ENDOCRINE DISRUPTION BY CADMIUM: MECHANISMS AND EFFECTS ON THE REPRODUCTIVE AXIS AND STRESS RESPONSE IN RAINBOW TROUT, *Oncorhynchus mykiss*

by

Helena Maria Lizardo Daudt

BSc, Universidade Federal do Rio Grande do Sul, Brazil, 1991
MSc, Universidade Federal do Rio Grande do Sul, Brazil, 1994

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APPROVAL

Name: Helena M.L. Daudt

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Title of Thesis:
Endocrine disruption by cadmium: mechanisms and effects on the reproductive axis and the stress response in rainbow trout, *Oncorhynchus mykiss*

Examining Committee:

Chair: Dr. C. Lowenberger, Assistant Professor

Dr. C. Kennedy, Associate Professor, Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. T. Williams, Professor
Department of Biological Sciences, S.F.U.

Dr. M. Veiga, Associate Professor
Department of Mining Engineering, U.B.C.

Dr. R. Nicholson, Associate Professor
Department of Biological Sciences, S.F.U.
Public Examiner

Dr. G. Iwama, Dean of Science and Professor
Faculty of Pure and Applied Sciences, Acadia University
External Examiner

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Date Approved

ii
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ABSTRACT

The purpose of this research was to investigate the sub-chronic effects of environmentally realistic cadmium (Cd) concentrations on the reproductive and developmental axes and stress response of rainbow trout with the intention of furthering our understanding on the endocrine-disrupting mechanism(s) of action of this metal in teleosts.

This study showed for the first time that very low Cd concentrations (0.05, 0.25, 0.50 and 2.50 µg/L) can result in a multifaceted toxicity paradigm, with important effects on the reproductive axis, and to a lesser extent on the stress response of rainbow trout.

Cd exposure has impaired three life-stages of trout: eggs, larvae and juveniles with juvenile being the most sensitive stage examined. The lowest concentrations used caused premature hatching and the highest caused delayed hatching. Interestingly final hatching success was not affected. 2.50 µg/L Cd negatively affected larval growth: larvae were shorter in length and weighed less compared to controls. Plasma sex steroid concentrations were increased in juveniles at all Cd concentrations after 28 days. The specific toxic site(s) of action of Cd on steroidogenesis was determined using an in vitro testicular cell preparation. Our results indicate that there are both stimulatory and inhibitory sites near the pituitary hormone receptor location, on the first steps of the pathway.

Similarly, the hypothalamus-pituitary-interrenals axis and stress response were both affected by Cd. Decreased plasma cortisol concentrations were observed at all concentrations from 7 to 40 days of exposure but they were not accompanied by interrenal impairment or alterations in other stress response parameters. However, interrenal steroidogenesis was impaired in a dose-dependent manner in vitro. One major inhibitory site(s) of action of Cd on the cortisol synthesis pathway is located after the pituitary hormone receptor location and prior to the cholesterol cleavage.
Negative effects of Cd at concentrations as low as 0.05 \( \mu g/L \) have never been reported in the literature. Since the guidelines for Cd are under review by the British Columbia Ministry of Environment, our results may add important data in determining new guideline values.

**Keywords**
Cadmium, rainbow trout, reproduction, stress response, endocrine disruption
Para Roque, meu esposo, e meus filhos, Gabriel e Jade;
minha família querida, que compartilhou comigo
cada momento desta caminhada, emprestando sorrisos
e fazendo com que tudo valesse a pena.

To Roque, my husband, and my two children,
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TABLE OF CONTENTS

Approval........................................................................................................................................ii
Abstract..........................................................................................................................................iii
Dedication.........................................................................................................................................v
Acknowledgements .....................................................................................................................vi
Table of Contents ........................................................................................................................viii
List of Figures..................................................................................................................................xi
List of Tables.....................................................................................................................................xii
Chapter 1 General Introduction and Literature Review .............................................................1
  1.1 Abstract .....................................................................................................................................1
  1.2 General introduction ...............................................................................................................3
  1.3 Literature review .....................................................................................................................5
    1.3.1 Cd in the environment .......................................................................................................5
    1.3.2 Cd uptake and toxic effects in fish .....................................................................................7
    1.3.3 Endocrine disruption ..........................................................................................................11
    1.3.4 Cd as an endocrine disruptor .........................................................................................13
    1.3.5 Major goals of the thesis .................................................................................................16
  1.4 References ..............................................................................................................................18

Chapter 2 Effects of cadmium chloride on the reproductive and developmental axes of rainbow trout, *Oncorhynchus mykiss* ..................................................27
  2.1 Abstract .....................................................................................................................................27
  2.2 Introduction .............................................................................................................................28
  2.3 Materials and methods ...........................................................................................................31
    2.3.1 Chemicals ..........................................................................................................................31
    2.3.2 Test organism ....................................................................................................................31
    2.3.3 Eggs and larvae assessment ...............................................................................................32
      2.3.3.1 Exposure system .........................................................................................................32
      2.3.3.2 Sampling and endpoints ..............................................................................................33
    2.3.4 Juvenile fish .......................................................................................................................34
      2.3.4.1 Exposure system .........................................................................................................34
      2.3.4.2 Sampling and endpoints ..............................................................................................35
    2.3.5 Aqueous Cd analysis .........................................................................................................36
    2.3.6 Statistical analysis .............................................................................................................36
  2.4 Results .......................................................................................................................................37
    2.4.1 Eggs and larvae ..................................................................................................................37
    2.4.2 Juvenile fish .......................................................................................................................39
Chapter 5  Biosynthetic capacity of rainbow trout (Oncorhynchus mykiss) interrenal tissue following cadmium chloride exposure

5.1 Abstract ........................................................................................................112
5.2 Introduction ..................................................................................................113
5.3 Materials and methods ..............................................................................116
  5.3.1 Fish ..........................................................................................................116
  5.3.2 Chemicals ................................................................................................116
  5.3.3 Preparation of interrenal tissue ...............................................................117
  5.3.4 Determination of stimulating or augmenting agent concentrations .......117
  5.3.5 Cd exposure ............................................................................................118
  5.3.6 Cell viability ............................................................................................118
  5.3.7 Cortisol determination ...........................................................................119
  5.3.8 Statistical analysis ..................................................................................119
5.4 Results ..........................................................................................................120
  5.4.1 Cell Viability ...........................................................................................120
  5.4.2 ACTH-stimulated, SC- and 25-OHC-augmented cortisol secretion .......120
  5.4.3 Cd effects on unstimulated and stimulated cortisol release ..................120
5.5 Discussion .....................................................................................................121
5.6 Figures ..........................................................................................................128
5.7 Tables ...........................................................................................................132
5.8 References ....................................................................................................135

Chapter 6  Summary and General Conclusions ..................................................139
LIST OF FIGURES

Figure 2.1 Cumulative percent hatching of rainbow trout eggs exposed to 0 (control), 0.05, 0.25 and 2.50 μg/L Cd from 273 to 328 °D. .................................48

Figure 2.2 Cumulative percentage of the various types of malformations for rainbow trout larvae exposed to 0 (control), 0.05, 0.25 and 2.50 μg/L Cd for 40 days. Spinal (■), cranio-facial (■) and yolk-sac malformations (■)..........................................................49

Figure 2.3 Growth measurements, A) length and B) weight, for rainbow trout larvae exposed to 0, 0.05, 0.25 and 2.50 μg/L Cd for 35 (■) and 56 days (□). .................................................................50

Figure 2.4 Sex steroids concentrations as % of control values in rainbow trout A) females and B) males exposed to 0, 0.05, 0.25 and 0.50 pg/L Cd for 7 (■) and 28 (□) days. .................................................................51

Figure 3.1 Initial steps of the 11-KT biosynthetic pathway (until pregnenolone formation) .................................................................78

Figure 3.2 In vitro 11-KT secretion (% of control—no Cd) of trout testicular tissue exposed to 0, 1, 10 and 100 μM Cd for 2 (■) and 18h (■). ......................79

Figure 3.3 In vitro 11-KT secretion (% of control—no stimulant) of trout testicular tissue stimulated with 0, 1, 10 or 100 IU/mL hCG (A); 0, 0.05, 0.5 or 5 mM dbcAMP (B) and 0, 0.05, 0.5 or 1.25 mM 25-OHC (C)...........80

Figure 3.4 In vitro 11-KT secretion (% of control—no Cd + stimulant) of trout testicular tissue exposed to 0, 1, 10 and 100 μM Cd for 18h and stimulated with A) 100 IU/mL hCG; B) 5 mM dbcAMP or C) 1.25 mM 25-OHC. .................................................................81

Figure 4.1 Plasma cortisol concentrations of trout exposed to Cd: A) 0.05 μg/L; B) 0.25 μg/L and C) 0.50 μg/L, for 7, 14, 21, 28 and 40 days..................103

Figure 4.2 Plasma cortisol concentrations for controls and trout exposed to Cd for 40 days and A) injected with ACTH or B) subjected to stress. ..............104

Figure 5.1 Initial steps of the cortisol biosynthetic pathway (until pregnenolone formation) ...........................................................................128

Figure 5.2 In vitro cortisol secretion in trout interrenal tissues stimulated with A) 0, 0.01, 0.1 and 1 IU/mL ACTH; B) 0, 0.05, 0.5 and 5 μM 25-OHC and C) 0, 0.016, 0.8 and 40 μL/mL SyntheChol®. ........................................129

Figure 5.3 In vitro cortisol secretion in trout interrenal tissues exposed to 0, 1, 10 and 100 μM Cd for 1 h. .................................................................130

Figure 5.4 In vitro cortisol secretion in trout interrenal tissues exposed to 0, 1, 10 and 100 μM Cd for 1 h and subsequently stimulated with A) 0.01 IU/mL ACTH, or augmented with B) 5 μM 25-OHC or C) 0.8 μL/mL SyntheChol®. ..................................................131
LIST OF TABLES

Table 2.1 Percent mortality in control and Cd exposed rainbow trout eggs and larvae (0, 0.05, 0.25 and 2.50 µg/L Cd) ..............................................................52
Table 2.2 Weight (mg) and length (cm) of rainbow trout larvae exposed to 0, 0.05, 0.25 and 2.50 µg/L Cd for 35 and 56 days ........................................53
Table 2.3 Mortality (%), weight (g) and GSI of juvenile rainbow trout exposed to 0, 0.05, 0.25, 0.50 and 2.50 µg/L Cd ..............................................54
Table 2.4 Plasma estradiol concentrations (pg/mL) of juvenile rainbow trout exposed to 0, 0.05, 0.25 and 0.50 µg/L Cd for 7, 14, 21 and 28 days ....55
Table 2.5 Plasma 11-KT concentrations (pg/mL) of juvenile rainbow trout exposed to 0, 0.05, 0.25 and 0.50 µg/L Cd for 7, 14, 21 and 28 days. n=3 to 5. ....56
Table 3.1 Viability (%) of trout testicular cells exposed to medium (controls) or exposed to hCG, 25-OHC or dbcAMP for 18 hours alone or in presence of Cd (1, 10 or 100 µM) ........................................................................82
Table 3.2 In vitro 11-KT secretion (ng/g wet tissue) of trout testicular tissue exposed to medium or three different concentrations of hCG, 25-OHC or dbcAMP for 2h. ................................................................................83
Table 3.3 In vitro 11-KT secretion (ng/g wet tissue) of trout testicular tissue exposed to medium (controls –no Cd) or 1, 10, 100 µM Cd for 2h or 18h ........................................................................................................84
Table 3.4 In vitro 11-KT secretion (ng/g wet tissue) of trout testicular tissue exposed to 0, 1, 10 or 100 µM Cd and stimulated/augmented with 100 IU/mL hCG, 5mM dbcAMP, or 1.25 mM 25-OHC for 18h. .......................85
Table 4.1 Hematocrit, plasma glucose and lactate, and liver glycogen in controls and trout exposed to Cd (0.05, 0.25 and 0.50 µg/L) for up to 40 days ...105
Table 4.2 Plasma cortisol concentrations (ng/mL) for controls and Cd exposed trout (0.05, 0.25 and 0.50 µg/L Cd) for 7, 14, 21, 28 and 40 days. ......................106
Table 4.3 Plasma cortisol concentrations (ng/mL) in control and exposed trout (0.05, 0.25 and 0.50 µg/L Cd) for 40 days following handling stress or ACTH injection ..................................................................................107
Table 5.1 Viability (%) of trout interrenal cells exposed to medium (controls) or exposed to ACTH, 25-OHC or SyntheChol® for 2h alone or in presence of Cd (1, 10 or 100 µM) .................................................................132
Table 5.2 In vitro cortisol secretion (ng/g wet tissue) of trout interrenal tissue exposed to medium (controls – no agent) or three different concentrations of ACTH, 25-OHC or SyntheChol® for 2h ......................133
Table 5.3 In vitro cortisol secretion (ng/g wet tissue) of trout interrenal tissue exposed to 0, 1, 10 or 100 µM Cd and stimulated/augmented with 0.01 IU/mL ACTH, 5 µM 25-OHC or 0.8 µL/mL SyntheChol® for 2h. 134
CHAPTER 1
GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Abstract

Endocrine-disrupting compounds (EDCs) are environmental contaminants that interfere in some way with endocrine function and can induce a number of biological responses and multigenerational effects at the individual and populational level. These hormonally-active compounds encompass a variety of chemical classes. Among them, metals and metal compounds have been shown to negatively impact numerous metabolic and physiological processes through effects on the endocrine system.

Cadmium (Cd), a very toxic metal, can act as an endocrine disruptor, interfering with biological functions such as reproduction, growth and development, osmoregulation and the ability to cope with stress in fish. Sensitivity to Cd varies between species of teleosts with salmonids being the most sensitive group as a whole. 120-h LC$_{50}$ values for salmonids vary between 0.35 and 1.1 µg/L, while values for non-salmonids can be 10,000-fold higher.

Sublethal Cd exposure can affect the reproductive axis and the stress response in fish however, results are inconsistent. Gonadal steroidogenesis has been shown to be a prime target of EDCs and it is of particular importance in the paradigm of Cd toxicity since contradictory effects have been reported. Cd and other metals have been shown to both stimulate and inhibit sex steroid production in vivo and in vitro depending on the
dose of metal, species and sex of organisms. The specific mechanism(s) of action of Cd on gonadal steroidogenesis are virtually unknown in fish.

The literature reports that Cd can also affect the organismal stress response in fish, but effects have been inconsistent. Generally, in the short term, Cd exposure increases plasma cortisol (stress hormone) concentrations. After longer exposures (> 1 month), plasma cortisol concentrations have been shown to return to baseline levels or to be persistently higher than baseline values. Field studies, however, have shown reduced plasma cortisol concentrations and/or a failure to respond to additional stress in fish from various different metal-contaminated (including Cd) sites.

Although the literature available indicates that sublethal concentrations of Cd can cause adverse effects on fish physiology through disruption of the endocrine system, laboratory studies have been inconsistent with regard to the modulation of plasma steroid concentrations (cortisol and sex steroids). In addition, neither the repercussions of altered steroidogenesis on the reproductive and developmental axes and stress response nor the mechanisms of action of Cd on the steroidogenic organs are well understood in fish.

Therefore, the larger goals of this thesis were twofold: to investigate the effects of Cd on 1) the reproductive and developmental axes and 2) the organismal stress response in rainbow trout, with the intention of furthering our understanding of the mechanism(s) of action of this endocrine disrupting metal in fish.
1.2 General introduction

Defining the extent to which environmental chemicals affect the functioning of the endocrine system and thereby contribute to adverse effects in wildlife and humans is a complex issue (Lister and Van der Kraak 2001). The endocrine system, primarily involved with chemical communication, serves to regulate and coordinate many physiological processes including reproduction, growth, maintenance of the internal environment, and energy availability (Van der Kraak et al. 1998). Hormones, messengers that interact with receptors in cells to trigger responses, facilitate communication by the endocrine system. Multiple hormones control complicated events through a number of different biochemical mechanisms. Moreover, there are multiple and interacting factors that govern the release of hormones within the endocrine tissues (Lister and Van der Kraak 2001).

Given the complexity of the endocrine system, it can be adversely affected through different processes by a wide range of substances [endocrine disrupting compounds (EDCs)]. A compound can bind to a receptor and act as an agonist, causing a response similar to the original hormone, or act as an antagonist, by preventing the normal response of the hormone. In addition, it can interfere with the synthesis, transport or availability, and metabolism of hormones or receptors (Van der Kraak et al. 1998, Lister and Van der Kraak 2001, Servos et al. 2001a). Moreover, intrinsic variables including, nutritional status, diet, age or gender, and environmental factors such as duration, timing and route of exposure may affect the adverse response to a specific EDC (Tillitt et al. 1998, Jalabert et al. 2000).
There are numerous chemicals in the environment that are hormonally active and these encompass a variety of chemical classes, including natural and synthetic hormones, plant constituents, pesticides, metals, industrial by-products and effluents (Stahlschmidt-Allner et al. 1997, Gray Jr et al. 1998, Hewitt and Servos 2001, Servos et al. 2001a). Among them, metals and metal compounds have been shown to negatively impact many metabolic and physiological functions through effects on the endocrine system (Hontela 1998, Depledge and Billinghurst 1999, Hewitt and Servos 2001).

Cadmium (Cd), a relatively rare heavy metal, can act as an endocrine disruptor interfering with different biological functions such as reproduction, growth and development, osmoregulation and the ability to cope with stress (Fu and Lock 1990, Pratap and Wendelaar Bonga 1993, Hontela 1998, Thompson et al. 2000, Jones et al. 2001). However, the reported effects of Cd on the reproductive axis and the stress response are inconsistent. Increases and decreases in hormone production have been described in fish with controversial repercussions on functional activities (Kime 1984, Fu and Lock 1990, Gill et al. 1993, Mukherje et al. 1994, Brodeur et al. 1998). In addition, the mechanism(s) of action of Cd in fish remain poorly understood (Arcand-Hoy and Benson 1998, Hontela 1998, Kime 1999).

Therefore, the purpose of this thesis was to investigate the effects of Cd on the reproductive axis and stress response with the intention of furthering our understanding on the mechanism(s) of action of this endocrine disrupting metal in fish.
1.3 Literature review

1.3.1 Cd in the environment

Cadmium (Cd) is a heavy metal closely related to zinc and is found wherever zinc occurs in nature (Friberg et al. 1974). It is accumulated as a by-product in the refining of zinc and other metals (May et al. 2001, Plachy 2003). Cd is released to the environment by several industries, especially those manufacturing plastics, photographic materials, paints, alkaline batteries and alloys (Canadian Environmental Protection Act 1994, US-EPA 2001, Plachy 2003). The burning of oil, waste and cigarette smoke also contributes to Cd’s input into the environment (Piasek and Laskey 1999). In addition, it is released by natural processes such as weathering and erosion of Cd bearing soil and rocks, forest fires and volcano emissions (Canadian Environmental Protection Act 1994, Environment Canada 2005).

Approximately 1,963 tonnes (t) of refined Cd are produced, 23 t are imported and 1580 t are exported each year in Canada (Environment Canada 2005). In the US, 600 t of refined Cd were produced, 10 t were imported and 400 t were exported in 2004 (Plachy 2005). Although quantitative releases were not identified for all sources, the available data indicate that an estimated 159 t of Cd are released annually into the Canadian environment, 82% (130 t) from base metal smelting and refining operations alone (Environment Canada 2005).

Cd-laden wastewater from base metal smelting and refining operations can cause many deleterious effects on both humans and wildlife. The classical *itai-itai* disease (literally ouch-ouch disease) was the first human poisoning outbreak reported in the literature due to Cd contamination of the Jinzu River (Japan, 1955). Cd released into the
river by a mining company caused a unique syndrome: softening of bones and pathological fractures (hence the name of the disease), kidney failure, pancreatic dysfunction and severe osteomalacia (Singhal and Merali 1979, Korte 1983). The contaminated river was used mainly for irrigation of the rice fields, but also drinking, washing, fishing and other uses by the downstream population. In 1968, the Japanese Ministry of Health and Welfare made an official announcement linking environmental factors, including Cd contamination of water, fish and rice and Itai-itai disease (Korte 1983).

In aquatic systems, Cd can be undetectable in the water phase while large concentrations can be found associated with bottom sediments and suspended particles (Canadian Environmental Protection Act 1994, May et al. 2001, Lydersen et al. 2002). Cd concentrations in surface waters are generally very low, ranging from < 0.1 to 8.9 μg/L with an average of 0.2 μg/L in British Columbia (Canadian Environmental Protection Act 1994). Even at low concentrations, Cd in the environment is of great concern since its bioavailability can vary widely depending on water chemistry.

