005). The muscle and brain of fish are highly protected tissues that accumulate only negligible levels of total and new Cd from both dietary and waterborne exposures (Shaffi et al., 1999; McGeer et al., 2000b; Chowdhury et al., 2004; Favorito et al., 2011). The detection of Cd in the control fish is to be expected in the kidney based upon previous literature that coincides with values currently shown (McGeer et al., 2000b). The presence of Cd in the control fish could be due to many factors including contamination of feed and residual Cd in tanks from previous studies.

Cortisol, the primary glucocorticoid in teleosts is regulated by the hypothalamus-pituitary-interrenal (HPI) axis and predominately produced through binding of ACTH to MC2R (Wendelaar Bonga, 1997; Mommsen et al., 1999; Barton, 2002; Aluru and Vijayan, 2009). During the 7 d exposure (0 h), no significant differences were observed in either plasma cortisol or ACTH values suggesting that juvenile rainbow trout can adapt to sublethal Cd concentrations, which has been previously recorded (McDonald and Wood, 1993; Hollis et al., 1999, 2001; McGeer et al., 2000a,b).

Although Cd accumulation in brain, liver and kidney were similar between control and 0.75 μg Cd/L treated fish, there was attenuation in ACTH levels and cortisol production in both Cd treated groups after 1 h of exposure to a secondary stressor. The attenuation in cortisol in the low Cd group, regardless of the insignificant accumulation in tissues could be due to the higher presence of dissolved Cd in the water compared to the control fish. Actual concentrations of dissolved Cd in the water within each fish tank were recorded as negligible values in control fish (<0 μg Cd/L) whereas Cd concentrations of 0.71 and 2.33 μg Cd/L were seen in fish exposed to low and high Cd respectively.

Upregulation of the HPI axis in response to acute stress is a well-documented process in teleosts (Mommsen et al., 1999; Aluru and Vijayan, 2009). Levels of CRF during an acute stress are increased and maintained in teleosts (Doyon et al., 2003; Bernier et al., 2004; Huising et al., 2004; Payne et al. 2004; Craig et al., 2005; Doyon et al., 2006) through GR receptor signaling (Alderman et al., 2012). Furthermore, CRF-BP, a known binding protein of CRF in mammals (Potter et al., 1991) that limits CRF available for binding to CRF receptors and initiating ACTH production is also thought to play a similar role in teleosts (Huising et al., 2004; Doyon et al., 2005; Alderman et al., 2008). Within the 7 d exposure, an overall increase in CRF mRNA levels was observed during acute stress in both control and Cd treated fish which coincided with a similar profile in CRF-BP mRNA levels suggesting that the stress response remained in tact at the level of CRF production and bioavailability. Production of CRF during response to a stressor results in the production of ACTH from the anterior pituitary (Mommsen et al., 1999). Within the hypothalamus, no significant changes were observed in POMCA or POMCB during Cd exposure (0 h), however, there was an attenuation of POMCB in both Cd groups 1 h after exposure to a secondary stressor. As previously mentioned POMC is the precursor for ACTH in teleosts (Mommsen et al., 1999) that is necessary to bind to MC2R and initiate corticosteroidogenesis (Aluru and Vijayan, 2008). Although ACTH was not measured in the pituitary, it is highly possible that a reduction in POMC would reflect in a change in ACTH production in the pituitary, which has been examined in mammals (Caride et al., 2010).

Despite the low Cd concentration in the brain relative to kidney, a disruption of MR protein expression was observed in the high Cd group. The physiological effects of cortisol on target tissues is mediated by both the glucocorticoid and mineralocorticoid receptor in teleosts (Ducouret et al., 1995; Colombe et al., 2000; Bury et al., 2003; Greenwood et al., 2003; Sturm et al., 2005; Prunet et al., 2006; Kiilerich et al., 2007; Milla et al., 2008; Alsop and Vijayan, 2008; Aluru and Vijayan, 2009; Kiilerich et al., 2011; Kim et al., 2011; Takahashi and Sakamoto, 2013). Within teleosts, MR has been shown to bind cortisol with a greater affinity than GR when cortisol is present at low concentrations (Bury et al., 2003; Sturm et al., 2005) and unlike GR, MR is more abundantly expressed in the brain (Johansen et al., 2011). However, the downstream impact of MR signaling in fish is not well known. Classically, it is presumed that cortisol binds to GR in the hypothalamus and pituitary to initiate the negative feedback system to suppress further cortisol production (Bradford et al., 1992; Mommsen et al., 1999; Aluru and Vijayan, 2009; Bury et al., 2003), but this has not been confirmed. Since MR is more abundantly expressed in the brain, it is probable that it is crucial in the negative feedback pathway, suggesting that Cd may be disrupting corticosteroid signaling in the brain through disruption of MR (Alderman et al., 2012; Alderman and Vijayan, 2012).

### 4.5.2 Cd-mediated interrenal axis dysfunction

Within the interrenal cells of rainbow trout, corticosteroidogenesis mediated through genomic signaling begins with ACTH binding to MC2R (Aluru and Vijayan, 2008). Previous work done on ACTH-mediated cortisol disruption by Cd shows inhibition of key genes by involved in steroid biosynthesis, particularly MC2R (Sandhu and Vijayan, 2011 [Chapter 3]). A similar inhibition was seen in MC2R mRNA levels in the present 7 d *in vivo* study within the interrenal cells of both Cd groups 1 h after being exposed to a secondary stressor suggesting that Cd is inhibiting the G-protein response associated with ACTH binding to MC2R.

Once ACTH binds to MC2R, the G protein response is initiated and activates the adenylyl cyclase pathway to produce cAMP which in turn activates protein kinase A, has been shown to phosphorylate StAR, culminating in the production of cortisol (Mommsen et al., 1999; Aluru and Vijayan, 2008; Manna et al., 2009). Previous *in vitro* work showed that stimulation of head kidney slices with 8-Bromo-cAMP abolished Cd-mediated inhibition of cortisol and MC2R (Sandhu and Vijayan, 2011 [Chapter 3]). A similar response was observed *in vivo* whereby stimulation of head kidney slices exposed to waterborne Cd showed an inhibition in cortisol production when stimulated with ACTH but not when stimulated with 8-Bromo-cAMP. This confirms previous assumptions that Cd-mediated cortisol disruption of the stress response is occurring upstream of cAMP production and points at the target being the MC2 receptor.

Recently, it has been recognized that the functionalization of MC2R requires the melanocortin receptor accessory protein (MRAP) (Clark et al., 2005; Metherell et al., 2005; Rumié et al., 2007; Sebag and Hinkle, 2007; Chan et al., 2009; Gorrigan et al., 2011; Reinick et al., 2012), a phenomenon similar to other G-protein coupled receptors (Brady and Limbird, 2002; Saito et al., 2004; Dupré et al., 2007; Matsunami et al., 2009). If MRAPs are not present, MC2R will not re-localize from the endoplasmic reticulum to the plasma membrane where it is required for ACTH binding (Liang et al., 2011). Within mammals and teleosts, two MRAPs have been identified, MRAP1 and MRAP2 (Sebag and Hinkle, 2007; 2009a,b, 2010; Webb et al., 2009), and in zebrafish three MRAPs have been identified (MRAP1, MRAP2a, MRAP2b) (Agulleiro et al., 2010). The increase in MRAP1 but not MRAP2 after 1 h of stressor exposure during 7 d Cd exposure and through ACTH stimulation of head kidney slices in control fishes *in vitro* suggests that MRAP1 works alongside MC2R to induce expression of MC2R along the plasma membrane. This coincides with previous work done in mammals, rainbow trout and frog cell lines where activation of MC2R has shown to require the presence of MRAP1 but not MRAP2 (Agulleiro et al., 2010; Liang et al., 2011; Roy et al., 2012). In fact, MC2R/MRAP2 complexes in cell lines have a lower MC2R activation by

ACTH in comparison to MC2R/MRAP1 complexes (Liang et al., 2011). Furthermore, transient transcfection of CHO cells with MC2R and MRAP1 was suppressed in the presence of Cd when stimulated with ACTH, suggesting for the first time that Cd targets the MC2R/MRAP complex to disrupt cortisol production, however the mechanism of action is unknown.

Within the reproductive axis, Cd has been shown to bind to estrogen receptors in both mammals and teleosts to inactivate or suppress genes regulated by estradiol or estrogens (Le Guével et al., 2000; Stoica et al., 2000; Johnson et al., 2003; Jezierska et al., 2009). Therefore, a possible mechanism of action could be that once MRAP1 translocates MC2R to the cell membrane, Cd competes with cortisol and inhibits downstream corticosteroidogenesis. However, we cannot rule out the possibility that Cd targets MRAP1, which would result in inefficient localization of the receptor and thus a decrease in ACTH binding receptors, resulting in a suppression of cortisol production.