Much of the literature in environmental biogeochemistry indicates that the speciation of metals in aquatic systems is of central importance to bioavailability. For example, acute tests in fish indicated the following order of increasing toxicity of Cd salts to *Pimephales promelas*: carbonate < sulphide < chloride = sulphate. The authors suggested that the observed order was a function of solubility and the presence of free Cd²⁺ (Erten-Unal et al. 1998). It is likely that many of the Cd toxic effects are related to the ionic size of Cd²⁺ (0.97 Å), which is very similar to the size of Ca²⁺ (0.99 Å) and
therefore facilitates competition with Ca\(^{2+}\) for binding sites in important molecules and may cause a great variety of deleterious effects.


1.3.2 Cd uptake and toxic effects in fish

The respiratory and gastrointestinal tracts are the two main routes of Cd’s absorption in vertebrates. In fish, the two main sites for Cd uptake are the gills and the intestine (Canadian Environmental Protect Act 1994, Kraal et al. 1995, May et al. 2001, Dang et al. 2001). The relative importance of each route seems dependent on the Cd source: either waterborne (gill route) or dietary sources (gastrointestinal route) (Szebedinsky et al. 2001, Xu and Wang 2002, Chowdhury et al. 2005, Long and Wang
Field studies indicate that the gastrointestinal route is the most important route of uptake (Besser et al. 2001, Bervoetes et al. 2001, Giguere et al. 2004). Thomann et al. (1997) highlights the importance of both water and food sources to determine Cd exposure risk, indicating that both should be considered for accurate risk determination.

Sensitivity to Cd varies between fish species with salmonids being the most sensitive group as a whole (Canadian Environmental Protection Act 1994). 120-h LC\textsubscript{50} values for Cd in salmonids (Oncorhynchus mykiss, Salvelinus confluentus and Oncorhynchus tshawytscha) have been reported to range from 0.35 to 1.1 $\mu$g/L in soft water (20 to 30 mg/L CaCO\textsubscript{3}) (Finlayson and Verrue 1982, Hansen et al. 2002). LC\textsubscript{50} values for non-salmonids are usually much higher than those reported for salmonids. For example, Yilmaz et al. (2004) reported a 96-h LC\textsubscript{50} of 30.4 mg/L in guppy (Poecilia reticulate). Labeo rohita, another non-salmonid fish, showed an even higher 96-h LC\textsubscript{50}: 89.5 mg/L in moderately hard water (165 mg/L CaCO\textsubscript{3}) (Dutta and Kaviraj 2001). Although hardness was elevated in this study compared with most of the previously cited studies, which may have protected fish against Cd toxicity in Labeo rohita, the reported LC\textsubscript{50} value increase was more than 10,000-fold, strongly suggesting a species-specific tolerance to Cd.

Liver and kidney are the main target organs of Cd in fish (Haux and Larsson 1984, de la Torre et al. 2000, Giguere et al. 2004) and much of the literature has focused on the adverse effects of this heavy metal on these two organs. Typically, Cd induces degenerative changes in both liver and kidney. Ultrastructural alterations include karyolysis, mitochondrial condensation, swelling and lysis, dilatation of rough endoplasmic reticulum and proliferation of vacuoles and lysosomes (Forlin et al. 1986,
Thophon et al. 2003, Rangsayatorn et al. 2004). This structural degeneration is supported by previous studies on enzymes activities in liver and kidney. Increased lysosomal activity is often associated with enhanced acid phosphatase activity in tissues undergoing cellular degeneration and necrosis due to exposure to toxic substances (Versteeg and Giesy 1986), a phenomenon observed in the kidney of Heteropneustes fossilis exposed to Cd (Sastry and Subhadra 1985). Moreover, necrosis in the liver and kidney usually results in the liberation of glutamate oxaloacetate (GOT) and glutamate pyruvate (GPT) transaminases into the blood and reduced enzyme activity in these organs (Santos et al. 1990), a result observed in fish exposed to Cd (Gill et al. 1991).

Cd may also interfere with energy mobilization in fish. Generally, Cd exposure increases plasma glucose and lactate concentrations, as part of a generalized stress response in the short term (up to 4 days of Cd exposure) (Haux and Larsson 1984, Tort and Torres 1988, Hontela et al. 1996). However, after longer Cd exposure regimens (30 days), plasma glucose concentrations decrease to baseline levels and liver glycogen depletion has also been reported (Hontela et al. 1996, Ricard et al. 1998). It has been suggested that glucose is used as an energetic substrate to repair the damage caused by Cd, and increased glycogenolysis, evident by liver glycogen depletion, may be involved on the maintenance of normal plasma glucose concentrations (Ricard et al. 1998). Similar effects have been described in early studies with mammals exposed to Cd (Singhal and Merali 1979).

Sublethal concentrations of Cd have also been shown to exert a wide range of behavioural changes in various fish species, from imbalanced swimming to decreases in the capture efficiency of prey (Bryan et al. 1995, Scherer et al. 1997, Yilmaz et al. 2004,
Riddell et al. 2005). Interesting behavioural effects of Cd have also been reported recently by Sloman et al. (2003a, 2003b, 2005) with respect to dominance hierarchies in rainbow trout. Exposed fish showed a decreased ability to compete with non-exposed fish, however, dominance hierarchies were formed more quickly in exposed groups compared to controls (Sloman et al 2003b, 2005). In contrast, the competition between pairs of exposed fish was less aggressive than between controls during dominance contests (Sloman et al. 2003b).

Cd may adversely affect reproductive processes and ultimately may contribute to declines in fish populations. Laboratory studies have shown morphological alterations, mainly degenerative lesions, in gonads of fish exposed to Cd (Sangalang and Freemann 1974, Victor et al. 1986, Singh 1989, Sindhe et al. 2002). Impaired gamete maturation and increased presence of atretic structures have also been observed (Saksena and Agarwal 1986, Baile and Kadu 1992, Sindhe et al. 2002). Field studies support these laboratory findings since similar effects have also been reported in fish from metal (including Cd) contaminated sites (Mousa and Mousa 1999, Levesque et al. 2003). Morphologically altered gonads may adversely modulate steroidogenesis producing abnormal sex steroid concentrations, effects that have also been demonstrated in vivo and in vitro for fish/cells exposed to Cd (Sangalang and Freemann 1974, Thomas 1989, Tilton et al. 2003). All these facts may be part of a complex paradigm leading to declines in fish populations. Farag et al. (2003) reported a decline in the biomass and density of fish in streams contaminated by heavy metals (including Cd), which could be in part, due to the effects of metals on fish reproduction.
Cd has also been shown to affect egg development and the growth of early-life stages. Generally, altered hatching and suppressed or retarded growth has been observed in fish exposed to low concentrations of Cd (< 1mg/L) (Rombough and Garside 1982, Brown et al. 1994, Jones et al. 2001). Increases in mortality and malformations have been reported after exposure to higher concentrations of Cd (> 1 mg/L) (Witeska 1995, Cheng et al. 2000, Williams and Holdway 2000, Chan and Cheng 2003).

1.3.3 Endocrine disruption

The publication of Our Stolen Future, by Colborn et al. (1996), heightened public attention to the potential adverse effects of very low concentrations of environmental pollutants on the reproduction and development of humans and wildlife; contaminants that can alter the normal function of endocrine systems (Servos et al. 2001a). These concerns prompted a number of governmental actions (US-EPA 1996, Kavlock 1999, Huet 2000, Environment Canada 2005) to assess and manage substances that “interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body, responsible for the maintenance of homeostasis and the regulations of development processes” (Kavlock 1999). The Organization of Economic and Cooperative Development and the International Program on Chemical Safety has defined an endocrine disrupting chemical as an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny or (sub) populations (Lister and Van der Kraak 2001).

While survival, growth, and reproduction of fish populations have been affected by a number of contaminants in a number of locations, it is difficult to determine the role
of endocrine-modulating compounds in these effects (Giesy and Snyder 1998). The classical case of Lake Apopka (Florida) alligators illustrates the potential population effects of EDCs, although even in this well-studied scenario, no single toxicant has been identified as causal for the decline in alligator populations. In this particular situation, correlations indicate that reproductive failure in Lake Apopka alligators was mediated by EDCs present in the environment as a result of an accidental spill from a chemical company (Matter et al. 1998). On the other hand, exposure to contaminants that affect the functioning of the endocrine system does not always result in an adverse outcome since the response depends on the level, duration, and timing of exposure (Lister and Van der Kraak 2001). Thus, in order to establish a causal link between exposure to a putative endocrine disruptive agent and changes in wildlife populations or communities, the presence of a chemical in association with an effect is not itself sufficient. Integrated research that incorporates measurement and evaluation of appropriate biomarker responses, chemical residues and population dynamics are necessary to understand the real significance of endocrine disruption in a given environment (Taylor and Harisson 1999).

Most of the literature regarding endocrine disruptors has focused on estrogen antagonists and agonists (Kendal et al. 1998, Taylor and Harisson 1999, Evanson and Van der Kraak 2001, Servos et al. 2001b), although the modulation of cortisol (Brodeur et al. 1997, Hontela 1998) and thyroid hormones (Zile 1992, Hontela 1996, Brown et al. 2002, McCormick et al. 2005) has also been examined, particularly in aquatic environments. In the field, chlorinated hydrocarbons, especially polychlorinated aromatic hydrocarbons (PAHs and pesticides), heavy metals, nonylphenol and
nonylphenol polyethoxylates (both present in final effluents of municipal treatment systems), and β-sitosterol (known to be present in paper mill wastewater), have been shown to be capable of causing endocrine disruption (Stahlschmidt-Allner et al. 1997, Hontela 1998, Taylor and Harrison 1999, Giesy and Snyder 1998, Servos et al. 2001b).

Although the recent literature has focused on potential endocrine-mediated effects of environmental contaminants in a wide range of organisms, many unresolved questions still persist. Servos et al. (2001b), analyzing the uncertainties associated with assessing the risk of an endocrine active substance in the Canadian environment, highlight large gaps in knowledge and difficulties in making scientific assessments based on endocrine mechanisms for these substances. The authors postulate that endocrine disruption is a mode of action and not a functional endpoint that needs to be considered carefully in problem formulation and the interpretation of the weight of evidence during a risk assessment.

1.3.4 Cd as an endocrine disruptor

Recent work has shown that Cd, along with all the other sublethal effects previously discussed, can also act as an endocrine disruptor in both mammals and teleosts. Most of the literature has focused on the hypothalamus-pituitary-gonadal (HPG) and the hypothalamus-pituitary-interrenal (HPI) axes (Fu and Lock 1990, Gill et al. 1993; Lebond and Hontela 1999, Thompson et al. 2000) and concerns regarding the successful development and reproduction of humans and wildlife (Hontela 1998, Henson and Chedrese 2004).
Both the HPG and HPI axes are controlled by the hypothalamus as a result of neural stimulation from the central nervous system. The messengers synthesized by the hypothalamus acts on the pituitary, controlling the synthesis and release of pituitary hormones including adrenocorticotropic hormone (ACTH) and gonadotropins (GtH) (Norris 1996, Arcand-Hoy and Benson 1998). ACTH is the main stimulant of cortisol secretion by interrenal cells in fish (Iwama 1998, Mommsen et al. 1999). GtH are the hormones principally involved in gametogenesis and steroidogenesis, stimulating the secretion of sex steroids by the gonads. The synthesis and release of these hormones are controlled by feedback systems (Arcand-Hoy and Benson 1998, Hontela, 1998).

Studies have shown that Cd can be linked to endocrine disruption on the reproductive axis in aquatic organisms (Sangalang and Freemann 1974, Mukherjee et al. 1994, Le Guevel et al. 2000). Gonadal steroidogenesis has been shown to be a prime target of EDCs (Arcand-Hoy and Benson 1998, Kime 1999, Jalabert et al 2000), and it is of particular importance in the paradigm of Cd toxicity. Cd and other metals have been shown to both stimulate and inhibit sex steroid production in vivo and in vitro depending on the concentrations of metal, species and sex of organisms (Thomas and Khan 1997, Sangalang and O’Halloran 1973, Sangalang and Freemann 1974, Kime 1984). Although the inhibition of gonadal steroidogenesis could be explained by morphological alterations observed in the gonads of exposed fish in laboratory and field studies (Sangalang and O’Halloran 1973, Mousa and Mousa 1999, Sepulveda et al. 2002, Levesque et al. 2003), the stimulation of steroidogenesis has been difficult to rationalize. It has been suggested that a sustained production of sex steroid hormones by the interrenals, combined with impairments of hormone clearance might account for elevated concentrations in the
plasma of exposed fish (Sangalang and Freeman 1974). In mammals, Laskey and Phelps (1991) postulate that Cd may act at multiple sites (inhibitory and stimulatory) in the intracellular signaling pathway of testosterone synthesis, thus the final steroid concentration being the sum of both inhibitory and stimulatory actions. Similarities between fish and mammals remain to be investigated at this point.

The literature reports that Cd can also affect the organismal stress response in fish, but effects have been inconsistent. Generally, in the short term, Cd exposure increases plasma cortisol (stress hormone) concentrations. Subsequent return to plasma cortisol baseline levels has been reported after longer exposures (> 1 month) (Pratap and Wendelaar Bonga 1990, Fu et al. 1990). However, a persistent, high concentration of cortisol or a secondary increase in plasma cortisol with time has also been observed (Gill et al. 1993, Brodeur et al. 1998). Field studies have shown reduced plasma cortisol concentrations and/or failure to respond to additional stress in fish from different metal (including Cd) contaminated sites (Hontela et al. 1995, Brodeur et al. 1997, Girard et al. 1998, Norris et al. 1999, Kakuta 2002). It has been suggested that a sustained stimulation of cortisol secretion and the high metabolic activity of the interrenal tissue may lead to a functional interrenal exhaustion (Brodeur et al. 1998), which could account for the low levels of cortisol and interrenal impairment observed in the field.

In summary, the available literature indicates that sublethal concentrations of Cd can cause adverse effects on fish physiology through disruption of the endocrine system, depending mainly on water quality, Cd concentration and species. Laboratory studies demonstrate that Cd modulates plasma steroid concentrations (cortisol and sex steroids), increasing or decreasing concentrations possibly in a dose- and time-dependent fashion.
In addition, neither the repercussions of the altered steroidogenesis on the reproductive axis and stress response nor the mechanisms of action of Cd on the steroidogenic organs are well understood in fish.

1.3.5 Major goals of the thesis

The major goals of this thesis were twofold: to investigate the effects of low concentrations of Cd on 1) the reproductive and developmental axes and 2) the organismal stress response in rainbow trout, Oncorhynchus mykiss, with the intention of furthering our understanding of the mechanism(s) of action of this endocrine disrupting metal in fish. These objectives were met using both in vivo and in vitro experiments. The main hypothesis of this study was that environmentally-realistic concentrations of Cd affect the reproductive and developmental axes and the stress response in rainbow trout by interfering with the HPG and HPI axis.

The specific objectives of these studies were to determine the effects of Cd on:

1) The reproductive and developmental axes. Although in vivo and in vitro studies have shown that Cd affects the production of steroids, the mechanisms of action of Cd on disrupted gonadal steroidogenesis have not been examined in teleosts. As well, the repercussions of steroid modulation on the reproductive axis and development are unclear. The objectives here were to investigate the effects of low concentrations of Cd on the reproductive and developmental axes through an examination of the three different early-life stages of fish: eggs (hatching), larvae (growth and incidence of malformations) and juvenile fish (growth and sex steroid modulation). The specific site(s) of action of
this metal in the signaling pathway of sex steroid production in fish were also investigated \textit{in vitro} using testicular cells.

2) The stress response. The effects of environmentally relevant concentrations of Cd on the stress response in fish are poorly understood. Cd exposure may modulate plasma cortisol concentrations by interfering with interrenal function and the ability to cope with additional stressors. The specific mechanisms involved in these processes are largely unknown in fish. The functioning of the HPI axis and the stress response was investigated here following Cd exposure. The effects of Cd on the organismal stress response were examined through measurements of plasma cortisol, lactate, glucose and liver glycogen content. In order to determine interrenal integrity and responsiveness, the ability of interrenals to respond to ACTH and handling stress \textit{in vivo} was examined. The specific mechanisms involved on the potential modulation of steroidogenesis were investigated \textit{in vitro}. 
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CHAPTER 2
EFFECTS OF CADMIUM CHLORIDE ON THE
REPRODUCTIVE AND DEVELOPMENTAL AXES OF
RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

2.1 Abstract

Cadmium (Cd) is an inorganic environmental pollutant previously found to disrupt reproduction. However, most of the literature has described the effects of high Cd concentrations on the reproductive and developmental axes, with few studies focusing on low environmentally-realistic concentrations. In this study, the sub-chronic effects of low concentrations of Cd (0.05, 0.25, 0.50 and 2.50 µg/L) on eggs, larvae and juvenile rainbow trout were investigated. Mortality, hatching rates, hatching success, larval malformations and growth were recorded for early-life stages of trout exposed to Cd. Premature hatching occurred at lower concentrations (0.05 and 0.25 µg/L Cd), however, delayed hatching was seen in the 2.50 µg/L Cd group, with more than 90% of hatching occurring on the last day of the hatching period. Surprisingly, no effects on final hatching success or larval malformations at any concentration of Cd were observed. Larval growth was negatively affected by Cd exposure in a concentration-dependent fashion. Larvae exposed to 2.50 µg/L Cd were 13.9 ± 0.8% shorter in length and weighed 22.4 ± 3.5% less than controls after 28 days of exposure. Early-life stages were less sensitive to Cd than juvenile fish, which showed 80% mortality at 2.50 µg/L Cd after 7 days of exposure. Plasma sex steroid concentrations (estradiol in females and 11-ketotestosterone in males) were elevated in exposed fish compared to controls in both males and females,
after 28 days of exposure to 0.05, 0.25 and 0.50 μg/L Cd. These results suggest that environmentally-realistic concentrations of Cd can affect the reproductive and developmental axes of rainbow trout impacting eggs, larvae and juvenile fish.

2.2 Introduction

Reproductive function involves various complex processes that are integrated through the nervous, endocrine and reproductive systems in vertebrates (Jalabert et al. 2000). The physiological mechanisms involved in these processes and their sensitivity to disruption have become an area of increasing concern since development and reproduction of humans and wildlife are considered to be potentially at risk from environmental pollutant exposure (Arcand-Hoy and Benson 1998, Kime 1999).

Exposure to aquatic contaminants that interfere with endocrine function, endocrine-disrupting compounds (EDCs), may affect one or several components of the piscine reproductive system including gonads, liver, sperm and eggs, reproductive behavior, and also may affect fish development, culminating in decrease in population survival (Arcand-Hoy and Benson 1998, Kime 1999). In fact, among vertebrates, fish as a group can be particularly vulnerable to environmental xenobiotics due to their permanent presence in the aquatic environment, the final repository of most of environmental pollutants.

Heavy metals have been linked to endocrine disruption in various aquatic systems (Hontela 1998, Depledge andBillinghurst 1999, Hewitt and Servos 2001). Among heavy metals, cadmium (Cd) in particular has been shown to modulate endocrine pathways in teleosts in vivo and in vitro (Thomas and Khan 1997, Hontela 1998). The hypothalamus-
pituitary-gonadal (HPG) axis is one prime target of Cd toxicity (Arcand-Hoy and Benson 1998, Henson and Chedrese 2004). Cd is known to act centrally in disrupting steroidogenesis at the level of the pituitary (Tilton et al. 2003), but can also act directly on steroidogenic tissues such as teleost gonads. Cd and other metals have been shown to both stimulate and inhibit sex steroid production depending on metal concentration, species and sex of the animal (Sangalang and O’Halloran 1973, Sangalang and Freemann 1974, Kime 1984, Thomas and Khan 1997).

Although studies have shown that Cd can act as an endocrine disruptor in gonadal steroidogenesis, the repercussions on reproduction and development are controversial, particularly when fish are exposed to environmentally-realistic concentrations of this metal. Cd concentrations in surface waters are generally very low, ranging from < 0.1 to 8.9 µg/L with an average of 0.2 µg/L in British Columbia (Canadian Environmental Protection Act 1994). Both reproductive dysfunction and normal reproduction have been reported after exposure to Cd environmentally-realistic concentrations. Benoit et al. (1976) studying the toxicity of Cd to three generations of brook trout (Salvelinus fontinalis), reported negative reproductive capabilities of first and second-generation adult males exposed to 3.4 µg/L Cd. Foran et al. (2002) reported elevated plasma estradiol concentrations in Japanese medaka, Oryzias latipes, females exposed to 1 µg/L in ovo and subsequently re-exposed as adults. However, adults whose parents were exposed to Cd (1, 5 and 10 µg/L) and those adults exposed to Cd early during development reproduced normally despite Cd exposure. Recently, Tilton et al. (2003) have studied the effects of Cd concentrations (1, 5 and 10 µg/L) on the reproductive axis of the same species. The authors postulated that although Cd may disrupt the HPG axis at
multiple sites in males and females, including sex steroid modulation, this impairment
does not cause reproductive dysfunction, since no developmental effects were detected or
impairment on reproductive capacity of adults was seen. However, developmental effects
such as altered growth after exposure to low concentrations of Cd have been previously
reported in the literature (Benoit et al. 1976, Rombough and Garside 1982, Hansen et al.
2002).