### 4.5.3 Conclusion

Exposure of juvenile rainbow trout to environmentally relevant concentrations of Cd over 7 days did not lead to significant accumulation in the brain, but there was significant accumulation in the kidney of juvenile rainbow trout exposed to 2.0 μg Cd/L. During the exposure period (0 h), no changes were observed in plasma cortisol, or ACTH levels, CRF, CRF-BP MRAP1 or MRAP2 mRNA levels or in GR protein expression in the brain of Cd exposed fish relative to control suggesting that juvenile rainbow trout are able to acclimate to Cd exposure. Significant changes in MR protein expression within the brain and MC2R mRNA levels in the head kidney suggests that Cd is impairing cortisol signaling at both the negative feedback level and interrenal steroidogenesis. Furthermore, exposure of Cd-treated juvenile rainbow trout to secondary stressor exposure similar to a predator-prey scenario did not impact CRF, CRF-BP, or POMCA mRNA levels suggesting that the genomic actions in the brain are intact. A decrease in POMCB 1 h after exposure to secondary stressor suggests that some alterations occur at the brain steroidogenic level that could potentially impact downstream sites that utilize precursors of POMC. Interestingly, we show that exposure of juvenile rainbow trout to a secondary stressor suppresses plasma ACTH and cortisol along with interrenal steroidogenesis and we propose that Cd-mediated cortisol disruption occurs through direct inhibition of MC2R and MRAP1 but not MRAP2. However, the mechanism of action remains to be elucidated.

## 4.6 Acknowledgements

# Chapter 5

# Cadmium disrupts glucocorticoid receptor signaling in rainbow trout liver

## 5.1 Overview

Although cadmium (Cd) has been shown to inhibit cortisol production in teleosts, the impact of this metal on target tissue cortisol action has not been ascertained. We tested the hypothesis that Cd disrupts cortisol signaling and the associated metabolic effects in rainbow trout liver. To test this, liver slices were incubated for 24 h in L-15 media containing 0, 250, 500 or 1000 nM Cd. Within each Cd concentration, liver slices were also exposed to cortisol (100 ng/ml) and mifepristone (glucocorticoid receptor [GR] antagonist; 1000 ng/ml) either alone or in combination. Cd accumulated in a dose-dependent manner in the liver. Cd at all concentrations abolished the cortisol-induced glucose production in the liver. The downregulation of GR expression seen with cortisol in control liver tissue was absent in the Cd treated liver slices. No changes were observed in heat shock protein 70 (HSP70) protein expression between sham and cortisol treated groups in the presence or absence of Cd, but there was a temporal increase of HSP70. Cortisol-induced upregulation of glucocorticoid-responsive genes, including phosphoenolpyruvate carboxykinase (PEPCK) and suppressors of cytokine signaling 1 (SOCS1), was completely abolished in Cd-treated liver slices. Also, cortisol-mediated upregulation of the activities of pyruvate kinase, PEPCK, glucokinase, hexokinase, aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase seen in control liver slices was absent with Cd treatment. Taken together, we demonstrate for the first time that Cd exposure impairs cortisol responsiveness of the liver associated with disruption of GR signaling and the accompanying suppression of liver metabolic capacity in trout liver.

## 5.2 Introduction

Many freshwater organisms are exposed to metal concentrations that are higher than what are considered to be natural background levels due to a continuous release of metals from natural and anthropogenic sources (Olsvik et al., 2000). Within a very short time frame, Cd can reach levels that are physiologically stressful to an organism and can possibly result in organismal death due to tissue accumulation (Gill and Pant, 1983; Gill et al., 1991; Spry and Wiener, 1991; Kamunde, 2009). During extended sublethal exposures, Cd accumulates in target tissues, including liver (Giles, 1988; McGeer et al., 2000b; Szebedinszsky et al., 2001; Hollis et al., 1999, 2000a, 2001). This metal exposure elicits a cellular stress response as indicated by the overexpression of heat shock protein 70 (Boone and Vijayan, 2002), a molecular chaperone essential for defense against proteotoxicity (Hightower, 1991; Iwama et al., 1998; 2006). Heat shock proteins (HSPs) are a family of proteins expressed in all cell types and are present in response to a wide array of biotic and abiotic stressors (Iwama et al., 1998; Feder and Hofmann, 1999). Hsp70, an important chaperone in preventing proteotoxicity and enhancing cell survival (Hightower, 1991;

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Mommsen et al., 1999; Vijayan et al., 2010), is a commonly used marker for cellular stress in response to a variety of stressors (Sanders, 1992; Sanders and Martin, 1993; Vijayan et al., 1994, 1997a,b, 1998; Williams et al., 1996; Duffy et al., 1999; Hassanein et al., 1999; Ackerman and Iwama, 2001; Basu et al., 2001), including Cd (Boone and Vijayan, 2002). However, the effect of Cd accumulation on liver function, and its mode of action, has not been previously investigated.

The liver plays a critical role in the metabolic adaptation to stress which involves energy substrate mobilization and reallocation to fuel the increased metabolic demand (Mommsen et al., 1999; Aluru and Vijayan 2009; Vijayan et al., 2010). As glucose is a key fuel, the stressor-induced production of this metabolite by the liver is essential for stress adaptation (Mommsen et al., 1999; Aluru and Vijayan, 2009). Glucose release from the liver is initially mediated by catecholamine-induced glycogenolysis, while the longer-term maintenance of glucose is thought to involve cortisol-induced gluconeogenesis (Mommsen et al., 1999; Aluru and Vijayan, 2009). The liver is an important target tissue for cortisol action allowing for the metabolic adaptation to stress (Mommsen et al., 1999; Aluru and Vijayan, 2009). The actions of cortisol are primarily mediated through genomic signaling via corticosteroid receptors in target tissues (Bern and Madsen, 1992; Mommsen et al., 1999; Colombe et al., 2000; Bury et al., 2003; Greenwood et al., 2003; Sturm et al., 2005; Prunet et al., 2006; Kiilerich et al., 2007, 2011; Alsop and Vijayan, 2008; Stolte et al., 2008; Aluru and Vijayan, 2009; Kim et al., 2011; Alderman et al., 2012; Takahasi and Sakamoto, 2013). In teleosts (except zebrafish, which only has a single GR; Alsop and Vijayan, 2008), multiple glucocorticoid receptors (GRs) and one mineralocorticoid receptor (MR) have been reported with wide tissue distribution, including the liver (Vijayan et al., 2005; Aluru and Vijayan, 2009). A key role for cortisol involves increasing the liver capacity for gluconeogenesis, which is mediated by GR signaling (Aluru and Vijayan, 2009). The upregulation of phosphoenolpyruvate carboxykinase (PEPCK), a key rate-limiting step in gluconeogenesis, activity and gene expression has been shown in response to acute stress and/or cortisol treatment and this is accompanied by higher activity of enzymes involved in amino acid metabolism, including alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) (Vijayan et al., 1994, 2003; Mommsen et al., 1999; Gravel and Vijayan, 2007; Wiseman et al. 2007; Aluru and Vijayan, 2009). This results is an overall increase in liver metabolic capacity during stress and leads to altered activities of enzymes involved in intermediary metabolism, including hexokinase (HK), glucokinase (GK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) (Mommsen et al., 1999; Aluru and Vijayan, 2009; Wiseman et al., 2007; Momoda et al., 2007).

Stressor-induced changes in either gluconeogenic or glycolytic capacity can provide valuable information on the effects of toxicants on metabolic performance (Hontela et al., 1992, 1997; Vijayan et al., 1997a,b, 2006; Ricard et al, 1998; Tintos et al., 2006, 2007; Gravel and Vijayan 2007; Hontela and Vijayan, 2008; Ings et al., 2011, 2012), and although Cd accumulates in the liver, its impact on liver function is unknown. To test this, sublethal concentrations of Cd, relevant to environmental levels that would accumulate within the liver, disrupt cortisol signaling and will impair the metabolic capacity of the liver. To test this, juvenile rainbow trout (*Oncorhynchus mykiss*) liver slices *in vitro* were exposed to 0, 250, 500 or 1000 nM of Cd either in the presence or absence of stress levels of cortisol (100 ng/ml). These studies were also carried out with GR receptor antagonist mifepristone (1000 ng/ml) to confirm that the observed metabolic effects were indeed GR mediated.

## 5.3 Materials and methods

### 5.3.1 Chemicals

2-Phenoxyethanol, protease inhibitor cocktail, bicinchoninic acid (BCA), adenosine 5'-triphosphate (ATP), L-alanine, aspartic acid, 2-deoxyguanosine-5-diphosphate, α-ketoglutaric acid, lactate dehydrogenase, malate dehydrogenase, manganese chloride, β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (β-NADH), phosphenolpyruvate, pyridoxal 5-phosphate, pyruvate kinase, pyruvic acid, sodium bicarbonate and mifepristone were purchased from Sigma-Aldrich (St.Louis, MO, USA). Cd (used as $CdCl_2$) and d-glucose were purchased from Bioshop (Burlington, ON, CAN). Trizol for RNA extraction and DNAse were purchased from Invitrogen (Carlsbad, CA, USA) and Fermentas (Pittsburgh, PA, USA), respectively. ECl Plus[TM] chemiluminescence kit was purchased from GE Health Care (Baie d'Urfe, QC, CAN). RNase free water for RNA extraction, cDNA synthesis kit and Sybr green were purchased from Qiagen (Toronto, ON, CAN). Nitric acid, potassium chloride, sodium bicarbonate were purchased from Fisher Scientific (Fairlawn, NJ, USA).

### 5.3.2 Fish and holding conditions

Juvenile rainbow trout (average body mass of 150-300 g) were purchased from Rainbow Springs Hatchery (Thamesford, ON, CAN) and maintained at the University of Waterloo aquatic facility. Fish were maintained in 2000 L tanks with a constant flow of aerated water at 13 °C, and under a 12 h light: 12 h dark photoperiod. Trout were acclimated for at least 3 weeks prior to experiment and were fed commercial trout feed (Martin Mills, Elmira, ON, CAN) to satiety once daily, 5 days a week. Experiments were approved by

the University of Waterloo Animal Care Protocol Review Committee and adhere to the guidelines established by the Canadian Council on Animal Care for the use of animals in teaching and research.

### 5.3.3 Experimental protocol

Juvenile rainbow trout that were fasted for 24 h prior to sampling were euthanized by an overdose of 2-phenoxyethanol. The liver of each fish was excised and washed twice using modified Hanks media (NaCl (136.9 mM), KCl (5.4 mM), $MgSO_4 \bullet 7H_2O$ (0.8mM), $Na_2HPO_4 \bullet 7H_2O$ (0.33 mM), $KH_2PO_4$ (0.44 mM), HEPES (5.0 mM), HEPES Na (5.0 mM), 5mM $NaHCO_3$ pH adjusted to 7.63) and sliced into fine sections (~1 μm thick and ~10 mm in diameter) using a precision cut liver slicer. The sections (weighing ~100 mg) were then placed into 6 well plates (Sarstedt, Inc., Newton, NC, USA) containing 2 ml of L-15 medium (Sigma-Aldrich, St Louis, MO, USA). The tissues were maintained for 2 h at 13 °C with gentle shaking, after which the media was removed and fresh L-15 media containing 0, 250, 500, or 1000 nM of Cd was added to each well. Liver slices were exposed to Cd concentrations for 1.5 h prior to the addition of either no cortisol (control), cortisol (100 ng/ml), mifepristone (1000 ng/ml) or a combination of cortisol and mifepristone. The concentration of cortisol and mifepristone used were based on previous studies in trout hepatocytes (Aluru and Vijayan, 2007; Ings et al., 2011) Mifepristone was added to liver slices 30 min prior to cortisol addition in the combination group. Liver tissue and media were sampled 24 h after exposure and stored frozen at -80 °C and -30 °C, respectively, until further analysis.

### 5.3.4 Liver cadmium accumulation

Cd concentrations were measured as previously described (Playle et al., 1993a, 1993b; McGeer et al., 2000b). Briefly, liver samples (30-50 mg) were weighed, thawed and digested using approximately 5 vol. of 1N TraceMetal grade $HNO_3$ and then baked for 3 h at 80 °C. After digestion, tissues were vortexed for 5 sec and then centrifuged for 2 min at 10,000 rpm (Spectrafuge 16M; Labnet International, Edison, NJ, USA) and left to settle. The supernatant was then diluted between 10 to 100 fold with 1% acidified ultrapure water. Cd was measured in the resulting sample using graphite furnace atomic absorption spectrophotometer (GFAAS; SpectraAA 880 GTA 100 atomizer, Varian, Mississauga, ON, CAN). The concentration of Cd in the liver is expressed as μg/g wet weight.

### 5.3.5 Media glucose analysis

Glucose released into L-15 media was measured enzymatically exactly as described previously (Bergmeyer, 1983; Birceanu, 2009).

### 5.3.6 RNA isolation and first strand cDNA synthesis

Total RNA from liver slices was extracted using Trizol RNA extraction reagent (Carlsbad, CA, USA). Briefly, 500 µl of Trizol was added to 30-50 mg of tissue. Tissues were sonicated for homogenization and chloroform was added to separate RNA and protein into phases. The samples were then spun at 12, 000 x g for 15 minutes and the aqueous phase (top layer) containing RNA was added to a new microcentrifuge tube. RNA was then precipitated using isopropanol and the dried pellet was redissolved in RNase free water.

RNA was then DNase treated using manufacturer's instructions (Fermentas, Pittsburgh, PA, USA). Briefly, 1 µg of RNA was treated with DNase and heated at 37 °C using an Eppendorf cycler. Afterwards DNase treatment was stopped using EDTA and heating the sample at 65 °C for ten minutes. cDNA was made from DNase treated RNA using a high capacity cDNA reverse transcription kit following manufacturer's instructions (Applied Biosystems, CA, USA).

### 5.3.7 Primers

Primers were designed to amplify fragments for suppressor of cytokines 1 (SOCS1), phosphoenolpyruvate carboxykinase (PEPCK) and elongation factor 1 alpha (EF1α) using previously described sequences (Aluru & Vijayan; 2007; Philip et al., 2012). Table 1 provides primer sequences, amplicon size, NCBI accession number and annealing temperature. As SOCS1 and PEPCK are glucocorticoid-responsive genes (Vijayan et al., 2003; Philip et al., 2012), their transcript abundances were used as markers of liver responsiveness to cortisol treatment.

**Table 1. Primer details.** Oligonucleotide primers [forward (F) and reverse (R)] for suppressor of cytokine signaling 1 (SOCS1), phosphoenolpyruvate carboxykinase (PEPCK) and elongation factor 1 alpha (EF1α) used in quantitative real-time PCR along with their melting temperature ($T_m$) and amplicon size.

| PRIMER | SEQUENCE | Amplicon Size (bp) | Accession # | $T_m$ (°C) |
|---|---|---|---|---|
| SOCS1 | F: TCAGCGTACGCATCGTCTAT | 120 | NM_001146166.1 | 60 |
| | R: CGGTCAGGCTTTTCTTAGAGG | | | |
| PEPCK | F: TGCTGAGTACAAAGGCAAGG | 154 | AF246149.1 | 60 |
| | R: GAACCAGTTGACGTGGAAGA | | | |
| EF1α | F: CATTGACAAGAGAACCATTGA | 95 | NM_00124339.1 | 56 |
| | R: CCTTCAGCTTGTCCAGCAC | | | |

### 5.3.8 Quantitative real-time polymerase chain reaction (qPCR)

qPCR was used to measure mRNA levels of SOCS1, PEPCK and EF1α (housekeeping gene) in the liver as previously described (Aluru & Viayan, 2008; Sandhu & Vijayan, 2011 [Chapter 3]; Philip et al., 2012). Briefly, a master mix containing Sybr green, RNase free water and the specific primer were added to 2 μl of sample. The samples were added to the wells of qPCR plates in triplicates. PCR was performed using iCycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 2 min at 94 °C followed by 40 cycles of 15 sec at 94 °C, 30 sec at desired melting temperature (Table 1), and 30 sec at 72 °C. PCR products were subjected to melt curve analysis to confirm presence of a single amplicon. Copy numbers of transcripts for each gene was determined with the threshold cycles (CT) using plasmid standard curves and were normalized to EF1α. EF1α transcript levels in all treatments were not variable, making it an appropriate housekeeping gene. Negative controls with no template were carried out for each gene analyzed.

### 5.3.9 Protein Immunoblot

Liver slices for protein analysis was carried out exactly as previously described (Ings et al., 2011a,b; Dindia et al., 2012). Briefly, protein concentration was measured using the bicinchoninic acid (BCA) method using bovine serum albumin as the standard. An 8% SDS-PAGE was used to determine GR and HSP70 protein expression in the liver in response to Cd exposure. Total protein (40 μg) was separated, using the discontinuous buffer system of Laemmli (1970) exactly as described before (Ings et al., 2011b; Dindia et al., 2012; Jeffrey et al., 2012). The separated proteins were transferred to a 0.45 μM nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) using the Transblot® SD semi-dry electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA) at 20 V for 25 min with a transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 10 % methanol). The membrane was blocked with 5% non-fat dry milk with 0.02% sodium azide made in 1 X TTBS (2 mM Tris, 300 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h at room temperature. The membranes were probed with polyclonal rabbit antibodies to either trout GR (1:1000 dilution; Sathiyaa and Vijayan, 2003; Ings et al., 2011a) for 1 h at room temperature or HSP70 (1:5000; Ings et al., 2011a) overnight for 18 h at 4 °C, followed by incubation with the secondary anti-rabbit horseradish peroxidase (HRP)-labeled secondary antibody (Bio-Rad, Hercules, CA, USA; 1:3000 dilution in 5% skim milk). Protein bands were detected using ECL Plus[TM] chemiluminescence (GE Health Care, Baie d'Urfe, QC, CAN) and imaged using Pharos FX Molecular Imager (Bio-Rad, Hercules, CA, USA). Equal loading was confirmed through incubation of membranes with Cy3[TM] conjugated monoclonal mouse β-actin antibody (Sigma-Aldrich 1:1000) for 1 h at room temperature.

### 5.3.10 Liver enzyme activity

Liver was homogenized in an enzyme buffer (50% glycerol, 21 mM $Na_2HPO_4$, 0.5 mM EDTA-Na, 0.2% BSA, 5 mM β-mercaptoethanol, pH 7.5) exactly as described previously (Vijayan et al., 2006; Ings et al., 2011b). Enzyme activities for each sample were measured in duplicate in 50 mM imidazole buffer (pH 7.4) at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA) exactly as described before (Ings et al., 2011b; Vijayan et al. 2006). Enzyme activity was expressed as micromoles of substrate consumed or product liberated per minute (U) per gram protein. The following assay conditions were used:

- **Hexokinase (HK: EC 2.7.1.1):** 1 mM glucose, 5 mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/mL lactate dehydrogenase (LDH) and 2.5 U/mL pyruvate kinase; reaction started with 1 mM ATP.