In order to establish a causal link between exposure to a putative endocrine
disruptive agent and changes at populational level, the presence of a chemical in
association with an effect, (e.g. modulation of sex steroids) is not itself sufficient. A
comprehensive approach, including investigations of different life stages and different
endpoints at each stage (e.g. analysis of alterations in liver, gonads and pituitary, as well
as plasma sex steroids and vitellogenin concentrations) is recommended when evaluating
the effects of xenobiotics on the reproductive systems of fish (Kime 1999). Integrated
research that incorporates measurements and the evaluation of appropriate biomarker
responses, chemical residues and population dynamics are necessary to enable the real
significance of endocrine disruption in a given environment (Taylor and Harisson 1999).
This comprehensive approach has been incorporated with more frequency into recent
investigations. Various researchers have examined EDCs and their effects on both
development and reproduction by examining various effects in eggs, larvae, juvenile and
mature fish. Among the measurable parameters of the reproductive axis, most studies
have focused on alterations in hatching, growth, morphology at the tissue and whole
organismal level, as well as modulation of plasma hormones concentrations (Teather et
The objective of the present study was to investigate the effects of subchronic environmentally-relevant concentrations of Cd on the reproductive axis of rainbow trout using a comprehensive approach measuring various parameters with the intention of furthering our understanding of the potential interferences of low concentrations on reproduction and development.

2.3 Materials and methods

2.3.1 Chemicals

Cadmium chloride (CdCl₂) and NaHCO₃ were purchased from Sigma Chemical Co. (St. Louis, MO). Tricaine methanesulfonate (MS222), Ovadine and nitric acid (environmental grade plus) were obtained from Argent Laboratories (Redmond, WA), Syndel International Inc (Vancouver, BC) and Anachemia Science (Vancouver, BC) respectively.

2.3.2 Test organism

Oncorhynchus mykiss eggs and sperm were obtained from one male and one female bred under standardized condition at Fraser Valley Trout Hatchery (Abbotsford, BC). The procedure for artificial insemination was based on Pankhurst et al. (1996). Briefly, ovulated fish were anesthesized, dried with a cloth, and eggs dry stripped into a shallow container by gently pressing the belly using constant pressure until no more eggs were obtained. Sperm from a male was poured onto the eggs using the same procedure. The sperm, eggs and ovarian fluid were gently mixed. Water was added after 5 min and fertilized eggs soaked for another 20 min. Subsequently, eggs were disinfected with 100
ppm of Ovadine (Syndel International Inc, Vancouver, BC) for 15 min, washed gently in water, and kept in cold water (approximately 8°C) until exposure.

Juvenile trout (68.40 ± 0.77 g) were obtained from Sun Valley Trout Farms (Mission, BC) and acclimated for at least 4 wk in 1200 L tanks. Tanks were supplied with dechlorinated municipal water at 8 ± 2°C. Fish were maintained under a 12:12 photoperiod and fed with commercial trout food *ad libitum* (3 mm pellets - Ewos Canada Ltd., Surrey, BC). Water conditions were: hardness 6.8 mg CaCO₃/L, dissolved oxygen concentration > 90% and pH 6.8.

2.3.3 Eggs and larvae assessment

2.3.3.1 Exposure system

Viable eggs (perfectly spherical and translucent) were randomly transferred as a monolayer into 8 different egg incubators at 4h post fertilization. Incubators were connected to four water reservoirs which supplied different Cd (0, 0.05, 0.25 and 2.50 μg/L as CdCl₂) concentrations to each incubator. Each egg incubator consisted of a modified polyethylene 3L bottle. Water up-welled into the chamber through a 3 cm inlet at the bottom of an incubator, through a 0.2 mm nylon mesh which supported the eggs, and exited through a screened outlet on the upper section of the incubator. Water was pumped from the water reservoir into each chamber and sent back to the reservoir by gravity. In this recirculating system, a constant water flow of 500 mL/min was attained. Each replicate incubator consisted of a single chamber containing 371 ± 5 eggs (2 chambers were used per concentration). Water in each reservoir was renewed (50%) every other day. The exposure conditions were based on guidelines provided by the
Organization for Economic Cooperation and Development (OECD-210 1992). Water temperature was 11.5±1.5°C.

After a 40 day exposure in an egg incubator, 60 hatched larvae at the swim-up stage (30 from each chamber) from each concentration were removed and transferred to individual aquaria (0.50 L) connected to reservoirs which supplied tanks with the same Cd concentrations as in the incubators. Larvae were maintained under a 12:12 photoperiod and fed *ad libitum* with commercial trout food (crushed 1.2 mm extruded pellets, Ewos Canada Ltd., Surrey, BC) every other day just before reservoir water renewal. Excess food and organic material in tanks was removed daily using a small net. Larvae then were exposed to Cd for a further 16 days.

### 2.3.3.2 Sampling and endpoints

Hatching was recorded as the number of hatched larvae per batch of eggs per degree-days (°D = days from fertilization x incubation temperature in °C) until a maximum of 20 larvae per chamber was recorded. Therefore, hatching was registered in “10% steps” based on the results of daily inspection according to Pickova et al. (1990). Hatching was recorded from 273 °D (first day of observed hatched larvae) until 328 °D (when all eggs were hatched). Mortality was recorded daily for the duration of the experiment. When heartbeat could be visually observed, cessation of beating was the criterion for mortality, prior to that it was opaqueness of embryos (Brauner and Wood 2002).

Sub-samples of 3 to 5 larvae per chamber/aquaria were taken after 35 (pre-swim-up stage) and 56 days of exposure for weight and length measurements. On day 56 fish
had reached approximately 2 cm in length. Larvae were sacrificed by immersion in buffered MS-222 (0.1 g/L).

Approximately 100 larvae at swim-up stage were sampled from each chamber at day 40 of exposure and analysed for morphological deformities assessment. Larvae were killed by immersion in buffered MS-222 (0.1 g/L) and immediately examined without preservation using a dissecting microscope. Three possible malformation categories were evaluated: spinal, craniofacial and yolk-sac according to von Westernhagen (1988) and Kennedy et al. (2000). Spinal deformities included abnormal curvature of spine, such as lordosis or scoliosis. Craniofacial abnormalities included ocular abnormalities (reduced eye diameter, absence or supranumerary eyes) and jaw malformations (size reduction, abnormal structure, or absence), and supranumerary heads. Yolk-sac abnormalities included incomplete yolk circulation or patches of necrotic tissue. With exception of one larvae exposed to 2.50 µg/L Cd that showed prominent spinal deformity (lordosis) and craniofacial abnormality (jaw malformation) and which was excluded from the study, all the other larvae showed one major type of malformation along with one or more minor deformities. Therefore, each animal was included in only one of the three malformation categories, which corresponded to the major deformity observed.

2.3.4 Juvenile fish

2.3.4.1 Exposure system

Juvenile fish were randomly divided into five groups, consisting of three replicate tanks for each group. Each flow-through tank (130 L) received a different concentration of Cd as CdCl₂: 0, 0.05, 0.25, 0.50 and 2.50 µg/L Cd. Tanks were subdivided into two compartments (n=3 for each compartment). Fish were exposed to Cd continuously for 28
days. The water flow in each tank was 900 mL/min and Cd concentrations were maintained using Mariotte bottles (Hontela et al. 1996). Fish were fed ad libitum (3mm pellet, Ewos Canada Ltd., Surrey, BC) every other day until 24 h before sampling. Excess food and all the organic material in tanks were removed daily with minimum disturbance to the fish.

2.3.4.2 Sampling and endpoints

One to three juvenile fish were sampled from each replicate tank in each treatment group on exposure days 7, 14, 21, and 28. At each sampling period, fish from alternate compartments were quickly removed, killed by over-anesthetization with buffered MS222 and body mass (wet weight) measured. Blood samples (1 mL) were taken by caudal puncture using a 1 mL heparinized syringe and placed into eppendorf tubes. Plasma was separated by centrifugation at 16,000 x g for 4 min and frozen at -80°C for sex steroid determination at a later date. Gonads were removed and weighed at 7 and 28 days exposure and gonadosomatic index (GSI: weight of gonads as percent of body weight) was determined. Mortality was recorded daily for the duration of the experiment. Overall mortality was calculated by the end of the experiment.

Concentrations of 11-ketotestosterone (11-KT) were determined in male plasma and estradiol concentrations in female plasma using commercially available EIA kits (Cayman Chemical Company, Ann Arbour, MI). Sub-samples of 50 μL of plasma were used for sex steroid analysis. Each 96-well steroid antibody coated plate was run with a standard curve based on a serial dilution of a stock solution (10 ng/mL for 11-KT and 100 ng/mL for estradiol) provided with the kit. Intra-assay and inter-assay coefficients of variation (CV) for 11-KT were 3.1% and 1.2% respectively (n=5). Intra-assay and inter-
assay CV for estradiol were 3.8% and 3.6% respectively (n=3). All measures were performed on a Spectra Max 420 microplate reader (Molecular Devices Corporation, Sunnyvale, CA) and absorbances read at 412 nm.

### 2.3.5 Aqueous Cd analysis

Water samples for metal analysis were collected in polyethylene bottles, preserved by acidification with concentrated nitric acid to pH < 2.0 and stored at 4°C until analysis. Aqueous Cd concentrations were measured using inductively coupled plasma - optical emission spectroscopy with Varian Vista PRO-axial view ICP-OES (detection limit 0.2 ng/mL Cd) by Cavendish Analytical Laboratories, Vancouver, BC. Since analytical confirmations were at least 80% of nominal values (0 = 0.0184 ± 0.0053 μg/L Cd, 0.05 = 0.0363 ± 0.0042 μg/L Cd, 0.25 = 0.2569 ± 0.0186 μg/L Cd, 0.50 = 0.4732 ± 0.0180 μg/L Cd and 2.50 = 2.1320 ± 0.0511 μg/L Cd) results are reported in terms of nominal concentrations (Tilton et al. 2003).

### 2.3.6 Statistical analysis

Statistical analyses were performed using JMP IN 4.0.3 (SAS Institute Inc., Cary, NC). The experimental data were analyzed by one-way or two-way ANOVA followed by Tukey-Kramer HSD tests. Hatching was assessed by repeated measures ANOVA followed by Pillai’s Trace test and subsequent ANOVA to locate the days when hatching was significantly different between groups. Differences were considered significant at p ≤ 0.05. Some of the data required natural logarithm transformation in order to meet the assumption of homogeneity of variance, although non-transformed data are shown in the figures. Results are expressed by means ± SEM. Since no significant differences
between body mass of male and female juvenile fish were observed, data from both sexes were pooled.

2.4 Results

2.4.1 Eggs and larvae

The mortality of eggs ranged from 11.94 ± 0.55 to 14.37 ± 1.03% (Table 2.1) and there were no significant differences between controls and any exposed group (F=1.17, d.f.=3, p=0.4255). Hatching occurred from 273 °D until 328 °D (Fig. 2.1). Hatching was significantly different between groups over time (F=67.78, d.f.=3, p=0.0004). Eggs exposed to 0.25 µg/L Cd began hatching earlier than all the other groups and, at 294 °D showed a significantly higher percent hatch compared to all other groups (F=17,074, d.f.=3, p< 0.0001). At 316 °D, all groups, with the exception of the 2.50 µg/L Cd group, had reached their highest percent hatch. Between 316 and 328 °D, almost all eggs from the 2.50 µg/L Cd group hatched and although delayed, hatching occurred to the same total percent hatch as the other groups. Cumulative percent hatch was not different between groups at 328 °D (85.63 ± 1.03, 86.13 ± 1.77, 85.64 ± 0.04 and 88.05 ± 0.55% for control, 0.05, 0.25 and 2.5 µg/L Cd groups respectively. F=1.17, d.f.=3, p=0.4255).

The mortality of larvae ranged from 2.89 ± 0.77 to 5.54 ± 1.16% with no significant differences between exposed and control groups (Table 2.1) (F=1.95, d.f.=3, p=0.2636). Total percent malformations in controls were 22.93 ± 2.29%. Malformations in Cd exposed groups were 13.85 ± 5.93, 20.38 ± 6.76 and 23.72 ± 6.76% for the 0.05, 0.25 and 2.50 µg/L Cd groups respectively. The one-way analysis of variance of percentage of malformations showed no significant differences between exposed and
control groups (F=0.61, d.f.=3, p=0.6451). Spinal malformations, cranio-facial and yolk-sac abnormalities were observed in all groups, with spinal being the most frequent. Among the spinal malformations observed, lordosis occurred in 87.33 ± 2.92% and scoliosis in 12.67 ± 2.92% of them. Supranumerary (two) heads was the major cranio-facial abnormality observed, with only one larva showing jaw malformation (2.50 µg/L group) and another showing three eyes (0.50 µg/L group). Among yolk-sac deformities, two larvae from the control group and one larva from the 2.50 µg/L group showed patches of necrotic tissue; all the others had incomplete yolk circulation. The percentages of the various types of malformations are summarized on Fig 2.2.

Weight and length of larvae were determined after 35 and 56 days of exposure to Cd (Fig. 2.3, Table 2.2). Changes in weight and length varied with time and Cd concentrations (weight, F = 2.88, d.f.= 3, p = 0.0411; length, F = 5.49, d.f.= 3, p = 0.0018). Length of controls was 2.22 ± 0.01 cm at 35 days and 2.54 ± 0.01 cm at 56 days. Length of larvae exposed to 0.05 and 0.25 µg/L Cd was not significantly different from controls at 35 days, however, larvae from the 2.50 µg/L Cd group were significantly shorter (approximately 10%) than all other groups at the same timepoint (F=18.42, d.f.=3, p< 0.0001). Length of larvae after 56 days showed similar trend (F=110.73, d.f.=3, p<0.0001). Weight of controls was 91.66 ± 1.71 mg and 95.80 ± 1.31 mg after 35 and 56 days respectively. Weight of larvae exposed to all Cd concentrations was not significantly different from controls at 35 days, however, larvae exposed to 2.50 µg/L Cd weighed significantly less than larvae exposed to 0.25 µg/L Cd (F=5.41, d.f.=3, p=0.0073). Larvae exposed to 0.25 and 2.50 µg/L Cd for 56 days weighed significantly less than larvae exposed to the same Cd concentrations for 35 days. In fact, larvae
exposed to 2.50 μg/L Cd weighed less than all the other groups after 56 days of exposure (F=15.96, d.f.=3, p<0.0001).

2.4.2 Juvenile fish

2.4.2.1 Mortality, weight and GSI

Total mortality was recorded per tank after 28 days of exposure, and no significant difference was seen between control and the 0.05, 0.25 and 0.50 μg/L Cd groups (F=0.4979, d.f.=3, p=0.6893) (Table 2.3). Mortality was 75.71 ± 6.49% for fish exposed to 2.50 μg/L Cd after 7 days of exposure. Since only 5 fish survived the first week, this group was discontinued and only mortality after 7 days was recorded.

Weight of controls was 70.13 ± 5.79 g after a 28 day exposure. No significantly differences were found between control and Cd-exposed groups (F=2.11, d.f.=3, p=0.1269)(Table 2.3). GSI ranged from 0.09 ± 0.01 to 0.15 ± 0.03 for females and from 0.04 ± 0.01 to 0.09 ± 0.04 for males with no treatment or time effect in both groups (females: F=3.67, d.f.=3, p=0.0269; males: F=0.84, d.f.=3, p=0.4812 (Table 2.3).

2.4.2.2 Plasma sex steroid concentrations

Sex steroid concentrations from fish exposed to varying concentrations of Cd for 7 and 28 days are shown in Fig. 2.4. Plasma estradiol concentrations in control female fish were 3.89 ± 0.44 pg/mL for the whole exposure period with no significant difference with time. Plasma estradiol concentrations varied with time and the different Cd concentrations (F = 6.60, d.f. = 9, p < 0.001). Plasma estradiol concentrations were higher than control values for the 0.05 and 0.50 μg/L Cd groups after 7 days (297.16 ± 47.62 and 323.15 ± 35.67 % of controls respectively) (F = 13.32, d.f. = 3, p = 0.0018). After 14
days of Cd exposure, estradiol concentrations in exposed fish ranged from $36.12 \pm 0.00$ to $145.07 \pm 13.08\%$ of controls with no significant difference between controls and Cd treated groups ($F = 6.02$, d.f.= 3, $p = 0.0084$). 21 days of exposure to Cd showed also no significant effect on estradiol concentrations with values ranging from $110.84 \pm 26.72$ to $184.36 \pm 42.21\%$ of controls ($F = 1.33$, d.f.= 3, $p = 0.3018$). By the end of the experiment (28 days) plasma estradiol concentrations were significantly higher in all Cd-exposed groups ($668.14 \pm 64.61$, $450.57 \pm 77.93$ and $548.68 \pm 73.64\%$ of controls for the 0.05, 0.25 and 0.50 µg/L Cd groups respectively) ($F = 15.03$, d.f.= 3, $p = 0.0005$). Plasma estradiol concentrations are shown in Table 2.4.

Plasma 11-KT concentrations in control fish were $13.39 \pm 2.51$ pg/mL for the whole exposure period with no significant difference with time. Plasma 11-KT concentrations varied with time and the different Cd exposure groups ($F = 5.86$, d.f.= 9, $p < 0.0001$). After 7 days of Cd exposure, male fish showed significantly higher 11-KT concentrations in the 0.25 µg/L Cd group compared to controls ($1296.34 \pm 136.29\%$ of controls) ($F = 35.22$, d.f.= 3, $p < 0.0001$). After 14 and 21 days of Cd exposure, levels of 11-KT in exposed fish ranged from $63.75 \pm 24.93$ to $179.17 \pm 77.15\%$ of controls with no significant difference between controls and any treatment groups (14 days: $F=0.20$, d.f.=3, $p=0.8925$; 21 days: $F = 2.56$, d.f.= 3, $p = 0.1035$). Plasma 11-KT concentrations were significantly higher in exposed vs. control fish for all concentrations of Cd after 28 days of exposure ranging from $501.80 \pm 36.15$ to $935.85 \pm 385.02\%$ of controls ($F = 9.02$, d.f.= 3, $p = 0.0060$). Plasma 11-KT concentrations are shown in Table 2.5.
2.5 Discussion

In this sub-chronic study, early-life stages (eggs and larvae) and juvenile fish were exposed to environmentally-realistic concentrations of Cd with the intention of assessing the potential for adverse effects on the reproductive and developmental axes of rainbow trout using a comprehensive approach. To the author's knowledge, this is the first study to demonstrate that low Cd concentrations affect rainbow trout reproductive and developmental parameters by interfering with egg and larval development and by modulating plasma sex steroid concentrations in juvenile fish.

Early life stages and juvenile fish responded very differently to Cd exposure. Cd was not acutely toxic to either eggs or larvae at the concentrations used, however, at the highest concentration tested (2.50 µg/L Cd), approximately 80% of juvenile fish died after 7 days of exposure. It is often assumed that the early-life stages are the most sensitive development stage of teleosts (von Westernhagen 1988, Kime 1999). However, the present mortality data clearly shows that juvenile trout are more sensitive to Cd than earlier life stages. Rombough and Garside (1980) also observed higher sensitivity to Cd in alevins compared to eggs of Salmo salar. In addition, Buhl and Hamilton (1991) demonstrated a higher sensitivity of juveniles compared to alevins in Oncorhynchus mykiss, O. kisutch and Thymallus arcticus. For example, these authors reported a 96-h LC₅₀ of 1.5 µg/L Cd for juvenile Oncorhynchus mykiss, compared to 37.9 µg/L Cd for alevins of the same species. It was suggested that a shift in the primary site of Cd uptake from the more robust integument in newly hatched larvae, to the fragile branchial epithelium in older fish may account for the increase in sensitivity in the later developmental stage (Rombough and Garside 1980). It is also conceivable that an
increase in the number of uptake sites, from the integument only in newly hatched larvae, to both the integumentary and branchial epithelium in yolk-sac larvae, to the integument, branchial and gastrointestinal routes in late larvae and juveniles, could influence sensitivity. In fact, Thomann et al. (1997) highlights the importance of different uptake routes in determining Cd exposure risk.

It has been demonstrated that most of the Cd present in carp eggs exposed to polluted river water was confined to the outer membranes with only a small amount passing into the embryo (van Anholt et al. 2002). After hatching however, the accumulation of Cd increased rapidly in larvae following yolk sac resorption and the onset of food uptake (Cd-contaminated organic matter and macroinvertebrates). Future investigations are necessary to address the relative importance of the various uptake routes to overall Cd toxicity and their importance to the susceptibility of different life stages of fish.