- **Glucokinase (GK: EC 2.7.1.2):** 15 mM glucose, 5 mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/mL lactate dehydrogenase (LDH) and 2.5 U/mL pyruvate kinase; reaction started with 1 mM ATP.

- **Pyruvate kinase (PK: EC 2.7.1.40):** 3 mM KCl, 10 mM $MgCl_2$, 0.12 mM NADH, 2.5 mM ADP, 20 U/mL LDH; reaction started with 2.5 mM PEP.

- **Lactate dehydrogenase (LDH: EC 1.1.1.27):** 0.12 mM NADH and reaction initiated with 1 mM pyruvic acid.

- **Phosphoenolpyruvate carboxykinase (PEPCK: EC 4.1.1.32):** 20 mM $NaHCO_3$, 1 mM $MnCl_2$, 0.5 mM phosphoenolpyruvate, and 0.12 mM NADH; reaction started with 5 mM deoxyguanosine diphosphate.

- **Alanine aminotransferase (AlaAT: EC 2.6.1.2):** 0.12 mM NADH, 200 mM l-alanine, 0.025 mM pyridoxal 5-phosphate, and 12 U/mL LDH; reaction started with 10.5 mM α-ketoglutarate.

- **Aspartate aminotransferase (AspAT: EC 2.6.1.1):** 7 mM α-ketoglutarate, 0.025 mM pyridoxal 5-phosphate, 0.12 mM NADH, and 8 U/mL malate dehydrogenase; reaction started with 40 mM aspartic acid.

### 5.3.11 Statistical analysis

All statistical analyses were performed using SigmaPlot 11 software (Systat Software Inc., San Jose, CA, USA) and data are shown as mean ± standard error of mean (SEM). Cd accumulation was tested

using one-way repeated measures analysis of variance (ANOVA). Glucose production, mRNA and protein levels and enzyme activities were analyzed using two-way repeated measures ANOVA. Paired t-test was performed to compare cortisol stimulated mRNA levels of PEPCK and SOCS1 and GR and hsp70 protein expression relative to control (sham) groups. Significant differences between treatment groups were compared using Holm-Sidak *post hoc* test or Student-Newman-Keul's test for rank comparisons. Statistics were performed either on raw or log- transformed data in order to meet the assumptions of normality and equal variance, although non-transformed data are shown in the figures. A probability level of $p<0.05$ was considered significant.

## 5.4 Results

### 5.4.1 Cadmium accumulation

A dose-dependent significant increase was observed in Cd accumulation within liver slices in response to 0, 250, 500 and1000 nM Cd exposure in the media over a 24 h period (Figure 1). Cd concentrations were measured as 0.48, 0.89 and 1.4 µg/g wet weight in tissues exposed to 250, 500 and 1000 nM Cd, respectively, and they were significantly higher than control liver slices (0.08 µg/g protein) (Figure 1).
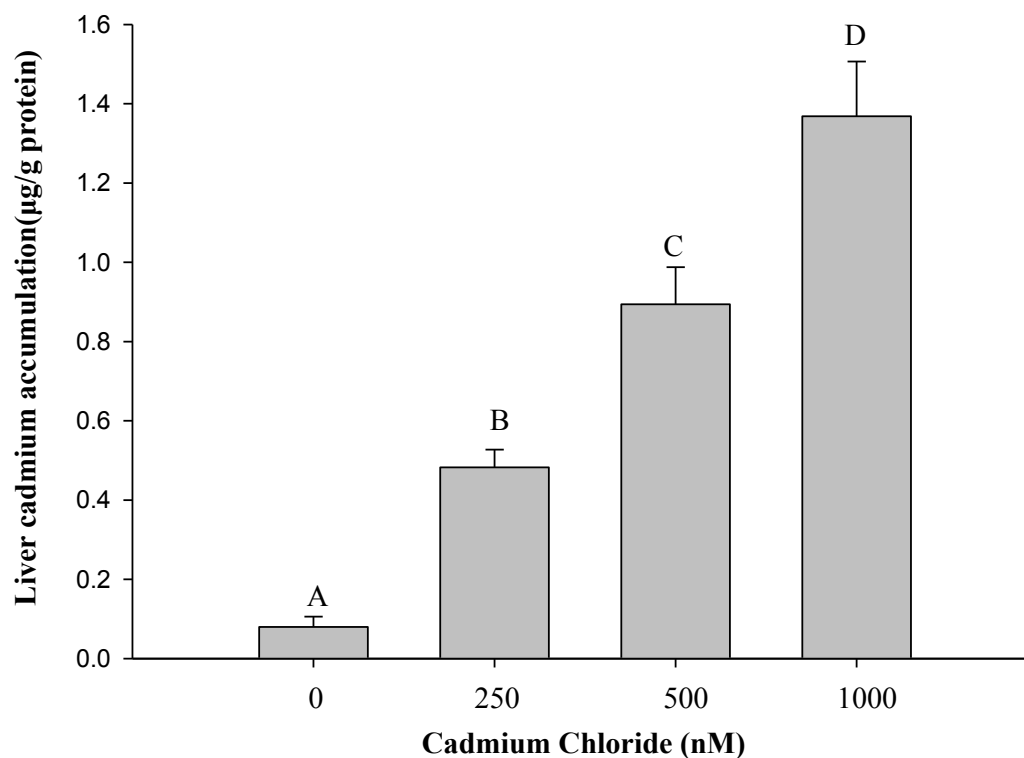
**Figure 1. Liver Cd accumulation.** Cd accumulation in liver slices treated with 0, 250, 500 or 1000 nM cadmium chloride. Values represented as mean ± SEM (n=6 fish). Bars with different letters are statistically significant (one-way repeated measured ANOVA; P<0.05).

### 5.4.2 Plasma glucose

No significant differences in plasma glucose were detected in liver slices treated with 250, 500 or 1000 nM of Cd relative to control (Figure 2). In the presence of cortisol, glucose levels were elevated by ~64% in liver slices only in the sham group and this elevation was abolished by mifepristone treatment (Figure 2). In the Cd groups the cortisol-induced glucose elevation observed in control liver slices stimulated with cortisol was completely abolished (Figure 2). Overall, glucose levels were significantly lower in all the Cd treatments compared to the control liver slices (Figure 2).

### 5.4.3 Transcript analysis

GR signaling in liver slices was confirmed by the significant increases in both PEPCK and SOCS1 mRNA levels in control liver slices stimulated with cortisol and the absence of this response in the presence of mifepristone (Figure 3A,B). In the Cd liver slices treated with 1000 nM Cd, no significant difference was observed in either PEPCK or SOCS1 mRNA levels in the presence of cortisol (Figures 3A,B).

### 5.4.4 Protein expression analysis

Liver slices showed a dose-related inhibition in GR protein expression with significant decreases observed in liver slices exposed to 500 (~20% lower) or 1000 nM (~30% lower) Cd compared to the sham group (Figure 4A). Liver slices treated with 0 nM of Cd showed a ~30% decrease in GR protein expression when stimulated with cortisol; however, no significant differences were observed in GR protein expression in liver slices exposed to 250, 500 or 1000 nM Cd and stimulated with or without cortisol (Figure 4A).

Overall, hsp70 protein expression was greater in unstimulated liver slices than liver slices stimulated with cortisol. Furthermore, an overall increase in hsp70 protein expression was observed in liver slices treated with 1000 nM Cd relative to control, 250 or 500 nM Cd (Figure 4B). There was no effect of cortisol on hsp70 expression in any of the Cd treatments (Figure 4B).
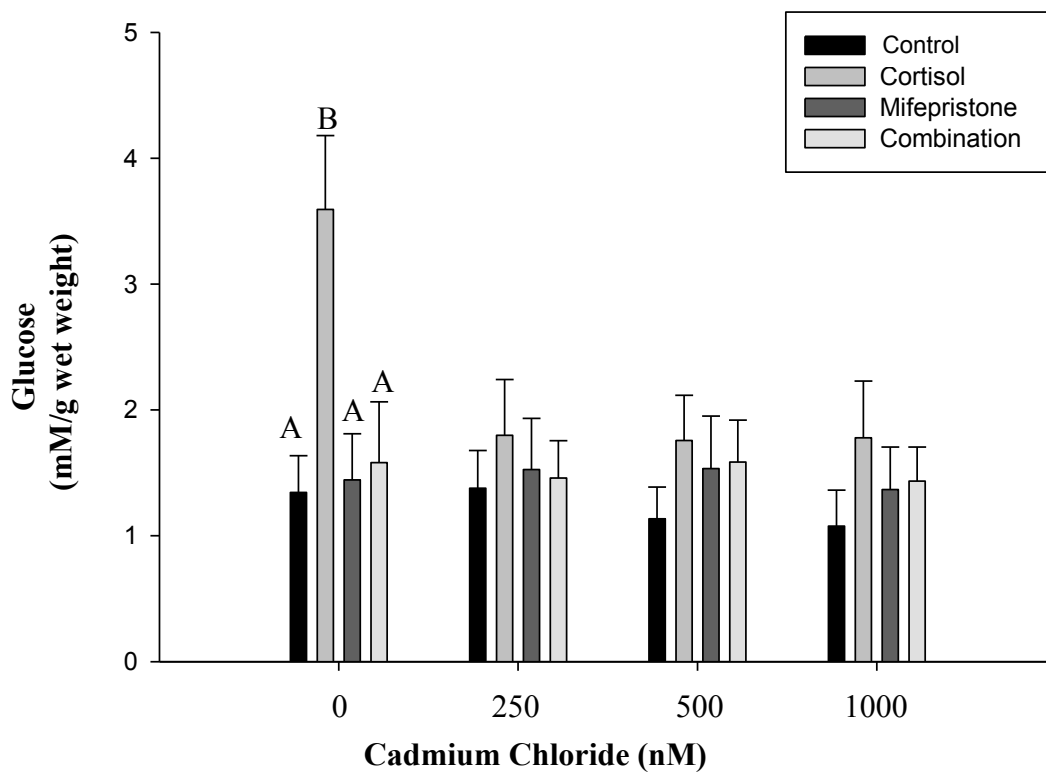
**Figure 2. Glucose production.** Glucose production in liver slices treated with 0, 250, 500 or 1000 nM cadmium chloride in control, 100 ng/ml cortisol, 1000 ng/ml mifepristone or a combination of cortisol and mifepristone groups. Values represented as mean ± SEM (n=6 fish). Bars with different letters are statistically significant (one way repeated measures ANOVA; P<0.05).