Cd affected the time to hatch differently for the varying concentrations used. Hatching began earlier than controls in eggs exposed to the two lower concentrations of Cd. However, delayed hatching was observed in eggs exposed to the highest concentration. Interestingly, the final percent hatch was the same in all the groups by 328 °D. Hatching started at 294°D in controls, with mass hatching at 316°D, slightly before previous reported data on hatching period for rainbow trout under normal conditions (approximately 330°D) (Lavrosky 1968, Wright et al. 2003). Changes in time to hatch are common in fish embryos exposed to sublethal concentrations of pollutants, and Cd has been shown to induce premature hatching in carp and herring, and delayed hatching in salmonids (von Westernhagen 1988). Delayed hatching also occurred in Cyprino
carpio eggs exposed to 10 to 50 μg/L Cd although no effects on eggs exposed to concentrations < 5 μg/L were observed (Witeska et al. 1995, Calta 2001). Despite differences in the sensitivity between those species (carp and trout), it seems that higher Cd concentrations delay hatching in fish. No clear explanation for this phenomenon exists, but it is possible that Cd may affect the activity of chorionase, the enzyme responsible for the dissolution of the egg envelope (Calta 2001). Our results also demonstrate an early hatching associated with the lowest Cd concentrations. Divalent cations such as Ca$^{2+}$ and Mg$^{2+}$ have been shown to stimulate and inhibit chorionase activity: high concentrations can inhibit activity and low concentrations can be slightly stimulating (Yamagamy 1973). Therefore, it is possible that Cd$^{2+}$ may both inhibit and stimulate chorionase activity, thus inducing early or delayed hatch depending on the concentration.

Body weight and length have been used as toxicity endpoints by several authors studying the effects of Cd on fish (Calta 2001, Nguyen and Janssen 2002, Tilton et al. 2003). Larval weight and length were negatively affected by the highest concentration of Cd and, to a lesser extent, by 0.25 μg/L Cd, suggesting a concentration-dependent response. In addition, a time-dependent effect on growth could also be identified: fish exposed to 0.25 and 2.50 μg/L Cd showed reduced weight with time. These results were significant despite the low growth rate observed in controls themselves, which was probably associated with the feeding regime used in our early-life stages experiments. Specific growth rate of 1-2% per day has been described for salmonids during early-life stages and under normal conditions (Uysal and Alpbaz 2002), a value higher than the 0.6% observed in our study. Feeding regime in fish toxicological studies may vary from
one to eight times each day to no feeding at all (Dave 1985, Hwang et al. 1995, Garcia et al. 1999, Williams and Holdway 2000, Foran et al. 2002, Nguyen and Janssen 2002, Ishibashi et al. 2004). Since Cd may be readily complexed with organic material present in the water thus decreasing toxicity (Van Ginneken et al. 2001, US-EPA 2001), the feeding regime in the early-life stages experiments was modified to be minimal, minimizing the presence of extra organic material, which might interfere with water quality (Manning et al. 1999). Independent of feeding regime, reduced larval growth was observed in *Salmo salar* exposed to 0.47 μg/L Cd (Rombough and Garside 1982), *Salvelinus fontinalis* exposed to 3.4 μg/L (Benoit et al. 1976) and *Cyprinus caprio* exposed to concentrations as low as 10 μg/L Cd (Calta 2001). Cd$^{2+}$ may reduce the uptake of Ca$^{2+}$, due their similar ionic size (0.97 and 0.99 Å respectively), and consequent competition for ionic binding sites in fish. Reduced Ca$^{2+}$ uptake may lead to a slowed growth in teleosts (Chang et al 1998, Meinelt et al. 2001). In addition, delays in growth hormone expression during rainbow trout development have been shown in Cd-contaminated water at a concentration of 100 μg/L Cd (Jones et al. 2001). These studies indicate that several mechanisms may be involved in Cd’s effects on rainbow trout larval growth.

Larval malformations were observed in both control and exposed groups with an average value of 20.2 ± 2.6 %, with no difference between groups. Compared to most literature reports, these values are high, although within the normal range reported for salmonids (0 to 21%) (Ciereszko et al. 1997, Kennedy et al. 2000, Aegerter and Jalabert 2004). In fact, the incidence of larval abnormalities under normal conditions is relatively common in cultured fish (Aubin et al. 2005). Regardless of the observed high percentage
of malformed larvae in controls, our results indicate that Cd does not act as a teratogen at the concentrations used. To the author’s knowledge, teratogenic effects in embryos exposed to Cd concentrations below 500 µg/L have never been cited in the literature, even though previous studies using higher Cd concentrations (mg/L range) have demonstrated a dose-dependent teratogenic effect in fish (Pragatheeswaran et al. 1989, Cheng et al. 2000, Nguyen and Janssen 2002, Chan and Cheng 2003).

Kime (1999) reviewed strategies for assessing the effects of pollutants on fish reproduction, and suggested several parameters to measure which would indicate gonadal dysfunction including both GSI and plasma sex steroid concentrations. GSI was not affected by the concentrations of Cd used in the present study. Similar results were observed in winter flounder (*Pleuronectes americanus*) exposed to 25 and 50 µg/L Cd (Pereira et al. 1993). On the other hand, in the present study, plasma sex steroid concentrations were significantly increased in juvenile fish following Cd exposure for 28 days, with values substantially higher than controls in both males and females exposed to all Cd concentrations. Plasma 11-KT (males) and estradiol (females) concentrations in mature salmonids are usually around 100 (11 KT) and 1 to 60 ng/mL (estradiol) respectively. In immature fish values below 1 ng/mL for both hormones have been reported (van Bohemen and Lambert 1981, Pottinger et al. 1996, McQuillan et al. 2003). Even at low concentrations, sex steroids are very important for sex differentiation and the onset of puberty in juvenile fish (Patinõ 1997, Consten et al. 2002). Previous studies with female *Micropogonias undulatus* exposed to 1 mg/L Cd for 40 days (Thomas 1989) and male *Salvelinus fontinalis* exposed to 1 µg/L Cd for 93 days (Sangalang and Freemann 1974) have resulted in elevated plasma sex steroid concentrations. However, some
studies have shown opposite results in vivo. For example, in female Oryzias latipes, plasma estradiol concentration was increased in fish exposed to 5 μg/L Cd for 49 days but decreased in fish exposed to 10 μg/L Cd for the same period of time (Tilton et al. 2003). Moreover, Singh (1989) reported reduced plasma testosterone and estradiol concentrations in Monopterus albus exposed to 3 mg/L Cd. In vitro studies show similarly inconsistent results: increases and decreases in sex steroid production following Cd exposure (Sangalang and O’Halloran 1973, Kime 1984, Thomas and Khan 1997). It is clear that Cd can modulate plasma sex steroid concentrations; however, the mechanism(s) of action of Cd on disrupted steroidogenesis in gonads has not been extensively examined in teleosts. In mammals, it has been suggested that Cd may act at multiple sites in the intracellular signaling pathway of testosterone synthesis, encompassing both stimulatory and inhibitory sites (Laskey and Phelps, 1991). Therefore, estimates of inhibition or stimulation of this hormone is likely a summation of both actions, a phenomenon that may also occur in teleosts. Another possible site of Cd action is at the pituitary and/or hypothalamic level. Studies in mammals have shown the modulation of pituitary hormones in vivo and in vitro (gonadotropins, prolactin, ACTH, growth hormone and thyroid-stimulating hormone) following exposure to Cd (Laflante and Esquifino 1999, Laflante et al. 2001, Laflante et al. 2002, Laflante et al. 2003, Pillai et al. 2003, Laflante et al. 2004). Cd has been shown to affect pituitary morphology (degenerative lesions) and to increase and decrease pituitary hormones (gonadotropins and prolactin) in vitro and in vivo in fish (Thomas 1989, Fu and Look 1990, Pundir and Saxena 1992, Mukherjee et al. 1994). In the field, fish from polluted sites (heavy metals, including Cd) have shown altered pituitary morphology (Mousa and Mousa 1999).
Seasonal variations in plasma concentrations of sex steroids have been linked to different phases of the reproductive cycle in rainbow trout: 11-KT peaks during the period of spermiation (Scott et al. 1980) and estradiol peaks during vitellogenesis, immediately before the ovulation period (Scott and Sumpter 1983). Modulation of plasma sex steroid concentrations by xenobiotics could affect the reproductive cycle by producing mature gametes outside the normal spawning season, and thus compromising the viability of offspring (Thomas 1989). Although plasma steroid modulation was accessed only in juvenile fish in the present study, the high levels of sex steroids observed for both sexes after 28 days of Cd exposures indicate that timing of reproduction could be affected in more mature fish.

The results of this study demonstrate that Cd can disrupt several components of the reproductive and developmental axes in rainbow trout following sub-chronic exposure to extremely low Cd concentrations. In fact, Cd acute and chronic toxicity tests that indicate that salmonids are the most sensitive fish group as a whole are supported by our data (Canadian Environmental Protection Act 1994, Hansen et al. 2002). Moreover, Cd impaired the three life-stages studied very differently, with juveniles being the most sensitive stage examined. Since environmentally-realistic concentrations of Cd were used, it is evident that additional field studies are needed to evaluate the potential impact of this metal on the reproductive success of fish and wildlife.
2.6 Figures

Figure 2.1 Cumulative percent hatching of rainbow trout eggs exposed to 0 (w-control), 0.05 ( ), 0.25 (a) and 2.50 (b) pg/L Cd from 273 'D to 328 'D. Statistical comparisons were made between concentrations at each time point. Treatments with different letters were significantly different from each other at p<0.05. Values are means ± SEM.
Figure 2.2 Cumulative percentage of the various types of malformations for rainbow trout larvae exposed to 0 (control), 0.05, 0.25 and 2.50 μg/L Cd for 40 days. Spinal (•), cranio-facial ( ) and yolk-sac malformations (•).
Figure 2.3 Growth measurements, A) length and B) weight, for rainbow trout larvae exposed to 0, 0.05, 0.25 and 2.50 µg/L Cd for 35 (a - n=5 to 6) and 56 days (b - n=11 to 18). Control values represent weight or length at each time point. Treatments with different letters were significantly different from each other at p < 0.05. Values are means ± SEM.
Figure 2.4 Sex steroids concentrations as % of control values in rainbow trout A) females and B) males exposed to 0 (control), 0.05, 0.25 and 0.50 μg/L Cd for 7 (■) and 28 (□) days. \( n=3 \) to 5. Treatments with different letters were significantly different from each other at \( p<0.05 \). Values are means ± SEM.
2.7 Tables

Table 2.1 Percent mortality in control and Cd exposed rainbow trout eggs and larvae (0, 0.05, 0.25 and 2.50 μg/L Cd). a: percent of incubated eggs. \( n = 371 \pm 5 \) eggs (X2 chambers), b: percent of hatched larvae. \( n = 340 \pm 5 \) larvae (X2 chambers).

<table>
<thead>
<tr>
<th>Life-stage</th>
<th>0 μg/L Cd</th>
<th>0.05 μg/L Cd</th>
<th>0.25 μg/L Cd</th>
<th>2.50 μg/L Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggs(^a)</td>
<td>14.37 ± 1.03</td>
<td>13.87 ± 1.77</td>
<td>14.36 ± 0.04</td>
<td>11.95 ± 0.55</td>
</tr>
<tr>
<td>Larvae(^b)</td>
<td>5.28 ± 1.16</td>
<td>2.90 ± 0.77</td>
<td>3.33 ± 0.64</td>
<td>5.54 ± 1.16</td>
</tr>
</tbody>
</table>
Table 2.2 Weight (mg) and length (cm) of rainbow trout larvae exposed to 0, 0.05, 0.25 and 2.50 μg/L Cd for 35 (n=5 to 6) and 56 days (n=11 to 18).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time (days)</th>
<th>0 μg/L Cd</th>
<th>0.05 μg/L Cd</th>
<th>0.25 μg/L Cd</th>
<th>2.50 μg/L Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mg)</td>
<td>35</td>
<td>91.66 ± 1.71</td>
<td>90.90 ± 1.59</td>
<td>97.41 ± 4.41</td>
<td>82.40 ± 1.87</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>95.80 ± 1.31</td>
<td>88.39 ± 1.60</td>
<td>87.94 ± 1.70</td>
<td>74.36 ± 3.31</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>35</td>
<td>2.22 ± 0.00</td>
<td>2.18 ± 0.01</td>
<td>2.22 ± 0.03</td>
<td>2.00 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>2.54 ± 0.01</td>
<td>2.54 ± 0.02</td>
<td>2.56 ± 0.01</td>
<td>2.19 ± 0.02</td>
</tr>
</tbody>
</table>
Table 2.3 Mortality (%), weight (g) and GSI of juvenile rainbow trout exposed to 0, 0.05, 0.25, 0.50 and 2.50 µg/L Cd. a: mortality (n=4 to 6) and weight (n=6 to 9) after 28 days of Cd exposure, b: GSI after 7 and 28 days of exposure (n=8 to 9 for females and 5 to 15 for males), c: mortality after 7 days of Cd exposure. This group was discontinued.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0 µg/L Cd</th>
<th>0.05 µg/L Cd</th>
<th>0.25 µg/L Cd</th>
<th>0.50 µg/L Cd</th>
<th>2.50 µg/L Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality (%) a</td>
<td>1.60 ± 1.60</td>
<td>4.25 ± 4.25</td>
<td>4.17 ± 2.88</td>
<td>7.38 ± 4.35</td>
<td>75.71 ± 6.49</td>
</tr>
<tr>
<td>Weight (mg) a</td>
<td>70.31 ± 5.79</td>
<td>75.11 ± 2.48</td>
<td>70.30 ± 4.54</td>
<td>65.42 ± 2.91</td>
<td>-</td>
</tr>
<tr>
<td>GSI females b</td>
<td>0.15 ± 0.03</td>
<td>0.09 ± 0.01</td>
<td>0.14 ± 0.03</td>
<td>0.10 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>GSI males b</td>
<td>0.06 ± 0.01</td>
<td>0.04 ± 0.00</td>
<td>0.09 ± 0.04</td>
<td>0.05 ± 0.00</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2.4 Plasma estradiol concentrations (pg/mL) of juvenile rainbow trout exposed to 0, 0.05, 0.25 and 0.50 μg/L Cd for 7, 14, 21 and 28 days. n=3 to 5.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>0 μg/L Cd</th>
<th>0.05 μg/L Cd</th>
<th>0.25 μg/L Cd</th>
<th>0.50 μg/L Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>2.28 ± 0.70</td>
<td>6.76 ± 1.08</td>
<td>2.12 ± 0.74</td>
<td>7.35 ± 0.81</td>
</tr>
<tr>
<td>14</td>
<td>5.05 ± 0.39</td>
<td>7.33 ± 0.66</td>
<td>4.40 ± 0.69</td>
<td>1.82 ± 0.00</td>
</tr>
<tr>
<td>21</td>
<td>4.72 ± 0.45</td>
<td>6.92 ± 1.75</td>
<td>9.26 ± 2.12</td>
<td>5.57 ± 1.34</td>
</tr>
<tr>
<td>28</td>
<td>1.04 ± 0.01</td>
<td>7.01 ± 0.08</td>
<td>4.73 ± 0.82</td>
<td>5.76 ± 0.77</td>
</tr>
</tbody>
</table>
Table 2.5 Plasma 11-KT concentrations (pg/mL) of juvenile rainbow trout exposed to 0, 0.05, 0.25 and 0.50 μg/L Cd for 7, 14, 21 and 28 days. n=3 to 5.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>0 μg/L Cd</th>
<th>0.05 μg/L Cd</th>
<th>0.25 μg/L Cd</th>
<th>0.50 μg/L Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>10.51 ± 0.15</td>
<td>10.65 ± 1.87</td>
<td>136.29 ± 52.48</td>
<td>4.94 ± 0.60</td>
</tr>
<tr>
<td>14</td>
<td>14.00 ± 0.75</td>
<td>22.15 ± 9.01</td>
<td>49.33 ± 20.35</td>
<td>25.70 ± 8.80</td>
</tr>
<tr>
<td>21</td>
<td>18.57 ± 11.58</td>
<td>33.28 ± 14.33</td>
<td>11.84 ± 4.63</td>
<td>29.10 ± 8.86</td>
</tr>
<tr>
<td>28</td>
<td>11.92 ± 7.05</td>
<td>59.84 ± 4.31</td>
<td>99.30 ± 0.00</td>
<td>93.35 ± 39.97</td>
</tr>
</tbody>
</table>
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CHAPTER 3
CADMIUM CHLORIDE-INDUCED DISRUPTION OF TESTICULAR STEROIDOGENESIS IN RAINBOW TROUT, ONCORHYNCHUS MYKISS

3.1 Abstract

Cadmium (Cd) is a known endocrine disruptor with the ability to affect the production of hormones involved in the regulation of reproductive processes. In the present study, the effects of CdCl₂ on unstimulated and stimulated testicular steroidogenesis were examined with the intention of furthering an understanding of the potential site(s) of action in the signaling pathway for 11-KT synthesis in teleosts. In short term (2-h) exposures, CdCl₂ stimulated 11-KT production (29.3 ± 10.3 and 28.2 ± 6.2 % over controls) in minced testicular tissues at 10 and 100 μM, respectively. However, 11-KT production was significantly lower than controls (54.0 ± 8.9, 61.7 ± 7.5 and 54.0 ± 4.6 %) when tissues were incubated for 18 h in the 1, 10 and 100 μM treatment groups. Incubation of testicular tissues with 100 IU/mL hCG and 5 mM dibutyryl-cAMP (dbcAMP), which activate rate-limiting steps in steroid synthesis, or 1.3 μM 25-hydroxycholesterol (25-OHC), which augments production, resulted in significant increases in steroidogenesis over controls. hCG-stimulated steroidogenesis was reduced to 53.8 ± 10.8 % and 61.9 ± 8.7% of stimulated controls when tissues were incubated with Cd at 1 and 10 μM, respectively. 11-KT production in dbcAMP-stimulated and 25-OHC-augmented tissues was not affected by Cd exposure. The results of this study indicate that one site of action of Cd in the signaling steroidogenic pathway is located
prior to cAMP formation. This impairment could be overcome when higher concentrations of Cd were used in hCG-stimulated cells, suggesting a presence of a stimulatory site at, or after, hCG receptor binding.

3.2 Introduction

Endocrine-disrupting compounds (EDCs) can obstruct wildlife reproduction through several modes of action, affecting both the structure and function of various components of the reproductive system. Many physiological processes, from sexual differentiation, to female and male gametogenesis can be impaired, due to disruptions among complex neuro-endocrine, endocrine and paracrine regulatory processes (Jalabert et al. 2000). Heavy metals such as Cd have been shown to act as EDCs in fish, and although no direct evidence exists, metals have been linked to endocrine disruption in teleosts in Canadian aquatic ecosystems (Hontela 1998, MacMaster 2001, Hewitt and Servos 2001).

The modulation of gonadal steroidogenesis (Arcand-Hoy and Benson 1998, Kime 1999, Jalabert et al. 2000) has been shown to be a prime target of EDCs. Testicular steroidogenesis is a complex process regulated mainly by pituitary hormones and testicular signaling systems (Schulz and Blum 1990, Schulz et al. 2001). 11-ketotesteosterone (11-KT), the major androgen in teleosts, 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). In salmonids, two pituitary hormones, gonadotropin I (GTI) and gonadotropin II (GTII) are the major secretagogues which stimulate 11-KT secretion by the testes (Swanson et al. 1991, Dickey and Swanson 1998). Cd has been shown to stimulate or inhibit gonadal steroid production in fish
depending on the dose and the sex of individuals (Thomas and Khan 1997). For example, Cd increases 11-KT and testosterone concentrations in vivo and in vitro in acute exposures (Sangalang and Freemann 1974, Kime 1984). However, following a long-term tank exposure, Cd inhibited the synthesis of 11-KT and testosterone by in vitro trout testicular preparations (Sangalang and O'Halloran 1973). These opposing effects of Cd on androgen production indicate that there are likely multiple effects of Cd on steroidogenesis which depend on exposure duration as well as concentration.

The mechanisms of action of Cd on disrupted steroidogenesis in testis have not been extensively examined in teleosts. In mammals, it has been suggested that Cd may act at multiple sites in the intracellular signaling pathway of testosterone synthesis (Laskey and Phelps 1991). Therefore, the purpose of this study was to investigate the effects of Cd on testicular steroidogenesis with the intention of furthering the understanding of the site(s) of action of this metal in the signaling pathway for 11-KT production in fish.

3.3 Materials and methods

3.3.1 Fish

Male rainbow trout (body weight 600 to 1200 g, gonadosomatic index >2) were obtained from Sun Valley Trout Farms (Mission, BC) and acclimated for at least 4 weeks in 1200 L tanks prior to experimentation. Tanks were supplied with dechlorinated municipal water (Burnaby, BC) at 17±1°C, hardness 6.8 mg CaCO₃/L, and pH 6.7. Fish were subjected to a 12:12 light:dark photoperiod and were fed with commercial trout
food (Ewos Canada Ltd., Surrey, BC; 2mm extruded pellets) until 48 hours prior to an experiment.