**Figure 3. GR-mediated PEPCK and SOCS1 liver mRNA levels.** Phosphoenolpyruvate carboxykinase (PEPCK) (A) and suppressor of cytokines 1 (SOCS1) (B) mRNA levels in liver slices treated with 0 or 1000 nM cadmium chloride in control and cortisol stimulated (100 ng/ml) groups. Values represented as mean ± SEM (n=6 fish). * significant different from the corresponding control (Paired t-test; $P<0.05$).
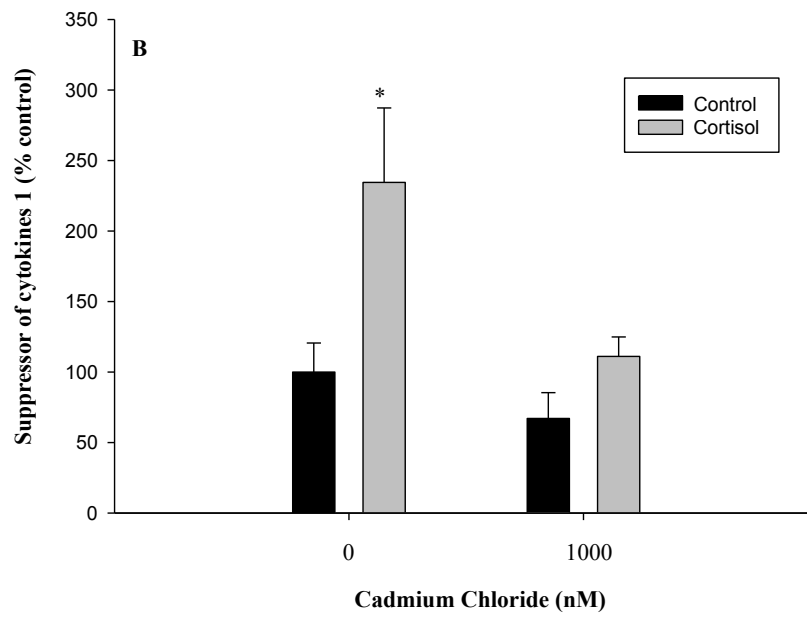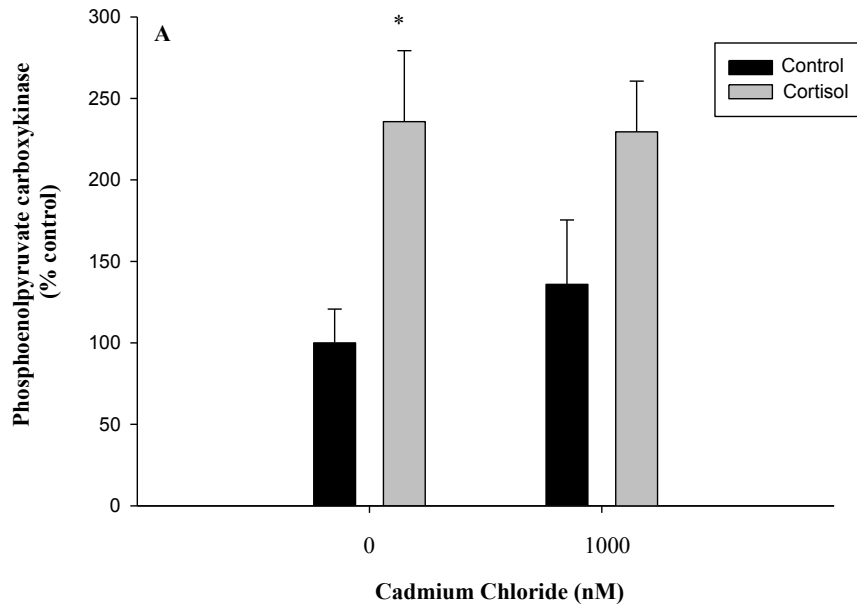
**Figure 4. Glucocorticoid receptor and heat shock protein 70 liver protein expressions.** Glucocorticoid receptor (GR; A) and heat shock protein 70 (hsp70; B) protein expression in liver slices treated with 0, 250,500 or 1000 nM cadmium chloride in control or cortisol stimulated (100 ng/ml) groups. Values represented as mean ± SEM (n=6 fish). * significant different from the corresponding control (Paired t-test; $P<0.05$);  different letters above lines represent significant differences in mRNA levels between Cd groups (two-way repeated measures ANOVA; $P<0.05$); inset represents significant differences between control and cortisol treatment (two-way repeated measures ANOVA; $P<0.05$); representative images for GR, hsp70 and β-actin are shown.

**Control**

0nM   250nM   500nM   1000nM

GR

β-actin

**Cortisol**

0nM   250nM   500nM   1000nM



**Control**

0nM   250nM   500nM   1000nM

hsp70

β-actin

**Cortisol**

0nM   250nM   500nM   1000nM
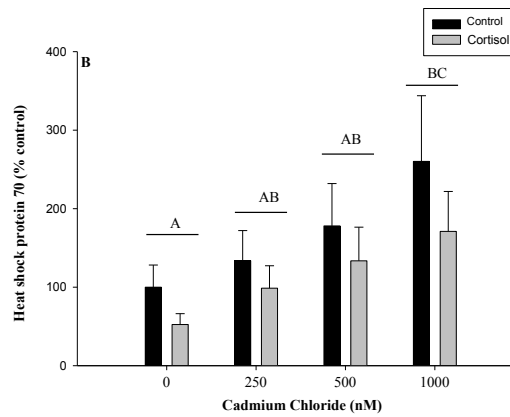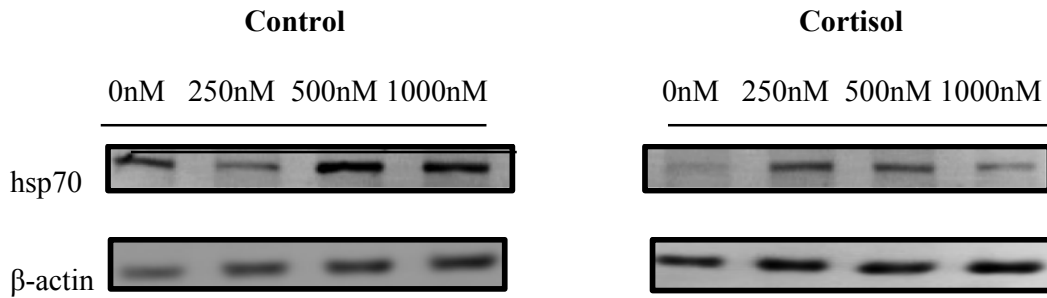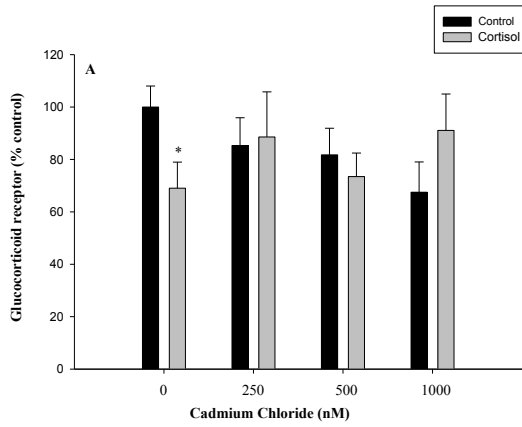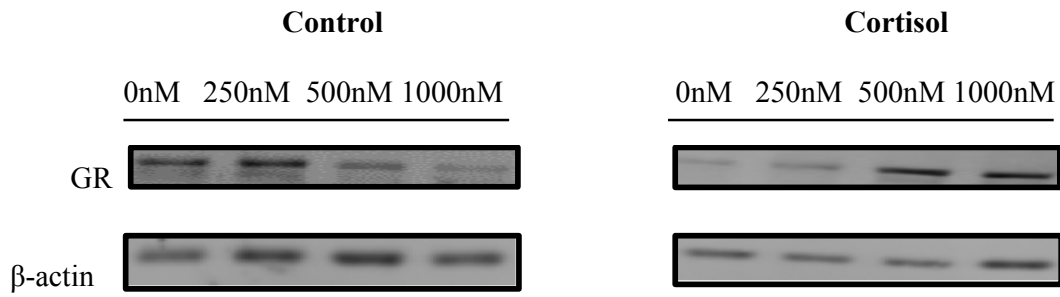
## 5.4.5 Enzyme activities

In the presence of Cd there was no significant change in hexokinase (HK) (Figure 5A), pyruvate kinase (Figure 5C), lactate dehydrogenase (LDH) (Figure 5A), or alanine aminotransferase (AlaAT) (Figure 5B) compared to the control group. In the Cd groups cortisol-induced enzyme elevation was abolished. A treatment effect was observed in GK activity in which activity was greater in liver slices treated with 500 and 1000 nM Cd compared to control (Figure 5B) decreased PEPCK activity occurred in 1000 nM-exposed liver slices compared to control. Furthermore, no significant differences in aspartate aminotransferase (AspAT) activity were observed in any Cd group except at 1000 nM where a ~33% decrease was observed (figure 6C). Cortisol treatment significantly increased the activities of HK, GK, PK, PEPCK LDH, AlaAT and AspAT enzyme activities in the control liver slices and these effects were abolished in the presence of mifepristone (Figures 5A-D, 6A, C, E). This cortisol-mediated enzyme activity changes were absent in the Cd treated groups (Figures 5A-D, 6B, D, F).

**Figure 5. Liver metabolic capacity.** Hexokinase (HK; A), Glucokinase (GK; B), Pyruvate Kinase (PK; C) and Phosphoenolpyruvate carboxykinase (PEPCK; D) enzyme activity in liver slices treated with 0, 250, 500 or 1000 nM cadmium chloride in control, cortisol (100 ng/ml), mifepristone (1000 ng/ml) or a combination of cortisol and mifepristone groups. Enzyme activity is expressed as micromoles of substrate consumed or product liberated per minute (U) per gram protein. Values represented as mean ± SEM (n=6 fish); bars with different letters within a Cd group is statistically different ($P<0.05$, one way repeated measures ANOVA; $P<0.05$); different letters above lines represent significant difference in enzyme activity between different Cd groups (two-way repeated measures ANOVA; $P<0.05$).
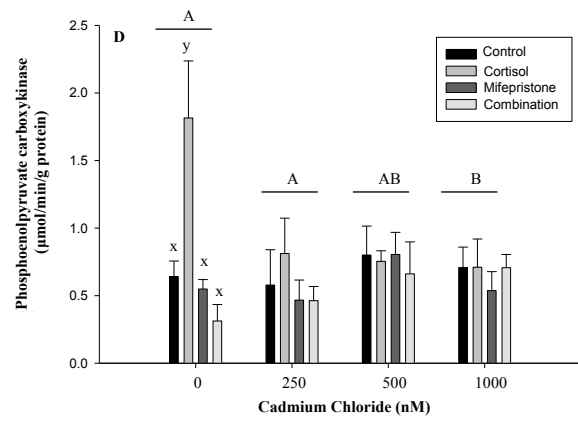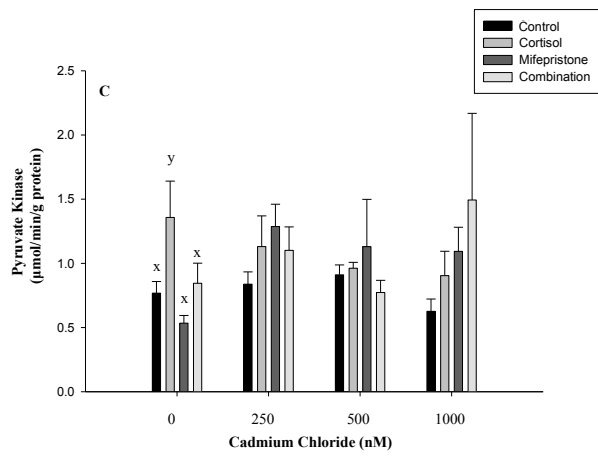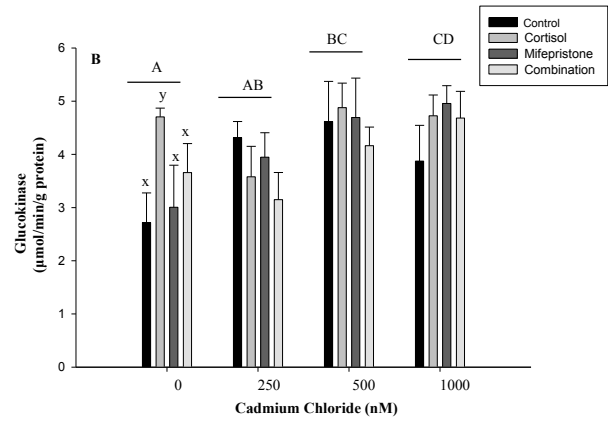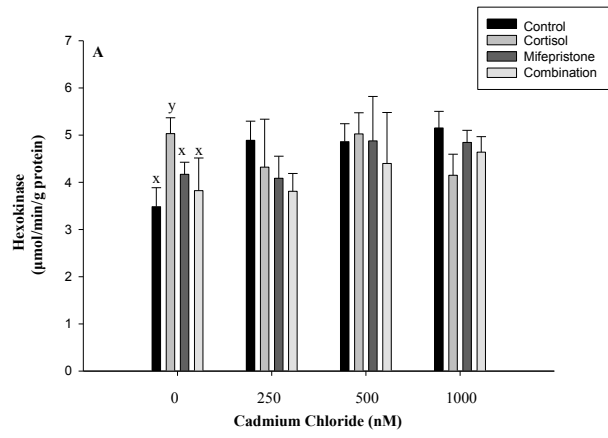
**Figure 6. Liver metabolic capacity.** Lactate dehydrogenase (LDH; A, B), alanine aminotransferase (AlaAT; C, D) and aspartate aminotransferase (AspAT; E, F) in control liver slices and liver slices treated with cortisol (100 ng/ml), mifepristone (1000 ng/ml) or a combination of cortisol and mifepristone (A, C, E) or in the presence of 0, 250, 500 or 1000 nM cadmium chloride (B, D, F). Enzyme activity is expressed as micromoles of substrate consumed or product liberated per minute (U) per gram protein. Values represent ± SEM (n=6 fish); bars with different letters are significant (repeated measures one-way ANOVA; $P<0.05$).
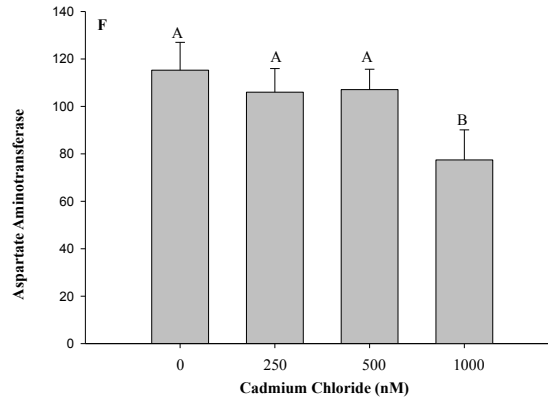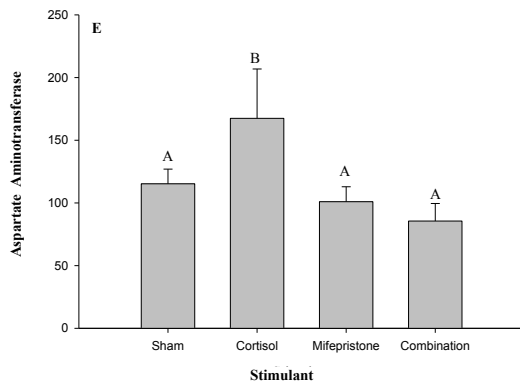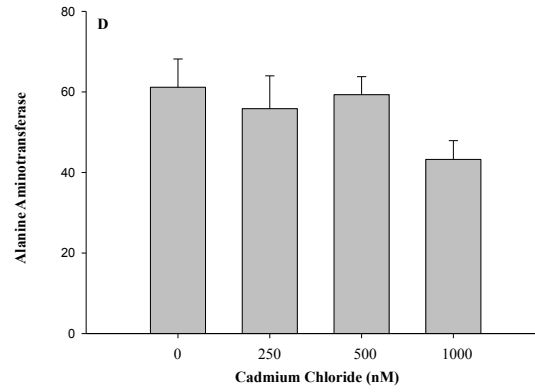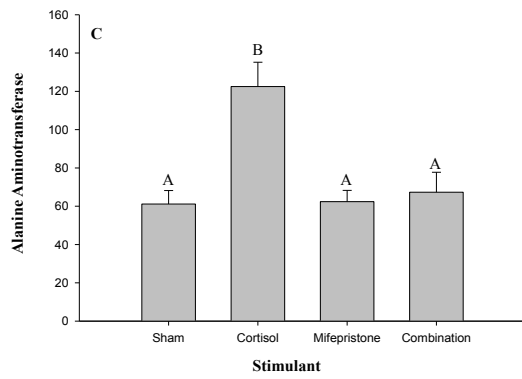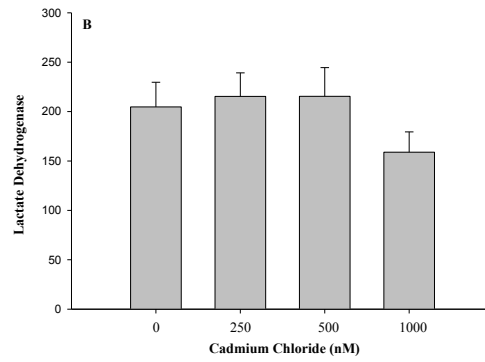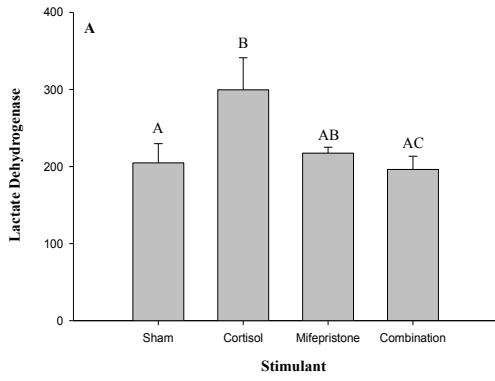
## 5.5 Discussion