### 3.3.2 Chemicals

Cadmium chloride (CdCl\textsubscript{2}), 25-hydroxycholesterol (25-OHC), hCG, N\textsuperscript{6},2\textsuperscript{'-o-}
dibutyryladenosine 3\textsuperscript{'}:5\textsuperscript{'}-cyclic monophosphate sodium salt (dbcAMP), Leibovitz medium (L-15), NaHCO\textsubscript{3}, Type IV collagenase and Trypan Blue were purchased from Sigma Chemical Co. (St. Louis, MO). Anhydrous D-glucose and tricaine methanesulfonate (MS222) were obtained from BDH Inc. (Toronto, ON) and Argent Laboratories (Redmond, WA), respectively.

### 3.3.3 Preparation of testicular tissue

Testicular tissues were prepared according to a modified method of Schulz and Blüm (1990). Trout were euthanized in 0.2 g/L buffered MS222 and the testes immediately removed and rinsed in ice-cold, well oxygenated (1% CO\textsubscript{2}, balance O\textsubscript{2}) L-15 medium supplemented with 5 mM D-glucose and 6 mM NaHCO\textsubscript{3}, pH 7.4. The tissue (51.1 ± 0.2 mg wet weight) was finely minced to approximately 1 mm\textsuperscript{3} in size and placed into glass vials containing 1 mL L-15 medium. The minced tissues were incubated at 15±1°C in the dark, with constant gentle shaking for 3 h. Following this, the supernatant was discarded and replaced with fresh L-15 medium. The tissue was then allowed to incubate for a further hour, the medium removed, stored at -80°C and subsequently analyzed for 11-KT in order to determine basal 11-KT secretion rates.
3.3.4 Determination of optimum stimulating or augmenting agent concentrations

The stimulating agents used in these experiments were hCG and dbcAMP, which activate the rate-limiting steps in steroid synthesis (Laskey and Phelps 1991, Mgbonyebi et al. 1994b). 25-OHC was used to augment unstimulated steroid secretion by entering the synthetic pathway distal to the rate-limiting steps controlled by gonadotropin (Laskey and Phelps 1991, Mgbonyebi et al. 1994a) (Fig. 3.1). When 11-KT secretion had reached basal levels (1-h incubation period), fresh L-15 medium was added and tissues were incubated for a 2-h period. The medium was again removed and replaced with either L-15 medium (control) or L-15 containing varying concentrations of stimulating or augmenting agents (1, 10 or 100 IU/mL hCG, 0.05, 0.5 or 5 mM dbcAMP and 0.05, 0.5 or 1.25 mM 25-OHC) and incubated for 18 h at 15±1°C in the dark, with constant gentle shaking (Schulz and Blüm 1990). The medium was subsequently removed and frozen at -80°C for further analysis. The concentration of agent which produced the maximum level of 11-KT secretion was used in subsequent experiments.

3.3.5 Cd exposure

Testicular tissues were prepared as described previously, and when basal 11-KT secretion levels were reached (1 h), medium was replaced with that containing CdCl₂ at concentrations of 0, 1, 10 or 100 μM. After a 2-h incubation period, medium was removed and stored at -80°C for 11-KT concentration determinations. In separate experiments, L-15 medium containing stimulant/augmenting agents alone or in combination with CdCl₂ (0, 1, 10 or 100 μM Cd) was added to tissues which were incubated for a further 18 h. The medium was then removed and stored at -80°C until analysis.
3.3.6 Cell viability

Cell viability of tissue following collagenase digestion (Louir 1999) was determined using Trypan Blue exclusion for each preparation in control tissue and that exposed to Cd and stimulant/augmenting agents. Type IV collagenase (2.5 mg/mL in L-15 medium) was added and the tissues allowed to incubate in 1mL of this solution for 1 h at 15±1°C with a constant gentle shaking. Cells were disaggregated by manual agitation and aliquots of cell suspension (50 μL) were added to a 0.4% Trypan Blue solution (50 μL). Cells were counted using a hemocytometer under light microscopy.

3.3.7 11-KT determination

Tissue suspension medium was centrifuged at 16,000 x g for 4 min to remove any excess testicular cells. Sub-samples of 50 μL of supernatants were assayed for 11-KT concentration using a commercially available EIA kit (Cayman Chemical Company, Ann Arbor, MI). Samples were checked for interference according to the manufacturer: two test samples were diluted to obtain two different concentrations. Final 11 KT concentrations were calculated and correlation between them was 12.7%, therefore purification was not required (correlations above 20% require purification). Each 96-well steroid antibody coated plate was run with a standard curve based on a serial dilution of a stock solution (10 ng/mL) provided with the kit with a reading taken at 412 nm. Intra-assay and inter-assay coefficients of variation were 3.1% and 1.2% respectively (n=5). All measures were performed on a Spectra Max 420 microplate reader (Molecular Devices Corporation, Sunnyvale, CA).
3.3.8 Statistical analysis

Statistical analyses were performed using JMP IN 4.0.3 (SAS Institute Inc., Cary, NC). Hormone concentrations and cell viability values were treated as a single-factor randomized complete block design and compared using ANOVA and Tukey-Kramer HSD tests for most of the experiments, except for the Cd-only exposures for which a two-factor (time and concentration) randomized complete block design was used. Differences were considered significant at p≤0.05. Some of the data required natural logarithm transformation in order to meet the assumption of homogeneity of variance, although non-transformed data are shown in the figures. Results are expressed as means ± SEM.

3.4 Results

3.4.1 Cell Viability

Cell viability in testicular tissues exposed to the stimulant/augmenting agents ranged from 88.2 ± 1.5 to 90.2 ± 1.3% (F = 0.3, d.f. = 3, p = 0.8379) (Table 3.1). Similarly, viabilities for tissue in the presence of CdCl₂ or both CdCl₂ and agents were also high, ranging from 79.7 ± 4.4 to 88.0 ± 1.2% with no significant difference between treatment groups (F = 1.8, d.f. = 15, p = 0.0574).

3.4.2 hCG, 25-OHG and dbcAMP effects on 11-KT secretion

Prior to Cd exposure, 11-KT secretion by testicular tissue was characterized in response to either hCG, dbcAMP stimulation or 25-OHC augmentation (Table 3.2). Baseline 11-KT production in testicular tissue incubated for 18 h in the absence of any agent was 16.3 ± 1.3 pg/mg wet tissue. The effects of hCG and dbcAMP-stimulation or 25-OHC-augmentation on 11-KT production expressed as a percent of baseline controls
is shown in Figure 3.2. The highest 11-KT concentrations produced (significantly different from controls) were with 100 IU/mL hCG (F=49.9, d.f.=3, p<0.0001), 5mM dbcAMP (F = 25.4, d.f.= 3, p<0.0001) and 1.25 mM 25-OHC (F = 105.6, d.f. = 3, p<0.0001).

3.4.3 Cd effects on unstimulated 11-KT secretion

11-KT secretion was 29.3 ± 10.3 and 28.2 ± 6.2% higher than controls in tissues exposed to 10 and 100 µM Cd, respectively, following a 2-h exposure (Fig. 3.2). After an 18-h incubation period, 11-KT secretion from testicular tissue was significantly lower than controls at all Cd concentrations (54.0 ± 8.9%, 61.7 ± 7.5% and 54.0 ± 4.6% of controls for 1, 10 and 100 µMCd respectively) (F = 14.9, d.f. = 3, p < 0.0001). Measured 11-KT concentrations are summarized on Table 3.3.

3.4.4 Cd effects on stimulated/augmented 11-KT secretion

hCG and dbcAMP-stimulated and 25-OHC-augmented 11-KT secretion from testicular tissue exposed to varying concentrations of Cd for 18 h are shown in Figure 3.3 and Table 3.4. With hCG stimulation, 11-KT secretion from tissues exposed to 1 and 10 µM Cd was significantly lower than controls (53.8 ± 10.8 and 61.9 ± 8.7 % of controls respectively); no difference was observed between tissues exposed to 100µM Cd and controls (F = 7.8, d.f. = 3, p = 0.0038). Although not significantly different from controls, 11-KT secretion in dbcAMP-stimulated tissue were 104.2 ± 9.7, 111.7 ± 10.3 and 80.7 ± 7.0% of controls after exposure to 1, 10 and 100 µM Cd, respectively (F = 3.7, d.f. = 3, p = 0.0396). 11-KT secretion in 25-OHC-augmented tissues were also not significantly affected by Cd exposure with values of 144.3 ± 17.6, 133.9 ± 9.8 and 89.6 ±
7.0% of control values following exposure to 1, 10 and 100 μM Cd, respectively (F = 7.2, d.f. = 3, p = 0.0051).

3.5 Discussion

Fish testicular tissue was exposed to Cd alone or in combination with various agents that stimulate or augment 11-KT biosynthesis (Fig 3.1), with the intention of increasing our knowledge regarding putative site(s) of action of this metal. hCG was used to test the integrity of the gonadotropin membrane receptor of testicular cells (Laskey and Phelps 1991). Although hCG is a mammalian hormone, it has been successfully used in in vitro fish studies to stimulate androgen production (Evanson and Van der Kraak 2001, Leatherland et al. 2003). The primary route by which steroidogenesis is mediated by GtH in the teleost testis is via the cAMP/protein kinase A signal transduction pathway (Wade and Van der Kraak 1991, Sakai et al. 1996). dbcAMP was therefore used to determine if Cd was acting at sites downstream from the membrane receptors (Laskey and Phelps 1991). 25-OHC passes through mitochondrial membranes without need for a membrane transporter (Leusch and MacLatchy 2003), is readily converted to steroid by cytochrome P450 side chain cleavage (P450scc) and subsequently to 11-KT. This cholesterol substrate derivative was expected to increase unstimulated 11-KT synthesis and was used to test for the integrity of the steroidogenic pathway after mitochondrial translocation (Evanson and Van der Kraak 2001, Leusch and MacLatchy 2003). To the author’s knowledge, this is the first study to demonstrate that Cd acts at multiple sites on fish testicular steroidogenic pathway.

Prior to Cd exposure, the response of the minced tissue to the different stimulants/augmentor was characterized with the intention to demonstrate that after the
manual disruption this preparation remained highly viable and responsive to these various agents. Cell viability was very high (more than 80%) after incubation with hCG, 25 OHC and dbcAMP alone, and all preparations were capable of producing 11-KT. The concentration of hCG that induced a maximal steroidogenic response (100 IU/mL) was similar to that determined for inducing ovarian follicle steroidogenesis in rainbow trout (Haddy and Pankhurst 1998). The dbcAMP concentration used in our study was approximately 50% of the concentration used to obtain maximal 11-KT secretion in testicular fragments of amago salmon, Oncorhynchus rhodurus (Sakai et al. 1996). In contrast, the concentration of 25-OHC that induced maximal steroidogenic response in our study was 100 times the dosage used to stimulate steroid production in goldfish ovarian follicles and Oryzias latipes testicular cells (Srivastava and Van der Kraak 1994). In vitro sensitivity to exogenous hormones is species/organ-specific, with genetic and biochemical factors playing a role in different responses, as observed in vivo during endocrine manipulations of spawning in cultured fish (Zohar and Mylonas 2001). Despite different in vitro sensitivity when compared with other cellular preparations, it was demonstrated that our minced tissue remained responsive to the different stimulators/augmentor and could be used in the following experiments.

Cell viability in testicular tissues exposed to Cd, the various stimulating agents, or both was high, suggesting no cytotoxic effects leading to cell death with the concentrations used. Laskey and Phelps (1991) also demonstrated no effects on viability in rat Leydig cells exposed to concentrations as high as 5 mM Cd. In addition, 25-50 μM Cd were not cytotoxic to R2C rat Leydig cell lines (Shiraishi et al. 1995). On the contrary, Yang et al. (2003) demonstrated a concentration-response relationship between
decline in cell viability and increased Cd concentration in rat Leydig cells after exposure to 10, 20 and 40 μM Cd. Despite the conflicting data regarding rat Leydig cell viability after Cd exposure, trout testicular cells appear less sensitive to Cd than rat cells.

The response of testicular tissue to Cd indicated that its effects on 11-KT secretion were both time and concentration-dependent. Following a 2-h exposure to Cd, 11-KT secretion levels significantly increased at the two highest concentrations, however, following an 18-h incubation period, tissues secreted significantly lower concentrations of 11-KT compared to controls. Similar contradictory trends have been reported in the literature with respect to short-term vs. long-term exposures. For example, 11-KT and testosterone production was significantly increased in rainbow trout testes *in vitro* following a 2-h exposure to concentrations of Cd between 50 and 500 μM (Kime 1984). Sangalang and O’Halloran (1973) observed lower 11-KT production in brook trout midtestes when fish were exposed to Cd in water for 24 h or when the testicular tissue was exposed to Cd for 4.5 h *in vitro*. Similarly, when rat Leydig cells were exposed to Cd for 24 h, significant decreases in testosterone production occurred (Yang et al. 2003). Laskey and Phelps (1991) postulated that Cd may act at multiple sites in rat Leydig cells, encompassing both stimulatory and inhibitory sites, a hypothesis that would explain the results of the present study. These authors have indicated that Cd inhibitory sites are likely to exist in the steroidogenic pathway proximal to cholesterol transport, and that stimulatory sites are likely in the cholesterol translocation steps or distal to that event. Moreover, Smida et al. (2004) reported a dual action of Cd on stable porcine granulosa cells: low concentrations activated and high concentrations inhibited expression of P450 scc gene and progesterone synthesis. The actions of Cd on steroid biosynthesis may be
complex, with multiple actions including those on plasma membranes (Nishiyama et al. 1985, Mgbonyebi et al. 1994b), adenyl cyclase (Mgbonyebi et al. 1994b) or endogenous mitochondrial cholesterol utilization through affects on calcium ion interactions, membrane proteins (e.g. P450scc) or lipids (Mgbonyebi et al. 1994a).

Synthesis of 11-KT involves the production of hormones by the hypothalamus, pituitary and testicular tissue (Fig 3.1). GTII, the major secretagogue for 11-KT in fish, binds to the plasma membrane of gonadal cells (hCG, a mammalian gonadotropin, elicits similar biological responses in mammals and fish, stimulating gonadal steroidogenesis [Evanson and Van der Kraak 2001, Schulz et al. 2001]). Ligand-receptor binding activates G protein, which in turn activates adenylate cyclase activity. Adenylate cyclase increases intracellular cAMP levels (Sakai et al., 1996), which stimulates cAMP-dependent protein kinase (PKA) activity required for steroid synthesis. The site of action of PKA is in the rate limiting step in steroid synthesis, namely the mitochondrial cholesterol translocation process (Planas 1997, Evanson and Van der Kraak 2001). After transport of cholesterol into the mitochondria, P450scc (activated by cAMP) converts cholesterol to pregnenolone which then leaves the mitochondrion and is subsequently converted to 11-KT (Gilman et al. 2003).

In the present study, hCG-stimulated 11-KT production was not maintained when tissues were exposed to 1 and 10 μM Cd. However, dbcAMP-stimulated and 25-OHC-augmented tissues maintained 11-KT production at control levels at all concentrations of Cd. Therefore, the steroid synthetic pathway was probably not affected after cAMP production. Similar results were observed in rats: dbcAMP-stimulated adrenal cells were also insensitive to Cd and steroid secretion was maintained at control levels following an
*in vitro* exposure (Mgbonyebi et al. 1994a). However, Laskey and Phelps (1991) reported that dbcAMP and hCG failed to restore testosterone secretion after exposure to high concentrations of Cd (1000 and 5000 μM) *in vitro* using rat Leydig cells, although no significant differences were observed for 1, 10 and 100 μM Cd exposure groups. Kawai et al. (2002) also reported that 8-bromo-cAMP did not reverse the decline in progesterone secretion by cultured human trophoblasts exposed to 20 μM Cd. It is conceivable that Cd effects are concentration-dependent and that higher Cd concentrations may affect additional sites of the pathway. However comparisons with our results are difficult since we have used a different range of Cd concentrations (lower concentrations than Laskey and Phelps 1991 and Kawai et al. 2002). On the other hand, our results do indicate that the Cd concentrations used affect the 11-KT biosynthetic pathway at or just subsequent to the ligand-receptor interaction and before cAMP production. Mgbonyebi et al. (1994b) have suggested that Cd interferes with cell regulation of adenylate cyclase-directed steroidogenesis in adrenal rat cells but has little effect on exogenously added dbcAMP-stimulated steroid secretion.

Interestingly, hCG stimulated 11-KT secretion to control levels when tissues were exposed to the highest concentration of Cd. Bisson and Hontela (2002) observed a similar phenomenon in trout interrenal cells exposed to atrazine, a commonly used herbicide: ACTH-stimulated cortisol production was below controls in cells exposed to low concentrations of atrazine but were above controls in cells exposed to the highest concentration. Both results suggest that the the membrane receptor was not negatively affected since hCG and ACTH could stimulate cellular secretion at the highest concentration used. Again, our results indicate that one affected site is after the
membrane receptor, probably at the G protein/adenyl cyclase complex as also suggested by Mgbonyebi et al. (1994b) in rat adrenal cells. It is possible that the complex is the site of both inhibitory and stimulatory effects. For example, studies have shown stimulation of adenylate cyclase systems by divalent cations such as Ca\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\), through a metal ion binding site that is independent of the catalytic site (Londos and Preston 1977, Mahaffee and Ontjes 1980). It is conceivable that Cd\(^{2+}\) can similarly stimulate adenylate cyclase at certain concentrations. In fact, several isoforms of adenyl cyclase co-exist in fish tissues (Wang et al. 2003) as described for mammalian testes (Simonds 1999). In mammals, nine isoforms of adenylate cyclase occur in different concentrations in various organs and are regulated by the different subunits of G protein, protein kinases, calmodulin or Ca\(^{2+}\), being stimulated or inhibited depending on the isoform (Simonds 1999). The effects of Cd exposure would depend on the presence of various isoforms, concentrations of the metal and the presence of modulators. Laskey and Phelps (1991) suggest that testosterone production after metal exposure is a sum of both inhibitory and stimulatory actions. Further investigations are necessary to determine if similarities with mammalian models exist.

The results of this study demonstrate that Cd exposure can both stimulate and inhibit testicular steroidogenesis in fish. Cd increases the steroidogenic biosynthetic capacity of testicular tissue in short term exposures, but decreases 11-KT secretion in long-term exposures, without affecting cell viability. These long-term inhibitory effects were restored by supplementation with dbcAMP and 25-OHC but not in hCG-stimulated cells at low Cd concentrations, indicating that an inhibitory site of action exists in the signaling steroidogenic pathway prior to cAMP formation. This impairment was reversed
at higher concentrations of Cd in hCG-stimulated cells, suggesting no impairment of the gonadotropin-receptor and the possible presence of a stimulatory site at or after hCG receptor binding. Considering the very low concentrations of the metal used in this experiment, the characterization of testicular tissue responses to the different stimulators and augmentor used, and the high viability of cells, this in vitro preparation may be viewed as an alternative fish model for sublethal investigations on Cd-impaired steroidogenesis in fish tissue.
3.6 Figures

Figure 3.1 Initial steps of the 11-KT biosynthetic pathway (until pregnenolone formation). Large arrows indicate the points of manipulation in our in vitro study. Based on Sakai et al. 1996, Planas 1997, Bauer et al. 2000, Evason and Van der Kraak 2001 and Gilman et al. 2003.
Figure 3.2 *In vitro* 11-KT secretion (% of control—no Cd) of trout testicular tissue exposed to 0, 1, 10 and 100 µM Cd for 2 (○) and 18h (●). Treatments with similar letters were not significantly different from each other at p < 0.05. n = 4. Values are means ± SEM.
Figure 3.3 *In vitro* 11-KT secretion (% of control—no stimulant) of trout testicular tissue stimulated with 0, 1, 10 or 100 IU/mL hCG (A); 0, 0.05, 0.5 or 5 mM dbcAMP (B) and 0, 0.05, 0.5 or 1.25 mM 25-OHC (C). Treatments with similar letters were not significantly different from each other at $p < 0.05$. $n = 4-6$. Values are means ± SEM. Shading represents the concentrations used in subsequent experiments.
Figure 3.4 *In vitro* 11-KT secretion (% of control—no Cd + stimulant) of trout testicular tissue exposed to 0, 1, 10 and 100 µM Cd for 18h and stimulated with A) 100 IU/mL hCG; B) 5 mM dbcAMP or C) 1.25 mM 25-OHC. Treatments with similar letters were not significantly different from each other at \( p < 0.05 \). \( n=4 \). Values are means ±SEM.
Table 3.1 Viability (%) of trout testicular cells exposed to medium (controls) or exposed to hCG, 25-OHC or dbcAMP for 18 hours alone or in presence of Cd (1, 10 or 100 μM). a: n=6-11, b: n=4.