From this study, we show that although exposure of liver slices to sublethal concentrations of Cd is not impacting liver function, there is a remarkable disruption of cortisol signaling. As expected, Cd accumulated within the liver slices in a dose-dependent manner. In field studies, Cd concentrations in the livers of fish range from 0.2 to 30 µg/g wet weight (Huguet et al., 1992; Rieberger, 1992; Jezierska and Witeska, 2001; Kraemer et al., 2006), suggesting that the accumulation of Cd seen in the liver slices in the present study were environmentally relevant.

The Hsp70 family is one of the most highly conserved heat shock proteins that is localized in the cytosol, mitochondria and endoplasmic reticulum, and is the prominent protein expressed following stressor exposure. The higher expression of hsp70 with Cd in the present study suggests an enhanced cellular stress response. The induction of Hsp70 is a common cellular stress response that is typically invoked in response to a variety of stressors to assist in the correct folding of nascent and stress-accumulated misfolded proteins, preventing protein aggregation or promoting degradation of selective misfolded or denatured proteins(Vijayan et al., 1997b, 1998, Ackerman and Iwama, 2001; Boone and Vijayan, 2002). As protein synthesis is energy demanding, the induction of hsp70 in Cd treated liver slices suggests an increase metabolic demand to the liver and may compromise other energy demanding pathways, especially in response to secondary stressor exposure (Boone and Vijayan, 2002). In both rats and fish, Hsp70 is associated with hepatic GR (Elez et al., 2001; Basu et al., 2003) and Cd can compete with and displace Zn from sulfhydryl groups, which are present in Hsp70 proteins (Wang and Rainbow 2006; Wang and Wang, 2008), suggesting that Cd could impact the GR/Hsp70 complex to disrupt GR signaling and the assocated metabolic capacity of the liver.

Elevation of glucose, the primary energy source during response to stress, aids in many physiological functions and adaptations. Furthermore, elevation of glucose due to circulating corticosteroids is a well-documented response in teleosts (Mommsen et al., 1999; Vijayan et al., 2010) that is in part due to an increase in the metabolic capacity of the liver through gluconeogenesis, and to a lesser extent by glycogenolysis (Vijayan et al., 1994, 2003, 2010; Mommsen et al., 1999). Elevation of glucose in control liver slices stimulated with cortisol implies that the target tissue effects mediated through cortisol were functional in these slices. Also, the abolishment of this response with mifepristone confirms that the glucose production by cortisol involves GR signaling (Aluru and Vijayan, 2007). Interestingly, Cd exposures completely eliminated the cortisol-induced glucose production in liver slices which points to an

inhibition in the well-observed GR-mediated glucose upregulation, highlighting for the first time that the disruption of glucose production in the liver may occur through disruption of GR signaling.

There is an increasing body of evidence suggesting that the cortisol stress axis, including target tissue cortisol action mediated by GR, are likely the target for many contaminants, including Cd (Simons et al., 1990; Dundjerski et al., 2000; Levesque et al., 2002; Vijayan et al., 2005, 2010; Ings et al., 2011a,b). The decrease in GR protein expression in Cd treated groups leads us to propose that disruption of GR signaling may be involved in the abolition of cortisol responsiveness in the liver. Further confirmation of impaired GR signaling was evident from the absence of an increase in PEPCK (key enzyme in gluconeogenesis) and SOCS1 (attenuates cytokine signaling) mRNA abundance, two genes that are glucocorticoid-responsive in trout (Sathiyaa and Vijayan, 2003, Vijayan et al., 2003, MackKenzie et al., 2006; Philip et al., 2012), in the presence of Cd further confirms that GR signaling is a target for Cd impact in trout.

Lack of glucose production in response to cortisol in Cd treated liver slices were further assessed by examination of key enzymes involved in the regulation of glucose production. Studies have shown an increase in activity or transcript abundance of key gluconeogenic enzymes that are regulated through cortisol, including phosphoenolpyruvate carboxykinase, which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate (Wiseman et al., 2007). Upregulation of PEPCK in liver slices was observed in both mRNA levels and enzyme activities in control liver slices stimulated with cortisol suggesting proper functioning of gluconeogenesis in the liver slices. Amino acid catabolism is another important method in providing substrate for hepatic gluconeogenesis, and has been assessed in response to contaminant and stress exposure by measuring the activity of aminotransferase enzymes, including alanine and aspartate aminotransferase, which convert their respective amino acids into pyruvate (Vijayan et al., 1994; Gravel and Vijayan, 2007; Tintos et al., 2007). Furthermore enzymes involved in glycolysis, such as the rate-limiting hexokinase (HK), pyruvate kinase (PK) as well as glucokinase (GK), have also been measured in response to acute stress, and have also shown to have an increased activity in both gene expression (Momoda et al., 2007; Wiseman et al., 2007) and enzyme activity (Gravel and Vijayan, 2007) to increase the energy demand associated with stressor adaptation. As expected glucokinase, hexokinase, alanine and aspartate aminotransferase, along with pyruvate kinase increased in liver slices stimulated with cortisol suggesting an overall increase in liver metabolic capacity with cortisol (Mommsen et al., 1999; Aluru and Vijayan, 2009). The metabolic changes associated with elevated cortisol points to an increased tissue glucose production (PEPCK activity) and an increase in glucose utilization (HK and GK activity) and

glycolytic capacity (PK activity). Interestingly, these cortisol-mediated effects were lacking in the Cd-treated group leading to the proposal that disruption of GR signaling by alteration in receptor expression may be a mechanism by which this metal impacts liver function. Increases in both hepatic and extra-hepatic glycolytic enzymes, such as hexokinase, pyruvate kinase and lactate dehydrogenase, have also been associated with acute stress and cortisol exposure, and are likely to deliver metabolic fuel required to manage the increased energy demand associated with stressor adaptation (Vijayan et al., 1994; Gravel and Vijayan, 2007). As cortisol is involved in all aspects of animal physiology, including extra-hepatic energy allocations, the disruption in GR signaling by Cd may have wide ranging effects on animal performances.

Taken together, we show that Cd accumulates within the liver in a dose-dependent manner and impairs the conserved glucose response to cortisol stimulation in trout liver. Glucose is a key fuel to meet the increase in energy demand required to cope with the stressor. The lack of cortisol-mediated gluconeogenic and glycolytic enzyme activities in the Cd treated fish suggests that this metal impairs cortisol responsiveness in the liver. Although the mechanism of action is unknown, we propose that the impact of Cd on liver function involves disruption of GR signaling in rainbow trout.

## 5.6 Acknowledgements

# Chapter 6

# Conclusions

## 6.1 Chapter conclusions

The overall goal of this thesis was to identify and characterize the impact of sublethal and environmentally relevant cadmium (Cd) concentrations on the stress performances in rainbow trout (*Oncorhynchus mykiss*), as well as to investigate the mechanism of action of Cd in disrupting cortisol production and target tissue cortisol action. To this end, a series of controlled laboratory studies were performed and the following conclusions from each chapter are stated.

1. Juvenile rainbow trout can adapt to subchronic exposure of environmentally relevant Cd concentrations; however, a metabolic disruption associated with an increased energy demand occurs within the liver and gills (Chapter 2). Cd impacts the stress performance through attenuation of the adaptive cortisol response to a secondary acute stressor (Chapter 2), leading to the hypothesis that Cd targets HPI axis functioning.

2. Cd targets key genes involved in corticosteroidogenesis to disrupt cortisol production, and mechanistically, this is likely occurring at the level of melanocortin 2 receptor (MC2R) activation (Chapter 3).

3. Cd exposure disrupts mineralocorticoid receptor protein expression in the brain, and possibly plays a role in disrupting the negative feedback cortisol regulation (Chapter 4). In the interrenal tissue, Cd-mediated disruption of MC2R is associated with disruption of melanocortin receptor accessory protein 1 (MRAP1) leading to the proposal that Cd directly targets MC2R/MRAP1 complex to disrupt corticosteroidogenesis (Chapter 4).

4. Cd impacts target tissue cortisol action by disrupting glucocorticoid receptor (GR) signaling and may compromise the metabolic actions of this hormone during stress in fish. (Chapter 5).