<table>
<thead>
<tr>
<th>Agent</th>
<th>0 μM Cd&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1 μM Cd&lt;sup&gt;b&lt;/sup&gt;</th>
<th>10 μM Cd&lt;sup&gt;b&lt;/sup&gt;</th>
<th>100 μM Cd&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>medium</td>
<td>88.4 ± 2.0</td>
<td>84.6 ± 1.2</td>
<td>82.2 ± 1.4</td>
<td>80.0 ± 1.8</td>
</tr>
<tr>
<td>hCG</td>
<td>90.1 ± 1.3</td>
<td>88.0 ± 1.2</td>
<td>85.7 ± 2.6</td>
<td>86.9 ± 2.9</td>
</tr>
<tr>
<td>25-OHC</td>
<td>88.2 ± 1.5</td>
<td>83.4 ± 1.5</td>
<td>83.7 ± 1.7</td>
<td>80.3 ± 2.6</td>
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<tr>
<td>dbcAMP</td>
<td>89.2 ± 2.8</td>
<td>87.3 ± 1.9</td>
<td>80.8 ± 1.8</td>
<td>79.7 ± 4.4</td>
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</tbody>
</table>
Table 3.2 *In vitro* 11-KT secretion (ng/g wet tissue) of trout testicular tissue exposed to medium (controls - no agent) or three different concentrations of hCG, 25-OHC or dbcAMP for 2h. n=4-6. a: C1, C2 and C3=1, 10 and 100 IU/mL respectively, b: C1, C2 and C3= 0.05, 0.5 and 1.25 mM respectively, c: C1, C2 and C3= 0.05, 0.5 and 5 mM respectively.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Control</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCG(^a)</td>
<td>16.9 ± 3.0</td>
<td>12.9 ± 3.5</td>
<td>14.6 ± 2.7</td>
<td>111.5 ± 21.8</td>
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<tr>
<td>25-OHC(^b)</td>
<td>15.6 ± 3.4</td>
<td>18.2 ± 4.9</td>
<td>31.6 ± 1.8</td>
<td>104.6 ± 39.3</td>
</tr>
<tr>
<td>dbcAMP(^c)</td>
<td>15.6 ± 3.0</td>
<td>20.8 ± 9.6</td>
<td>21.9 ± 2.6</td>
<td>179.0 ± 27.7</td>
</tr>
</tbody>
</table>
Table 3.3 *In vitro* 11-KT secretion (ng/g wet tissue) of trout testicular tissue exposed to medium (controls – no Cd) or 1, 10, 100 μM Cd for 2h or 18h. n=4.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0 μM Cd</th>
<th>1 μM Cd</th>
<th>10 μM Cd</th>
<th>100 μM Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>4.7 ± 1.1</td>
<td>4.4 ± 1.0</td>
<td>6.3 ± 1.7</td>
<td>6.1 ± 1.6</td>
</tr>
<tr>
<td>18</td>
<td>19.2 ± 2.6</td>
<td>10.9 ± 2.5</td>
<td>12.2 ± 2.7</td>
<td>10.1 ± 1.0</td>
</tr>
</tbody>
</table>
Table 3.4 *In vitro* 11-KT secretion (ng/g wet tissue) of trout testicular tissue exposed to 0, 1, 10 or 100 µM Cd and stimulated/augmented with 100 IU/mL hCG, 5mM dbcAMP, or 1.25 mM 25-OHC for 18h. n=4.

<table>
<thead>
<tr>
<th>Agent</th>
<th>0 µM Cd</th>
<th>1 µM Cd</th>
<th>10 µM Cd</th>
<th>100 µM Cd</th>
</tr>
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<tbody>
<tr>
<td>hCG</td>
<td>140.6 ± 52.1</td>
<td>87.0 ± 45.7</td>
<td>97.1 ± 40.1</td>
<td>119.4 ± 49.7</td>
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<tr>
<td>25-OHC</td>
<td>79.0 ± 3.0</td>
<td>102.6 ± 39.5</td>
<td>103.2 ± 47.2</td>
<td>78.7 ± 38.4</td>
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<tr>
<td>dbcAMP</td>
<td>151.4 ± 47</td>
<td>155.5 ± 55.8</td>
<td>166.0 ± 60.7</td>
<td>119.2 ± 53.6</td>
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3.7 References


Sangalang GB, O'Halloran MJ (1973) Adverse effects of cadmium on brook trout testis and on *in vitro* testicular androgen synthesis. Biol Reprod 9:394-403


CHAPTER 4
IN VIVO EFFECTS OF SUB-CHRONIC EXPOSURE TO CADMIUM CHLORIDE ON THE STRESS RESPONSE OF RAINBOW TROUT, ONCORHYNCHUS MYKISS

4.1 Abstract

The physiological response to stressors can be disrupted in wildlife species, including teleosts, by contaminants such as metals in the environment. Acute cadmium (Cd) exposure can illicit a generalized stress response in the short-term, although contradictory response is seen in sub-chronic and chronic studies. In the present study, the effects of CdCl₂ on plasma cortisol, glucose, lactate and liver glycogen in rainbow trout were examined. Fish were exposed to waterborne Cd at 0, 0.05, 0.25 and 0.50 µg/L for 7 to 40 days. Exposure resulted in a decrease in plasma cortisol concentration at all Cd concentrations examined from 7 to 40 days with values ranging from 7.7 ± 3.6 to 57.3 ± 10.8 % of controls, but not in a concentration-dependent manner. Fish exposed to Cd for 40 days were subjected to ACTH or handling stress challenge to examine interrenal impairment. Cd-exposed fish responded similarly to ACTH administration, increasing 10 to 30-fold, and to a handling stress, increasing 2 to 3-fold compared to controls, as measured by plasma cortisol concentrations; indicating no interrenal impairment. The results of this study indicate that environmentally realistic concentrations of Cd persistently decrease plasma cortisol levels after sub-chronic exposure without interrenal impairment.
4.2 Introduction

The physiological response to intrinsic or extrinsic stimuli that disturb homeostasis (stressors), evolved as a compensatory and/or adaptive process which enables animals to readjust their biological activities to overcome threats or interferences to the dynamic equilibrium of the organism (Wenderlaar Bonga 1997).

In general, the stress response in fish is similar to that of mammals with three levels of response: primary, secondary and tertiary (Wenderlaar Bonga 1997, Iwama 1998, Van Ham et al. 2003, Huisng et al. 2005). Primary responses include the activation of brain centers resulting in the release of stress hormones, catecholamines and corticosteroids by interrenal tissue; secondary responses encompass effects of these hormones at blood and tissue levels; and tertiary responses, such as reduced growth and changes in metabolic rates, occur at the level of whole organism and/or population (Barton and Iwama 1991, Iwama 1998, Iwama et al. 1999, Huisng et al. 2005).

During the primary response, catecholamine hormones (CAs), predominantly epinephrine in teleosts, are released rapidly following stressor stimuli by chromaffin cells (Barton 2002). CAs are associated with immediate reactions to stressors including increases in cardiac output, ventilation and oxygen uptake, and hyperglycemia (Wenderlaar Bonga 1997, Barton 2002). Cortisol, the major corticosteroid in teleosts, is released following CA release and is frequently associated with chronic stress (Barton and Iwama 1991). The messenger synthesized by the brain (corticotropin-releasing hormone) acts on the pituitary, controlling the synthesis and release of pituitary hormones including adrenocorticotrophic hormone (ACTH), which is the main stimulant of cortisol secretion by interrenal cells in fish (Norris 1996, Hontela 1998, Barton 2002). Most fish
species show their highest cortisol plasma increase within 0.5 to 1h after a stressful disturbance (Barton and Iwama 1991). Elevation of cortisol is important for central nervous system activation, increasing blood glucose concentration, and elevating mean blood pressure, all of which are important for coping with stress (Bamberger et al. 1996, Vijayan et al. 1997b, Mommsen et al. 1999, Ackerman et al. 2000). It is generally agreed that important metabolic roles of cortisol during stress include glucose-regulation and glycogen-repletion processes, both of which are important pathways for the recovery from stress. Cortisol may also play a role in the peripheral mobilization of substrates, lactate particularly, providing precursors for hepatic gluconeogenesis (Vijayan et al. 1997b, Mommsen et al. 1999).

Besides its importance in coping with stressors, the two major actions of cortisol are in the regulation of hydromineral balance and normal energy metabolism. Cortisol has stimulatory effects on branchial Na⁺ and Cl⁻ extrusion and uptake (depending on the osmotic habitat) (Dange 1986); and on ion-transporting enzymes activity such as Na⁺/K⁺-ATPase (Wenderlaar Bonga 1997, Shrimpton and McCormick 1999). Under resting conditions, cortisol may be responsible for sustaining plasma normoglycemia as observed in mammals (Mommsen et al. 1999). Elevations of cortisol concentrations during chronic stress may interfere with osmotic and ionic balance by decreasing gill corticosteroid receptor concentration and thus decreasing Na⁺/K⁺-ATPase activity (Shrimpton and McCormick 1999).

Environmental contaminants including metals have been shown to interfere with the organismal stress response in fish. Metals may increase plasma cortisol concentrations in laboratory studies, although alterations in plasma glucose and lactate,
and liver glycogen have not always been observed (Bleau et al. 1996, Ricard et al. 1998, Dethloff et al. 1999). Among metals, cadmium (Cd) has been shown to alter plasma cortisol concentrations in fish, even at environmentally-realistic concentrations (≤ 10 μg/L Cd - Canadian Environmental Protection Act 1994). Cd exposure at those low concentrations can increase plasma cortisol concentrations in the short-term. Cortisol levels return to baseline values that are persistent, even after continuous subchronic exposure (Pratap and Wendelaar Bonga 1990, Fu et al. 1990). During subchronic Cd exposure (30 days), a secondary rise of plasma cortisol concentrations and an increased responsiveness of interrenal tissue to ACTH can occur (Brodeur et al. 1998). In addition, plasma glucose, lactate and liver glycogen have been reported as increasing, decreasing or not being affected at all after Cd exposure, data which does not provide conclusive evidence regarding stress metabolic responses associated with Cd (Lowe-Jinde and Niimi 1984, Fu et al. 1990, Pratap and Wendelaar Bonga 1990, Ricard et al. 1998, De Smet and Blust 2001, Cicik and Engin 2005).

Although results to date are inconsistent, it is clear that Cd interferes with the organismal stress response in fish after acute and sub-chronic exposure. Field studies suggest that its effects in the long-term are, however more consistent. Several teleost species from sites contaminated by mixtures of PAH, PCBs, bleached kraft mill effluent (BKME) and heavy metals exhibit an impaired interrenal response to additional stress, although the direct involvement of Cd as a causative agent cannot be established due to the presence of other pollutants (Hontela et al. 1995, Brodeur et al. 1997, Girardi et al. 1998, Laflamme et al. 2000, Levesque et al. 2003, Gravel et al. 2005). Observations that the impairment of cortisol secretion detected at field sites contaminated by metals is only
evident in adult fish four years and older support the long-term exposure hypothesis (Brodeur et al. 1997). The effects of Cd on several biochemical parameters influenced by cortisol such as plasma glucose and liver glycogen are inconsistent in those field studies (Hontela et al. 1995, Girard et al. 1998, Laflamme et al. 2000, Kakuta 2002, Levesque et al. 2002, Gravel et al. 2005).

The purpose of this study was to investigate the effects of environmentally-realistic concentrations of Cd on the organismal stress response of rainbow trout. The specific objectives of the present study were: (i) to determine the dynamics of plasma cortisol concentrations during long-term low-level Cd exposure, (ii) to determine the extent of interrenal impairment by activating the HPI axis by ACTH and through a handling stress, and (iii) to measure secondary biochemical effects during Cd exposure (plasma glucose, lactate, and liver glycogen).

4.3 Materials and methods

4.3.1 Fish

Immature rainbow trout, Oncorhynchus mykiss (68.40 ± 0.77 g) were obtained from Sun Valley Trout Farms (Mission, BC) and acclimated for at least 4 wk in 1200 L tanks prior to experimentation. Tanks were supplied with dechlorinated municipal water (Greater Vancouver Regional District), at 8 ± 2 ºC, photoperiod of 12h light:12 h dark, hardness 6.8 mg CaCO₃/L, dissolved oxygen concentration > 90% and pH 6.8.

4.3.2 Chemicals

Cadmium chloride (CdCl₂), porcine adrenocorticotropic hormone (ACTH) and NaHCO₃ were purchased from Sigma Chemical Co. (St. Louis, MO). Tricaine
methanesulfonate (MS222) and nitric acid (environmental grade plus) were obtained from Argent Laboratories (Redmond, WA) and Anachemia Science (Vancouver, BC) respectively.

### 4.3.3 Exposure and sampling

Fish were randomly divided into four groups, each consisting of three replicate tanks. Fish were exposed to Cd as CdCl₂ (0, 0.05, 0.25 and 0.50 µg/L) in flow-through tanks (130 L). The lowest concentrations used (0.05 and 0.25 µg/L Cd) are below the repeated lowest-observed-effect-concentration (LOEL: 0.47 µg/L) for freshwater fish in soft water (Canadian Environmental Protection Act 1994). Water flow in each tank was 0.9 L/min and Cd was delivered using Mariotte bottles (Hontela et al. 1996). Fish were fed *ad libitum* every other day with commercial trout food (3mm pellets - Ewos Canada Ltd., Surrey, BC) until 24h before sampling. Excess food and all the organic material in the tanks were removed daily by careful forced tank drainage. A small net was used when necessary with minimal disturbance to fish.

Fish were exposed to Cd up to 40 days and were sampled on days 7, 14, 21, 28 and 40. Fish were quickly removed and euthanized by immersion in buffered MS-222 (100 mg/L). Blood samples (1 mL) were taken by caudal puncture, centrifuged for 4 min at 10,000 x g and hematocrit was determined. Plasma was frozen at -80 °C until analysis. Livers were dissected and immediately frozen at -80 °C for further analysis.

In a separated set of experiments, fish were exposed to Cd for 40 days and were sampled as described above, following one of three treatment groups: 1) baseline controls; 2) intraperitoneal ACTH administration [4 IU/100 g – approximately 0.3 mL]
total volume. ACTH was diluted in saline (10 IU/mL) immediately prior administration] according to the procedure of Girard et al. (1998) and sampled 2 h later and 3) subjected to a 3-min handling stress, according to the procedure of Wilson et al. (1998), and immediately sampled as above.

4.3.4 Analysis

Plasma was analyzed for glucose and lactate concentrations using enzymatic colorimetric assays (Sigma-Aldrich Canada, Oakville, Ontario). Glucose and lactate were assayed using 2.5 μL of plasma sample with 250 μL of reagent, with a reading taken at 520 and 340 nm respectively. Assays were run in 300 μL Falcon flat-bottom 96-well microplates (BD Biosciences, Oakville, ON). Each plate was run with three different standard solutions in duplicate (Sigma-Aldrich Canada, Oakville, ON). Intra- and inter-assay coefficients of variations (CV) for glucose were 3.2% and 3.7% respectively (n=5). Inter-and intra-assay CV for lactate were 3.6% and 6.1% respectively (n=4). Sub-samples of 50 μL were used for cortisol analysis. Cortisol was assayed using an ELISA kit (Neogen Corporation, Lexington, KY). Each 96-well cortisol antibody coated plate was run with a standard curve based on a serial dilution of 1 μg/mL stock solution (provided with the kit) with a reading taken at 650 nm. Intra-assay and inter-assay CV were 4.4% and 6.7% respectively (n=4). All measures were performed on a Spectra Max 420 microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Liver glycogen was measured according to a modified method of Sweeting (1989). Briefly, liver tissues were homogenized in citrate buffer (0.1 M citric acid, 0.1 M sodium citrate, 2.5 g/L sodium fluoride, pH 4.2), centrifuged at 3,000 x g for 12 min and an aliquot of the supernatant assayed for glucose. In another aliquot of the homogenized tissue,
amyloglucosidase (1 mg/mL) was added, incubated for 20 min at 55°C, recentrifuged at 3,000 x g for 5 min and the supernatant again assayed for glucose. Glycogen was determined from the difference between glucose values (glucose values before amyloglucosidase digestion subtracted from glucose values after digestion).

Water samples for metal analysis were collected in polyethylene bottles, preserved by acidification with concentrated nitric acid to pH < 2.0 and stored at 4°C until analysis. Aqueous Cd concentrations were measured using inductively coupled plasma - optical emission spectroscopy with Varian Vista PRO-axial view ICP-OES (detection limit 0.2 ng/mL Cd) by Cavendish Analytical Laboratories, Vancouver, BC. Since analytical confirmations were at least 80% of nominal values (0 = 0.0184 ± 0.0053 μg/L Cd, 0.05 = 0.0363 ± 0.0042 μg/L Cd, 0.25 = 0.2569 ± 0.0186 μg/L Cd and 0.50 = 0.4732 ± 0.0180 μg/L Cd), results are reported in terms of nominal concentrations (Tilton et al. 2003).

4.3.5 Statistical analysis

Statistical analyses were performed using JMP IN 4.0.3 (SAS Institute Inc., Cary, NC). The experimental data were analyzed by one-way or two-way ANOVA followed by Tukey-Kramer HSD test. Differences were considered significant at p ≤ 0.05. Some of the data required natural logarithm transformation in order to meet the assumption of homogeneity of variance, although non-transformed data are shown in figures. Results are expressed by means ± SEM. Since no significant differences between male and female fish were observed, data from both sexes were pooled.
4.4 Results

Cd exposure did not affect hematocrit, plasma glucose, lactate or liver glycogen concentrations over the course of the exposure period (Table 4.1) (hematocrit, $F = 1.41$, d.f. = 12, $p = 0.1683$; plasma glucose, $F = 2.06$, d.f. = 12, $p = 0.0249$; plasma lactate, $F = 1.23$, d.f. = 12, $p = 0.2749$; liver glycogen, $F = 2.11$, d.f. = 12, $p = 0.0243$).

Plasma cortisol concentrations in control fish ($22.09 \pm 3.61$ ng/mL) did not significantly change over the course of the exposure period. Plasma cortisol concentrations of Cd-exposed fish changed with time and Cd concentrations ($F = 7.56$, d.f. = 19, $p < 0.0001$), however, values were significantly lower than controls at all time points, for all the different Cd-exposed groups ($0.05 \mu g/L$ Cd, $F = 11.37$, d.f. = 9, $p < 0.0001$; $0.25 \mu g/L$ Cd, $F = 19.18$, d.f. = 9, $p < 0.0001$; $0.50 \mu g/L$Cd, $F = 11.68$, d.f. = 9, $p < 0.0001$) (Table 4.2). Plasma cortisol concentrations were $29.9 \pm 4.5\%$, $30.4 \pm 4.6\%$ and $26.6 \pm 5.5\%$ of controls for the 0.05, 0.25 and 0.50 $\mu g/L$ Cd groups, respectively, after 40 days of exposure (Fig 4.1).

An injection of ACTH and a handling stress resulted in significantly higher plasma cortisol levels in both control fish and those exposed to Cd compared to unexposed and unstressed fish (ACTH, $F = 36.65$, d.f. = 4, $p < 0.0001$; handling stress, $F = 28.29$, d.f. = 4, $p < 0.0001$) (Fig 4.2, Table 4.3). No significant differences in response to either ACTH or a handling stress were seen between controls and Cd-exposed fish. ACTH administration resulted in significantly higher concentrations of plasma cortisol compared to handling stress ($F = 33.67$, d.f. = 11, $p < 0.0001$).
4.5 Discussion

The dynamics of plasma cortisol concentrations and the subsequent biochemical and physiological responses during long-term, low-level exposure to chemical stressors are still poorly understood in fish (Hontela 1998). In the present study, rainbow trout were exposed to environmentally realistic concentrations of Cd in vivo for 40 days, with the intention of increasing our knowledge regarding the effects of this metal on baseline stress parameters and the ability to mount a stress response. Our data shows that low Cd concentrations in soft water caused a persistent suppression in baseline plasma cortisol concentrations for the entire exposure period. This consistent depression was not accompanied by alterations in baseline hematocrit, plasma glucose, lactate or liver glycogen concentrations. Since exposed fish were able to respond to a handling stress and ACTH administration in a manner similar to control fish, it is likely that interrenal tissue was not affected by Cd at the concentrations used.

Acute elevations in plasma cortisol have been described as part of the general stress response to different stressors, including Cd, in fish (Wenderlaar Bonga 1997, Hontela 1998). This acute increase in plasma cortisol concentrations typically returns to baseline levels, although the reported time frame for the return varies between 7 and 35 days (Fu et al. 1990, Pratap and Wendelaar Bonga 1990, Brodeur et al. 1998). The specific mechanisms responsible for this return are not completely understood, however two main hypotheses have been postulated: acclimation or functional interrenal exhaustion (Fu et al. 1990, Brodeur et al. 1998, Hontela 1998). Fu et al. (1990) suggested that the gradual return of plasma cortisol concentrations to baseline levels after 35 days of exposure was part of a general adaptation response. This phenomenon coincided with a
return of plasma Ca\(^{2+}\), Na\(^{+}\) and glucose to near normal levels. Brodeur et al. (1998) reported a different pattern, with a return of plasma cortisol concentrations to baseline levels after 7 days of exposure and a second rise after 30 days. These authors also observed in fish exposed for 30 days an increased ACTH responsiveness of interrenal tissue \textit{in vitro}. It was suggested that the sustained cortisol secretion and the high interrenal activity may eventually lead to functional exhaustion, a hypothesis supported by observations of interrenal impairment in fish subjected to life-long exposure to heavy metals in the field (Brodeur et al. 1997, Hontela 1998).