## 6.2 General conclusion

The generalized stress response is a well-conserved phenomenon amongst vertebrates. Upregulation of cortisol through the HPI or HPA axis is crucial to help the organism cope with the stressor, primarily through its role in intermediary metabolism. Taken together, these studies suggest that exposure of juvenile rainbow trout to sublethal concentrations of Cd leads to an overall metabolic disruption and impairment of the stress response (Figure 1). A major role for cortisol is to facitlitate energy reallocation by

altering tissue metabolic capacities to meet the increased energy demand associated with stress. Disruption of this highly conserved adaptive stress response may compromise the reestablishment of homeostasis.

From a biological standpoint, the changes observed in both the metabolic capacity and stress response in juvenile rainbow trout may have ramifications that go beyond what was measured in these studies since cortisol also plays an important role in other processes, including growth, reproduction osmoregulation and immune function. These physiological changes could result in alterations in fish behaviours and ultimately result in reduced population sizes and an inability to adapt and survive exposure to multiple stressors, ultimately affecting fitness. From an ecological standpoint, changes at the biological level could alter ecological systems including nutrient cycling, vegetation, productivity, trophic interactions, and biodiversity (Hilderbrand et al., 2004; McIntyre et al., 2007). In North America, salmonids are commercially valuables as they are greatly consumed by humans and also support recreational fishing activities (Guido, 2011). Therefore, disruption of the stress response due to waterborne Cd exposure could result in a decrease in commercially available salmonids, resulting in an economic loss.

Toxicity of heavy metals as well as other pollutants in the aquatic environment is rapidly becoming an important field. Waterborne Cd exposure can result in metabolic changes as well as the inability to escape a predator and also reduce reproductive capacity implying the importance to water managers in regulating Cd concentrations. Although a lot of preliminary work has been done to assess the impact of toxicants on endocrine disruption, for most, the mechanism of action is unknown. From our studies, we can conclude that environmentally relevant concentrations of Cd impairs the melanocortin 2 receptor activation and, thereby, inhibits corticosteroidogenesis (Figure 1). Our results suggest that Cd is downregulating MC2R activation by impact the melanocortin receptor accessory protein, specifically, MRAP1 (Figure 1), but the mechanism by which Cd target MRAP1 and/or MC2R activation remains to be determined.

**Figure 1**. Schematic diagram of Cd-mediated cortisol disruption of the stress response in rainbow trout from each research chapter. Green arrows represent a typical stress response as outlined in chapter 1 (introduction), whereas red arrows show sites of Cd-mediated disruption as outlined in data chapters (chapters 2-5). Within chapter 2, a Cd-mediated disruption was observed in liver glycogen, glycolysis and glucocorticoid receptors *in vivo* along with glycolytic disruption in the gill and alterations in steroidogenic genes in the head kidney (chapter 2). Further disruption was observed in cortisol, glucose and lactate in response to a secondary stressor. Within chapter 3, Cd exposure *in vitro* disrupted ACTH-mediated cortisol production, and further disruption was observed in various corticoisteroidogenic genes including MC2R, StAR and P450scc. Within chapter 4, Cd-mediated disruption was observed in brain mineralocorticoid receptors *in vivo* and a further disruption was observed in plasma cortisol and ACTH levels in response to a secondary stressor. Further analysis showed a disruption in MC2R and MRAP1 mRNA levels both *in vivo* and *ex vivo*. Finally in chapter 5, disruption of target tissue responses, specifically glycolysis and gluconeogenesis were observed in the liver suggesting that Cd is impacting cortisol signaling through downregulation of the glucocorticoid receptor.

 (Abbreviations: Cd – Cadmium, SC – superior collicus, HB – hindbrain, MB – midbrain, OT – optic tectum, PO – preoptic area, H – hypothalamus, P – pituitary, T – telencephalon, ON – optic nerve, OB – olfactory bulb, ACTH – adrenocorticotropin hormone, MC2R – melanocortin 2 receptor, MRAP1 – melanocortin receptor accessory protein 1, ATP – adenosine triphosphate, StAR – steroidogenic acute regulatory protein, P450scc – cytochrome P450 side chain cleavage enzyme, 3β – HSD – 3-beta-hydroxysteroid dehydrogenase).
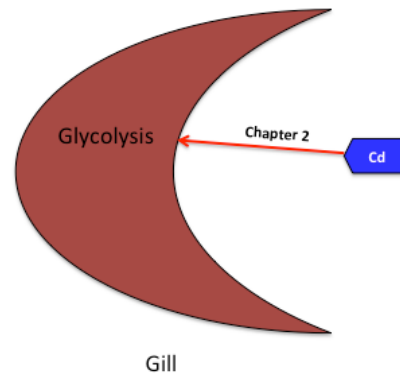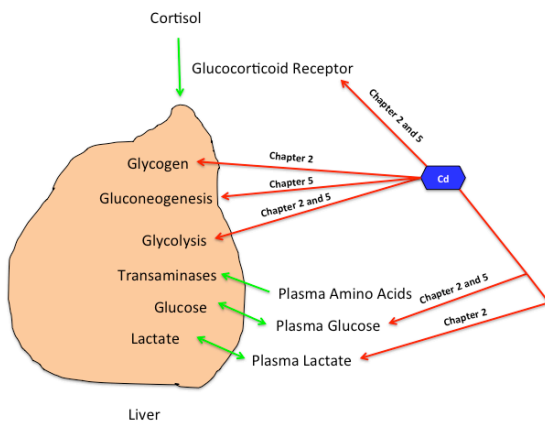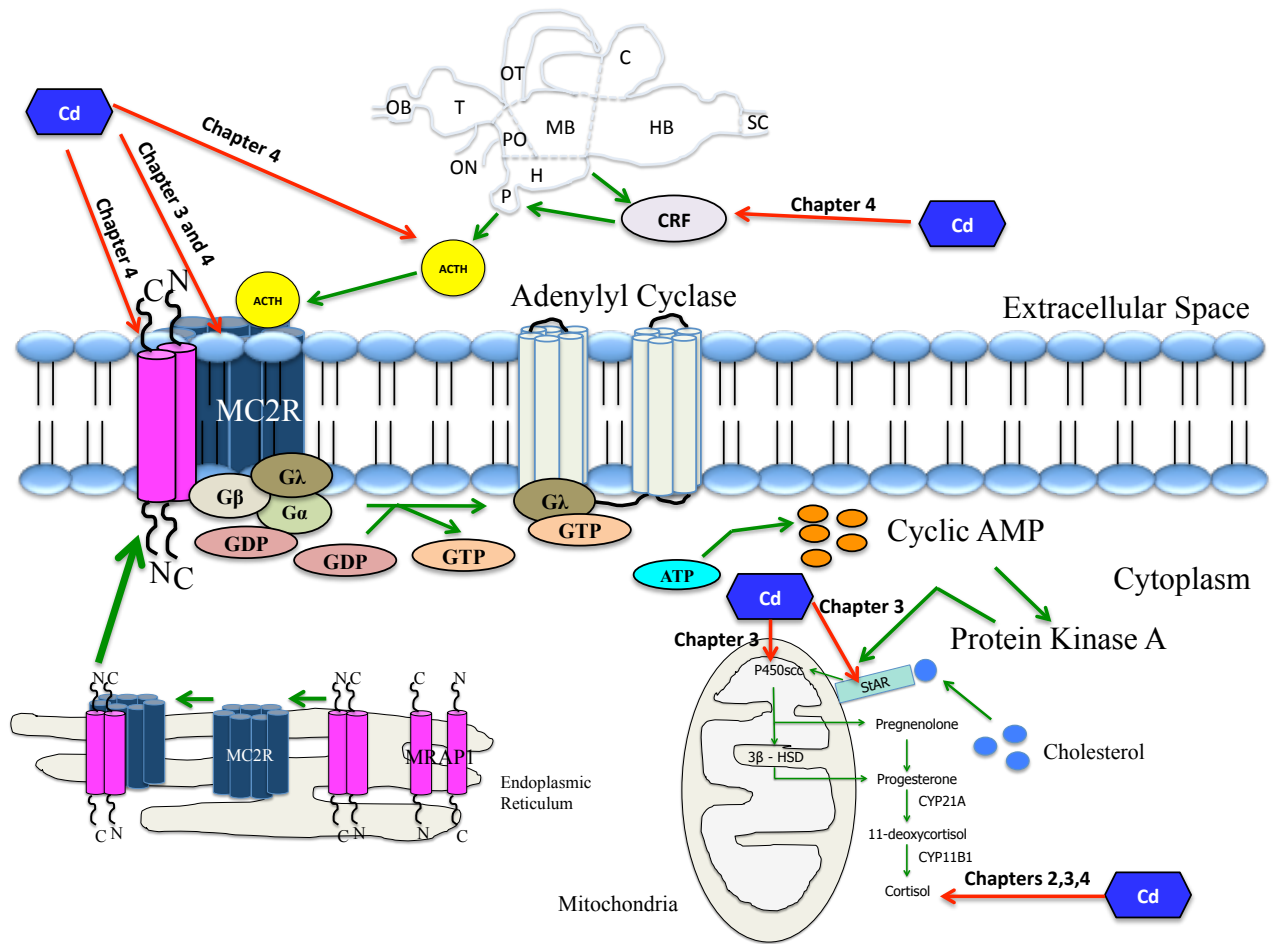
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