The functional tests that challenged the HPI axis after 40 days of exposure showed that Cd-exposed fish responded in a similar fashion to controls. ACTH and handling stress were used to test if low plasma cortisol concentrations in fish exposed to Cd were due to a decreased interrenal capacity to generate cortisol during the stress response \textit{in vivo} (Girard et al. 1998, Laflamme et al. 2000). Lower plasma cortisol concentrations and decreased interrenal responsiveness to secondary stress have been reported in fish from contaminated sites (including Cd), when compared with fish from reference sites (Hontela et al. 1995, Brodeur et al. 1997, Laflamme et al. 2000, Nolan et al. 2003, Gravel et al. 2005). However, the low Cd concentrations used in our study do not appear to affect interrenal tissue, since ACTH and handling stress resulted in increase in plasma cortisol concentrations similar to control fish. Therefore, our results do not give support to the ‘functional exhaustion’ hypothesis postulated by Hontela (1998).

The mechanisms responsible for the persistently lower plasma cortisol concentrations in Cd-exposed fish are not known at present. However, it is conceivable that an increase in metabolism and clearance of cortisol could be involved. Induction of
Phase I and II hepatic enzymes and lower plasma sex steroid concentrations have been reported in fish exposed to PAH and metals (Johnson et al. 1988). In addition, Vijayan et al. (1997a) observed increased cortisol uptake and clearance in fish exposed to a PCB congener. Regardless the mechanisms involved on lowering baseline plasma cortisol concentrations, this effect may negatively impact fish normal functions, since cortisol participates in the regulation of hydromineral balance and energy metabolism under resting conditions (Dange 1986, Wenderlaar Bonga 1997, Shrimpton and McCormick 1999).

Waterborne Cd exposure did not alter hematocrit in the present study, results which are similar to other studies (Fu et al. 1990, Ricard et al. 1998, De Smet and Blust 2001). In addition, neither plasma glucose or lactate concentrations, or liver glycogen content was affected by Cd exposure at any of the concentrations used. Comparable results have been reported in laboratory and in field studies (Pratap and Wendelaar Bonga 1990, Ricard et al. 1998, De Smet and Blust 2001, Kakuta 2002). The baseline glucose, lactate and liver glycogen response in fish exposed to Cd may be a direct result of the low cortisol concentrations as suggested by Afonso et al. (2003) for female fish exposed to bleached kraft mill effluent. The authors postulated that the lack of cortisol response might be an adaptative mechanism to prevent excess energy utilization during periods of stress (observed at cellular level for the same fish that showed no other metabolic alterations in their study). Although the cellular stress response was not investigated in our study, a similar mechanism could be involved. However, the low circulating cortisol levels in exposed fish do not necessarily indicate absence of an organismal stress response. It is possible that plasma cortisol concentrations increased earlier in Cd-
exposed fish. In fact, Brodeur et al. (1998) have shown an acute increase in plasma cortisol concentrations after 2 days of Cd exposure and a subsequent decrease to baseline levels after 7 days of exposure.

It is possible that the chemical characteristics of the exposure water, particularly the very low hardness (6.8 CaCO$_3$/L), played an important role in the observed cortisol modulation. It is well established that Cd toxicity is dependent on water quality, with hardness, as calcium ions, an important modifying factor. Generally, as hardness decreases Cd toxicity increases (Hollis et al. 2000, US-EPA 2001, Hansen et al. 2002). Although hardness was not always reported, apparently none of the above Cd studies were conducted in highly soft water such as in the present study, where the protective effect of calcium ions may be much diminished. It could be speculated that the high Cd toxicity due to low hardness of exposure water possibly caused an intense acute cortisol response, and induced an adaptative mechanism to prevent excess energy utilization in long-term. Future acute studies are necessary to elucidate this point.

The results of this study demonstrated that environmentally-realistic Cd concentrations for up to 40 days persistently lowered resting plasma cortisol concentrations. The reduced plasma cortisol levels after sub-chronic Cd exposure were likely not associated with interrenal impairment. To the authors’ knowledge, this was the first time that negative effects were found at Cd concentrations below 0.47 $\mu$g/L, the lowest-observed-adverse-effect-concentration (LOAEL) for freshwater fish in soft water (Canadian Environmental Protection Act 1994). The observed results are particularly relevant in BC, where the guidelines for Cd are under review by the BC Ministry of
Environment. Our results may add important data in determining new aquatic guidelines values.
4.6 Figures

A)

B)

C)

Figure 4.1 Plasma cortisol concentrations of trout exposed to Cd: A) 0.05 µg/L; B) 0.25 µg/L and C) 0.50 µg/L, for 7, 14, 21, 28 and 40 days. Control values (-----) represent plasma cortisol concentrations in fish not exposed to Cd at each time point. Treatments with different letters were significantly different from each other at p < 0.05. All cortisol concentrations were significantly different from controls. n = 6 to 9. Values are means ± SEM.
Figure 4.2 Plasma cortisol concentrations for controls and trout exposed to Cd for 40 days and A) injected with ACTH or B) subjected to stress. Baseline values represent plasma cortisol concentrations in fish not exposed to Cd and not stressed or ACTH injected. Treatments with similar letters were not significantly different from each other at p < 0.05. n = 3 to 5. Values are means ± SEM.
4.7 Tables

Table 4.1 Hematocrit, plasma glucose and lactate, and liver glycogen in controls and trout exposed to Cd (0.05, 0.25 and 0.50 µg/L) for up to 40 days. n=25-45.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0 µg/L Cd</th>
<th>0.05 µg/L Cd</th>
<th>0.25 µg/L Cd</th>
<th>0.50 µg/L Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td>35.73 ± 0.96</td>
<td>33.91 ± 0.93</td>
<td>34.80 ± 0.82</td>
<td>34.05 ± 0.91</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>101.81 ± 5.89</td>
<td>108.21 ± 4.84</td>
<td>99.42 ± 6.32</td>
<td>103.01 ± 5.11</td>
</tr>
<tr>
<td>Lactate (mg/dL)</td>
<td>10.16 ± 0.94</td>
<td>10.09 ± 0.83</td>
<td>12.69 ± 0.92</td>
<td>12.88 ± 0.74</td>
</tr>
<tr>
<td>Liver glycogen (mg/g liver)</td>
<td>10.45 ± 1.97</td>
<td>15.60 ± 1.87</td>
<td>10.50 ± 2.42</td>
<td>11.81 ± 1.95</td>
</tr>
</tbody>
</table>
Table 4.2 Plasma cortisol concentrations (ng/mL) for controls and Cd exposed trout (0.05, 0.25 and 0.50 µg/L Cd) for 7, 14, 21, 28 and 40 days. n= 6 to 9.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>0 µg/L</th>
<th>0.05 µg/L</th>
<th>0.25 µg/L</th>
<th>0.50 µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>25.99 ± 5.77</td>
<td>7.27 ± 3.05</td>
<td>10.86 ± 2.64</td>
<td>6.00 ± 2.46</td>
</tr>
<tr>
<td>14</td>
<td>20.37 ± 7.24</td>
<td>4.57 ± 1.74</td>
<td>11.11 ± 2.05</td>
<td>9.31 ± 3.29</td>
</tr>
<tr>
<td>21</td>
<td>22.58 ± 8.06</td>
<td>2.60 ± 0.70</td>
<td>2.52 ± 0.95</td>
<td>3.25 ± 0.99</td>
</tr>
<tr>
<td>28</td>
<td>20.55 ± 9.13</td>
<td>6.67 ±1.68</td>
<td>1.58 ± 0.74</td>
<td>3.44 ± 1.51</td>
</tr>
<tr>
<td>40</td>
<td>21.32 ± 13.95</td>
<td>12.21 ± 2.30</td>
<td>6.72 ± 1.60</td>
<td>6.49 ± 4.25</td>
</tr>
</tbody>
</table>
Table 4.3 Plasma cortisol concentrations (ng/mL) in control and exposed trout (0.05, 0.25 and 0.50 μg/L Cd) for 40 days following handling stress or ACTH injection. n=3-5.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 μg/L Cd</th>
<th>0.05 μg/L Cd</th>
<th>0.25 μg/L Cd</th>
<th>0.50 μg/L Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH injection</td>
<td>237.71 ± 43.87</td>
<td>261.29 ± 28.66</td>
<td>195.08 ± 30.21</td>
<td>147.88 ± 37.40</td>
</tr>
<tr>
<td>Handling stress</td>
<td>49.93 ± 4.64</td>
<td>67.17 ± 6.96</td>
<td>41.65 ± 5.70</td>
<td>69.25 ± 1.30</td>
</tr>
</tbody>
</table>
4.8 References


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CHAPTER 5
BIOSYNTHETIC CAPACITY OF RAINBOW TROUT (ONCORHYNCHUS MYKISS) INTERRENAL TISSUE FOLLOWING CADMIUM CHLORIDE EXPOSURE

5.1 Abstract

The disruption of endocrine systems in wildlife species, including teleosts, by contaminants such as metals is presently of major environmental concern. Recently, it has been shown that cadmium (Cd) exposure can result in significant reductions in corticosteroid secretion by fish interrenal steroidogenic cells, likely through an inhibition of intracellular cortisol synthesis. In the present study, the effects of CdCl₂ on unstimulated and stimulated interrenal steroidogenesis were examined with the intention of furthering an understanding of the site(s) of Cd action. CdCl₂ alone reduced cortisol secretion in minced interrenal tissues to 58.9 ± 8.9 % and 55.0 ± 9.5 % of control values when exposed to 10 and 100 µM Cd, respectively. Incubation of interrenal tissues with 0.01 IU/mL adrenocorticotropic hormone (ACTH), which activates rate-limiting steps in steroid synthesis, resulted in significant stimulation of steroidogenesis in controls (1105 ± 22.7 %). ACTH-stimulated steroidogenesis was reduced to 15.3 ± 5.7 %, 20.4 ± 10.7 % and 11.4 ± 10.3% of controls when tissues were incubated with Cd with 1, 10 and 100 µM, respectively. Maximal rates of unstimulated cortisol secretion were achieved by augmentation with 5 µM 25-hydroxycholesterol (25-OHC) or 0.8 µL/mL SyntheChol NS0 supplement (SC). Steroidogenesis augmentation by 25-OHC was reduced by 43.1
± 7.2 %, 44.9 ± 5.3 % and 52.4 ± 6.7 % of control values when tissues were incubated with 1, 10 and 100 μM Cd, respectively. Interestingly, cortisol secretion was significantly higher in tissue exposed to 1 and 10 μM Cd when compared to control tissue augmented by SC. The results of this study show that Cd affects both stimulated and unstimulated steroidogenesis in teleosts, and that one major site(s) of inhibitory action of Cd on the cortisol synthesis pathway is likely between the steps of cAMP formation and prior to cytochrome P450 side chain cleavage.

5.2 Introduction

Cadmium (Cd) is a non-degradable cumulative pollutant in aquatic ecosystems that is primarily associated with sediments and suspended particles (May et al. 2001, Lyndersen et al. 2002). Mean Cd concentrations in surface waters are usually low, however, concentrations near industrial sites can be as high as 400 μg/L (Page 1981, Taylor et al. 1990, Thorton 1992). Cd bioavailability varies widely with water chemistry, and even at low water concentrations, toxicity can be substantial. Cd is acutely toxic to fish at low concentrations in soft (minimal calcium carbonate concentrations) or alkaline water (Lydersen et al. 2002). For example, mean 120-h LC50 values for bull and rainbow trout were 0.83 and 0.44 μg/L, respectively, at pH 7.5 and 30 mg/L CaCO3 (Hansen et al. 2002).

In most natural waters, heavy metals are usually present at sublethal concentrations (Meranger et al. 1979, Harrison 1986). Sublethal Cd toxicity is well documented and has been shown to negatively impact a number of biological processes including tissue metabolism (Vaglio and Landriscina 1999, Almeida et al. 2002), ion and

Cd is also a known endocrine disruptor in both mammals and teleosts. One primary target of disruption is the hypothalamus-pituitary-interrenal (HPI) axis which mediates corticosteroid release in fish. Cd is known to act centrally to disrupt steroidogenesis at the level of the pituitary (Tilton et al. 2003), but can also act directly on steroidogenic tissues including teleost interrenals. Cortisol (the major corticosteroid in teleosts) concentrations are regulated by the HPI axis (Hill and Fromm 1968, Schreck 1990). Adrenocorticotropic hormone (ACTH), a pituitary hormone, is the dominant secretagogue which stimulates the interrenal tissues to release cortisol (Schreck et al. 1989, Kloas et al. 1994, Iwama 1998). In mammals, glucocorticoids are vital for maintaining stress-related homeostasis, and elevated cortisol is important for central nervous system activation, increasing blood glucose concentration, and elevating mean blood pressure, all of which are important for coping with stress (Bamberger et al. 1996, Mommsen et al. 1999). Although the precise mechanisms in fish are unclear, it is generally agreed that important metabolic roles of cortisol during stress include glucose-regulation and glycogen-repletion processes, both of which are important pathways for the recovery from stress (Vijayan et al. 1997, Mommsen et al. 1999). Cortisol may also play a role in the peripheral mobilization of substrates, providing precursors for hepatic gluconeogenesis (Vijayan et al. 1997, Mommsen et al. 1999).

Effects of Cd on plasma cortisol concentrations reported in the literature are inconsistent. It is clear that Cd exposure at low sublethal concentrations illicits a
generalized stress response in the short-term. Generally, acute and subchronic Cd exposures increase plasma cortisol concentrations, which subsequently return to baseline levels (Pratap and Wendelaar Bonga 1990, Fu et al. 1990, Ricard et al. 1998, Brodeur et al. 1998), although the time frame for return to pre-exposure concentration varies widely. It is likely that the transient stress response with subchronic exposures is an acclimation of the corticosteroid response to Cd. Brodeur et al. (1998) showed that interrenal tissue of trout was still responsive to the actions of ACTH, and that the response was actually enhanced following exposure to 1 μg/L Cd for 30 days.

Cd may act directly as an endocrine disruptor in longer-term exposures (>30 days) by targeting pituitary or adrenocortical tissues. Several teleost species from sites contaminated by mixtures of PAH, PCBs, bleached kraft mill effluent and heavy metals exhibit an impaired adrenocortical response to stress, although the direct implication of Cd as a causative agent cannot be established due to the presence of other pollutants (Brodeur et al. 1997, Hontela et al. 1997, Lappivaara 2001). However, observations that the impairment of cortisol secretion detected at field sites contaminated by metals is only evident in adult fish four years and older, or young fish older than one year, support the long-term exposure hypothesis (Brodeur et al. 1997, Gravel et al. 2005).

In addition to field studies, several *in vitro* studies have demonstrated endocrine impairment in teleost interrenal cells exposed to Cd. These studies suggest that the site of action of Cd is in the intracellular signaling pathway for cortisol synthesis located downstream from cAMP formation and prior to pregnenolone synthesis (Lebond and Hontela 1999, Lacroix and Hontela 2004). However, there is evidence that additional sites of action of Cd in steroidogenic tissues may also exist. For example, in Y-1 mouse
adrenal tumor cells, Cd affected unstimulated adrenal cell steroid secretion in vitro, through a separate mechanism from that which affects stimulated (ACTH) steroid secretion (Mgbonyebi et al. 1994). In teleosts, the specific cellular site(s) of action of Cd on tissue steroidogenesis remain unknown. This study was performed to investigate the effects of Cd on interrenal steroidogenesis with the intention of furthering our understanding of the mechanism(s) of action of this endocrine disrupting metal in fish.

5.3 **Materials and methods**

5.3.1 Fish

Mature male and female rainbow trout (600 to 1200 g) were obtained from Sun Valley Trout Farms (Mission, BC) and acclimated for at least 4 weeks in 1200 L tanks prior to experimentation. Tanks were supplied with dechlorinated municipal water at 17±1°C, hardness 6.8 mg CaCO$_3$/L and pH 6.7. Fish were subjected to a 12:12 light:dark photoperiod and fed with commercial trout food (Ewos Canada Ltd., Surrey, BC; 3mm extruded pellets) until 48 hours prior to an experiment.

5.3.2 Chemicals

Cadmium chloride (CdCl$_2$), porcine adrenocorticotropic hormone (ACTH), 25-hydroxycholesterol (25-OHC), SyntheChol NS0 Supplement® (SC), Leibovitz medium (L-15), NaHCO$_3$, Type IV collagenase and Trypan Blue were purchased from Sigma Chemical Co. (St. Louis, MO). Anhydrous D-glucose and tricaine methanesulfonate (MS222) were obtained from BDH Inc. (Toronto, ON) and Argent Laboratories (Redmond, WA), respectively.
5.3.3 Preparation of interrenal tissue

Interrenal tissues were prepared according to a modified method of Wilson et al. (1998). Trout were euthanized in 0.2 g/L buffered MS222 and dissected. The head kidney, containing interrenal tissue, was immediately dissected out and rinsed in ice-cold, well oxygenated (1% CO₂, balance O₂) L-15 medium supplemented with 5 mM D-glucose and 6 mM NaHCO₃, pH 7.4. Tissue was placed into fresh medium and finely minced to approximately 1 mm³. Aliquots of interrenal tissue (17 to 24 mg wet weight) were added to vials containing 1 mL complete medium and incubated at 15 ± 1°C in the dark, with constant gentle shaking for 2 h. This 2-h preincubation stabilization period was required for cells to attain basal cortisol secretion levels (Laflamme et al. 2000). Mitochondria apparently contain adequate endogenous cholesterol supplies for cytochrome P450scc to sustain a low steroidogenic activity during unstimulated periods (Young and Hall 1971, Mason et al. 1978). Following stabilization, the supernatant was discarded and replaced with fresh L-15 medium containing stimulating, augmenting agents or Cd as described below.

5.3.4 Determination of stimulating or augmenting agent concentrations

The stimulating agent used in these experiments, ACTH, activates rate-limiting steps in steroid synthesis (Mgbonyebi et al. 1994). The augmenting agents, SC and 25-OHC, are exogenous substrates that increase unstimulated cortisol secretion by entering the synthetic pathway distal to the rate-limiting steps controlled by ACTH (Calogero et al. 1993, Mgbonyebi et al. 1994) (Fig 5.1). In order to determine the appropriate concentration of either agent type that results in maximal, enhanced cortisol secretion, interrenal tissues were collected and prepared as above. When cortisol secretion had
reached basal levels, fresh L-15 medium was added and tissues were incubated for a further hour. The medium was again removed and tissues were incubated in medium alone (control) or medium containing different concentrations of an agent: 0.01, 0.1 or 1 IU/mL ACTH; 0.016, 0.8 or 40 μL/mL SC or 0.05, 0.5 or 5 μM 25-OHC. Following a 2-h incubation period, medium was removed and frozen at -80°C for further cortisol analysis. The concentration of agent determined to produce maximum cortisol secretion was used in all subsequent experiments.

5.3.5 Cd exposure

In order to determine the effects of Cd alone on cortisol secretion, interrenal tissues were prepared as described above. When basal cortisol secretion levels were reached, medium was replaced with that containing CdCl₂ at concentrations of 0, 1, 10 or 100 μM. After an 1-h incubation, medium was removed and stored at -80°C for further cortisol analysis. In order to determine the potential sites of Cd impairment on stimulated and unstimulated cortisol synthesis, medium was replaced with fresh L-15 containing CdCl₂ at various concentrations and one of the agents. Tissues were incubated for a further 2 h after which the medium was removed and frozen at -80°C until analyzed.

5.3.6 Cell viability

Cell viability was determined using Trypan Blue exclusion for each preparation in control tissue and that exposed to Cd and/or agents following collagenase digestion (Lebond and Hontela 1999). Type IV collagenase (2.5 mg/mL in medium) was added and the tissues allowed to incubate for 1 h at 15±1°C with constant gentle shaking. Cells were disaggregated by manual agitation and aliquots of cell suspension (50 μL) were
added to a 0.4% Trypan Blue solution (50 μL). Cells were counted using a hemocytometer under light microscopy.

5.3.7 Cortisol determination

Tissue suspension medium was centrifuged at 16,000 x g for 4 min to remove any interrenal cells. 50 μL of supernatants were assayed for cortisol using an ELISA kit (Neogen Corporation, Lexington, KY). Each 96-well cortisol antibody coated plate was run with a standard curve based on a serial dilution of 1 μg/mL stock solution (provided with the kit) using L-15 medium as diluent, with a reading taken at 650 nm. Intra-assay and inter-assay CV were 4.4% and 6.7% respectively (n=4). All measures were performed on a Spectra Max 420 microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

5.3.8 Statistical analysis

Statistical analyses were performed using JMP IN 4.0.3 (SAS Institute Inc., Cary, NC). All cortisol secretion and cell viability values were treated as a single-factor randomized complete block design and compared using ANOVA and Tukey-Kramer HSD tests. Differences were considered significant at p < 0.05. Some of the data required natural logarithm transformation in order to meet the assumption of homogeneity of variance, although non-transformed data are shown in the figures. Results are expressed as means ± SEM. Since no significant difference between males and females was observed, data from both sexes were pooled.
5.4 Results

5.4.1 Cell Viability

There were no effects of CdCl₂ or any agent on cell viabilities at the concentrations used (Table 5.1). Cell viability of interrenal tissues exposed to the various agents ranged from 96.7 ± 0.6 to 97.7 ± 0.4% (F = 0.5, d.f.= 3, p = 0.6874). Similarly, viabilities for tissue in the presence of CdCl₂ alone or in combination with agents were also high, ranging from 93.9 ± 1.0 to 96.4 ± 1.0% (F = 0.5, d.f.= 15, p = 0.9223).

5.4.2 ACTH-stimulated, SC- and 25-OHC-augmented cortisol secretion

Baseline cortisol secretion by interrenal tissue prior to Cd exposure incubated for 2 h in the absence of any agent was 139 ± 37.7 ng/g wet tissue. The amount of cortisol produced during the incubation period was significantly greater than controls for most of the concentrations of agents used (Fig. 5.1 and Table 5.2). Maximal cortisol secretion was seen with 0.01 IU/mL ACTH (an increase of 1105 ± 227%; F = 21.6, d.f.= 3, p < 0.0001), 5 µM 25-OHC (397 ± 116%; F = 5.3, d.f.= 3, p = 0.0093), and 0.8 µL/mL SC (269 ± 54.5 % over controls; F = 5.3, d.f.= 3, p = 0.0065).

5.4.3 Cd effects on unstimulated and stimulated cortisol release

Cd affected cortisol secretion from interrenal tissues (Fig. 5.2). Baseline cortisol secretion was significantly lower than controls (58.9 ± 8.9 and 55.0 ± 9.5% of controls) when tissues were exposed to 10 and 100 µM Cd, respectively (F = 10.1, d.f.= 3, p = 0.0006). Actual cortisol concentrations after 1 hour of incubation were 53.6 ± 6.2, 45.4 ± 5.1, 31.1 ± 4.6 and 29.6 ± 7.0 ng/g wet tissue for controls, 1, 10 and 100 mM Cd groups respectively.
Cd also affected ACTH-stimulated and SC- and 25-OHC-augmented cortisol release from interrenal tissue (Table 5.3). The data are expressed as a percent of ACTH-stimulated, SC- or 25-OHC-augmented controls (Fig. 5.3). Cortisol secretion from ACTH-stimulated tissue and exposed to 1, 10 and 100 \( \mu \text{M} \) Cd was significantly lower than controls (15.3 ± 5.7, 20.4 ± 10.7 and 11.4 ± 10.3 % of controls, respectively; \( F = 36.6, \text{d.f.}= 3, p < 0.0001 \)). As well, cortisol secretion was significantly lower in tissues exposed to Cd in 25-OHC-augmented tissue compared to controls (43.1 ± 7.2%, 44.9 ± 5.3% and 52.4 ± 6.7% of controls; \( F = 25.3, \text{d.f.}= 3, p < 0.0001 \)). For both the ACTH and 25-OH treatment groups, no strict concentration-response relationship for cortisol inhibition was seen. SC-augmented cortisol release was significantly elevated by Cd exposure in the 1 and 10 \( \mu \text{M} \) Cd exposure groups (222 ± 42.2% and 199 ± 39.3% over controls, respectively; \( F = 9.03, \text{d.f.}= 3, p = 0.0012 \)), however, SC did not significantly affect SC-augmented cortisol release at 100 \( \mu \text{M} \).

5.5 Discussion

The essential roles of steroid hormones in critical organism functions such as homeostasis, reproduction and the stress response, and the known effects of Cd on steroidogenic tissues, prompted this investigation into the cortisol secretion process in teleost interrenal cells. Earlier work has shown that at least one of the intracellular sites of action of Cd in fish is on the first steps of the signaling pathway of stimulated cortisol synthesis (Lebond and Hontela 1999, Lacroix and Hontela 2004). Complementary work using mouse adrenal cells indicates that CdCl\(_2\) non-lethally affects stimulated as well as unstimulated steroidogenic pathway sites. We exposed interrenal tissue to Cd alone, in conjunction with the stimulating agent adrenocorticotrophic hormone (ACTH), the
primary secretagogue for cortisol secretion in these cells, or the augmenting agents SyntheChol NS0 Supplement® (SC) and 25-hydroxycholesterol (25-OHC). SC and 25-OHC are substitutes for endogenous synthetic-sequence substrates that were expected to increase unstimulated corticosteroid synthesis in interrenal tissue. It was assumed that specific steps inhibited by Cd would be bypassed if these compounds acted distal to Cd-affected site(s). SyntheChol® is pure synthetic cholesterol that behaves similarly to animal-source cholesterol in cell culture (Talley et al. 2003). The proprietary formulation we have used, SyntheChol NS0 Supplement®, enhances the solubility of the SyntheChol® molecule in aqueous medium (Talley et al. 2003), likely increasing cholesterol diffusion into the mitochondrion. 25-OHC is a relatively soluble cholesterol derivative that readily diffuses into the mitochondrion, bypassing the rate-limiting cytoplasmic and mitochondrial cholesterol transport steps (Jefcoate et al. 1974).

Cell viabilities in interrenal tissues exposed to all concentrations of Cd, ACTH, SC and 25-OHC or combinations were consistently high. Other studies have found low cellular toxicity values using similar Cd concentrations (Lebond and Hontela 1999, Lacroix and Hontela 2004). \( \text{LC}_{50} \) values for dispersed interrenal cells (10.8 mM) were 60-fold higher than \( \text{EC}_{50} \) values for the inhibition of corticosteroid secretion by these same tissues (Lebond and Hontela 1999) indicating that sublethal endocrine disruption can occur at concentrations well below those which result in tissue death.

The response of this minced tissue preparation to the stimulating or augmenting agents demonstrates that after manual disruption, tissues remained responsive and functional. The concentrations of ACTH and 25-OHC found to induce maximal cortisol secretion in the present study (Fig. 5.2) were approximately 100-fold lower than those
reported in other studies (Lebond and Hontela 1999, Lacroix and Hontela 2001, Lacroix and Hontela 2004). These differences may be attributed to the method of interrenal tissue preparation, since the partially preserved architectural structure of tissues in the present study may positively affect steroidogenesis activity in vitro compared to dispersed cell preparations. Hines and Azziz (1999) found that the complex architecture of the human adrenal cortex plays a role in regulating steoidogenesis, for example, their results show that dehydroepiandrosterone sulphate production was quite sensitive to architectural disruption, increasing as the degree of tissue disruption increased.

The characterization of the interrenal response to SC augmentation was particularly interesting since this recently synthesized compound was used for the first time to our knowledge in an in vitro fish system. The physico-chemical behavior of SC is similar to that of animal-derived cholesterol and has been shown to support mouse myeloma cells (NS0 cell line) that are cholesterol auxotrophs (Talley et al. 2003). In the present study, SC-augmented steroidogenesis occurred maximally at 0.8 μL/mL without stimulation with ACTH, an effect which was attenuated at the highest SC concentration used, perhaps suggesting low levels of cytotoxicity without a decline in measured cell viability. Free excess animal-derived cholesterol has been shown to be toxic to cells (Azhar et al. 2003). Although it has been claimed that SC exhibits similar behavior to cholesterol in cell culture (Talley et al. 2003), this may not be precisely the case in fish interrenal tissues. The transport of cytoplasmic cholesterol to the inner mitochondrial membrane by StAR protein does not occur during unstimulated steroidogenesis in mammals (Mgbonyebi et al. 1994). Experimental data suggests that StAR structure and function are highly conserved in vertebrates (Bauer et al. 2000, Kusakabe et al. 2002) and
the present results suggest that SC not only entered the mitochondrion, but was also a
substrate for P450scc. SC enhanced cortisol secretion, likely bypassed the rate-limiting
cholesterol transport step. Moreover, SC may be a better candidate for steroidogenesis
augmentation in fish in vitro systems when compared to 25-OHC.

Cortisol secretion, but not cell viability was impaired in a concentration-dependent manner by exposure to Cd alone. Statistically significant impairment occurred at 10 μM Cd in unstimulated cells, a value which is lower than any reported in the literature. Lebond and Hontela (1999) and Lacroix and Hontela (2004) observed Cd-induced impairment in cortisol secretion (after ACTH stimulation), at concentrations ≥100 μM. The higher sensitivity of our interrenal cells further suggests that preserved architecture in this preparation may play a significant role in fish steroidogenesis and its response to toxicants in vitro.

Cortisol synthesis is a complex process involving the production of hormones by
the hypothalamus, pituitary and interrenal tissues. ACTH, the major secretagogue of
cortisol, binds to a receptor on the plasma membrane of the interrenal steroidogenic cells,
increasing adenylate cyclase activity and intracellular cyclic 3′, 5′-adenosine
monophosphate (cAMP) concentrations, steroid synthesis and steroid secretion
(Grahame-Smith et al. 1967, Miller 1988). In the present study, ACTH-stimulated
cortisol release in the presence of Cd was significantly lower than in unexposed controls.
Similarly, Lebond and Hontela (1999) found that ACTH-stimulated cells secreted less
cortisol when exposed to 100 μM Cd, however, the concentration of Cd that impaired
cortisol secretion was significantly lower in the present study (1 μM). It is suggested that
the site of toxic action of Cd is located downstream from cAMP formation since ACTH
and cAMP supplementation fail to restore cortisol secretion in dispersed interrenal cells following Cd exposure.

It has also been suggested that the target of Cd is prior to pregnenolone synthesis, since supplementation of cells with this compound following Cd-induced impairment restores steroidogenesis in vitro (Lacroix and Hontela 2004). Phosphorylated steroidogenic acute regulatory protein (StAR protein) promotes the intramitochondrial delivery of cholesterol to the steroidogenic machinery in mammals (Kallen et al. 1998, King et al. 2004). In fish, recent studies strongly suggest that StAR is also involved in the regulation of steroidogenesis (Bauer et al. 2000, Kusakabe et al. 2002) and that cAMP-dependent protein kinase (PKA) has a stimulatory effect on the mitochondrial cholesterol translocation step (Lacroix and Hontela 2001). After the transport of cholesterol into the mitochondria, P450scc activated by cAMP converts cholesterol to pregnenolone, which leaves the mitochondrion. Pregnenolone is then subsequently converted to the final corticosteroid, cortisol, for secretion (Mommsen et al. 1999).

Monohydroxylated cholesterol derivatives have been shown to diffuse to P450scc located on the matrix surface of the inner membrane without special transport requirements. Interrenal cells and their mitochondria readily use exogenous 25-OHC as substrate for cortisol synthesis. Because 25-OHC bypasses StAR protein transport, it was used to determine if Cd potentially affects P450scc function. In the face of noncompromised P450scc, 25-OHC was expected to augment cortisol secretion (Lacroix and Hontela 2001), as it did in the present study. However, cortisol levels were significantly decreased after Cd exposure and subsequent 25-OHC augmentation. Similar results were observed in mouse adrenal cells where CdCl₂ inhibited 25-OHC
conversion to hydroxylated progesterone, a cortisol precursor (Mgbonyebi et al. 1994). These authors suggested that Cd may interfere with 25-OHC availability to the P450scc active site in the inner mitochondrial membrane or with the substrate binding process. In fact, there is evidence that hydroxylated cholesterol may interact with P450scc in a different manner when compared to cholesterol, altering its conversion to pregnonelone (Mason et al. 1978).

In the present study, SC augmented and increased cortisol secretion, even after Cd exposure, similar to pregnonelone augmentation (Lacroix and Hontela 2004), indicating that P450scc function was likely not affected by lower concentrations of Cd as it is suggested by the results with 25-OHC. However, SC-augmented cortisol secretion at 100 μM Cd was significantly lower than at 1 and 10 μM, indicating that at higher concentrations, Cd may affect steroidogenesis, perhaps through effects on P450scc. Since SC restored cortisol secretion and 25-OHC did not, the synthetic cholesterol used in this study may represent a better precursor than 25-OHC for examining Cd steroidogenic effects in vitro.

The results of this study show that Cd can impair the biosynthetic capacity of interrenal tissue at concentrations well below those affecting cell viability and values reported previously in the literature. This effect can be restored by synthetic cholesterol but not 25-OHC, indicating that two sites of action may exist, but that the major site of action of Cd is in the signaling steroidogenic pathway, likely prior to the P450scc step in the synthetic pathway. Cd may act downstream to cAMP formation (Lebond and Hontela 1999); therefore future studies are required to examine the importance of PKA and StAR protein function in Cd-induced steroidogenic impairment. In mammals, PKA is
responsible for the phosphorylation of a series of proteins including cholesterol ester hydroxylase, which upon activation catalyzes the conversion of cholesterol esters (the main source of stored cholesterol in the adrenal cells) to cholesterol (Azhar et al. 2003). As well, ACTH stimulates de novo StAR synthesis via cAMP/PKA (Mommsen et al. 1999, Lacroix and Hontela 2001, Kusakabe et al. 2002).
5.6 Figures

Figure 5.1 Initial steps of the cortisol biosynthetic pathway (until pregnenolone formation). Large arrows indicate the points of manipulation in our \textit{in vitro} study. Based on Lebond and Hontela 1999, Lacroix and Hontela 2001, Lacroix and Hontela 2004 and Geslin and Auperin 2004.
Figure 5.2 *In vitro* cortisol secretion in trout interrenal tissues stimulated with A) 0, 0.01, 0.1 and 1 IU/mL ACTH; B) 0, 0.05, 0.5 and 5 μM 25-OHC and C) 0, 0.016, 0.8 and 40 μL/mL SyntheChol®. Control values represent tissues incubated in medium without any stimulating or augmenting agent. Treatments with similar letters were not significantly different from each other at p < 0.05. Numbers in bars represent sample sizes and shaded bars represent the concentration used in subsequent experiments. Values are means ± SEM.
Figure 5.3 *In vitro* cortisol secretion in trout interrenal tissues exposed to 0, 1, 10 and 100 μM Cd for 1 h. Control values represent tissues incubated in medium without Cd. Treatments with similar letters were not significantly different from each other at p < 0.05. n = 5. Values are means ± SEM.
Figure 5.4 *In vitro* cortisol secretion in trout interrenal tissues exposed to 0, 1, 10 and 100 μM Cd for 1 h and subsequently stimulated with A) 0.01 IU/mL ACTH, or augmented with B) 5 μM 25-OHC or C) 0.8 μL/mL SyntheChol®. Control values represent tissues stimulated or augmented by an agent without Cd exposure. Treatments with similar letters were not significantly different from each other at p < 0.05. n = 4-5. Values are means ± SEM.
### 5.7 Tables

**Table 5.1**: Viability (%) of trout interrenal cells exposed to medium (controls) or exposed to ACTH, 25-OHC or SyntheChol for 2h alone or in presence of Cd (1, 10 or 100 μM). a:n=6 to 8, b:n=4 to 5.

<table>
<thead>
<tr>
<th>Agent</th>
<th>0 μM Cd&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1 μM Cd&lt;sup&gt;b&lt;/sup&gt;</th>
<th>10 μM Cd&lt;sup&gt;b&lt;/sup&gt;</th>
<th>100 μM Cd&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>medium</td>
<td>97.0 ± 0.6</td>
<td>95.8 ± 0.6</td>
<td>95.3 ± 0.7</td>
<td>96.1 ± 1.4</td>
</tr>
<tr>
<td>ACTH</td>
<td>97.3 ± 0.6</td>
<td>95.1 ± 1.0</td>
<td>95.1 ± 1.0</td>
<td>94.5 ± 1.5</td>
</tr>
<tr>
<td>25-OHC</td>
<td>97.7 ± 0.4</td>
<td>94.1 ± 2.1</td>
<td>94.3 ± 1.4</td>
<td>93.9 ± 1.0</td>
</tr>
<tr>
<td>SyntheChol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96.7 ± 0.6</td>
<td>95.7 ± 1.5</td>
<td>96.4 ± 1.0</td>
<td>94.8 ± 2.1</td>
</tr>
</tbody>
</table>
Table 5.2 *In vitro* cortisol secretion (ng/g wet tissue) of trout interrenal tissue exposed to medium (controls – no agent) or three different concentrations of ACTH, 25-OHC or SyntheChol® for 2h. n = 4 to 8. a: C1, C2 and C3 = 0.01, 0.1 and 1 IU/mL respectively; b: C1, C2 and C3 = 0.05, 0.5 and 5 μM respectively; c: C1, C2 and C3 = 0.16, 0.8 and 40 μM/mL respectively.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Control</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>234.3 ± 40.2</td>
<td>2133.7 ± 310.1</td>
<td>1118.8 ± 260.3</td>
<td>1481.3 ± 351.7</td>
</tr>
<tr>
<td>25-OHC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>107.5 ± 64.7</td>
<td>130.9 ± 91.8</td>
<td>162.1 ± 61.0</td>
<td>255.9 ± 116.7</td>
</tr>
<tr>
<td>SyntheChol&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>254.5 ± 40.1</td>
<td>472.5 ± 116.7</td>
<td>624.6 ± 183.0</td>
<td>430.6 ± 121.7</td>
</tr>
</tbody>
</table>
Table 5.3 *In vitro* cortisol secretion (ng/g wet tissue) of trout interrenal tissue exposed to 0, 1, 10 or 100 μM Cd and stimulated/augmented with 0.01 IU/mL ACTH, 5 μM 25-OHC or 0.8 μL/mL SyntheChol® for 2h. n = 4 to 5.

<table>
<thead>
<tr>
<th>Agent</th>
<th>0 μM Cd</th>
<th>1 μM Cd</th>
<th>10 μM Cd</th>
<th>100 μM Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>1750.7 ± 404.9</td>
<td>292.7 ± 161.1</td>
<td>426.9 ± 223.9</td>
<td>187.2 ± 177.3</td>
</tr>
<tr>
<td>25-OHC</td>
<td>117.7 ± 33.0</td>
<td>57.1 ± 21.7</td>
<td>55.2 ± 17.7</td>
<td>64.9 ± 23.9</td>
</tr>
<tr>
<td>SyntheChol®</td>
<td>42.1 ± 3.9</td>
<td>93.8 ± 26.2</td>
<td>84.0 ± 18.6</td>
<td>18.7 ± 6.9</td>
</tr>
</tbody>
</table>
5.8 References


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CHAPTER 6
SUMMARY AND GENERAL CONCLUSIONS

The major goals of this thesis were twofold: to investigate the effects of environmentally-realistic Cd concentrations on 1) the reproductive and developmental axes and 2) the organismal stress response of rainbow trout. This main finding of the thesis was that extremely low Cd concentrations resulted in a multifaceted toxicity paradigm, with time- and concentration-dependent effects on the reproductive and developmental axes, and to a lesser extent on the stress response.

A direct link between exposure to environmental contaminants and disruption in the reproductive and developmental axes in wildlife is often difficult to establish, however, laboratory studies can provide useful information on mechanisms of action to begin to establish cause-effect relationships. Much of the literature describes the effects of high concentrations of xenobiotics with studies using environmentally-realistic concentrations being rare. Studies using realistic concentrations may yield better insights about the association between chemical concentrations and physiological dysfunction in the environment. In the present study, different life stages of rainbow trout were impaired by concentrations of Cd that have been observed in the environment. Cd concentrations in surface waters are generally very low, ranging from < 0.1 to 8.9 μg/L with an average of 0.2 μg/L in British Columbia (Canadian Environmental Protection Act 1994). Eggs were affected to a lesser extent than the other life-stages, since the observed premature or delayed hatching did not affect final hatching success. In contrast, larval growth was
reduced in a concentration-dependent manner. Similar effects for eggs and larvae exposed to heavy metals, including Cd, have been reported, however, the concentrations causing effects in our study were below the LOEL (0.47 µg/L) reported for Cd (Canadian Environmental Protection Act 1994). This is particularly relevant since the LOEL is often used to determine guideline concentrations for water quality in many countries, including Canada. The prominent impact of Cd on juvenile fish has also been previously reported, although at much higher Cd concentrations.

Separate studies have also shown that Cd can modulate fish sex steroid concentration in vivo and in vitro, with both increases and decreases being reported. Taken together, those separate studies suggested that time of Cd exposure may play an important role in sex steroid modulation. Through our in vivo and in vitro studies a pattern of Cd effects on sex steroid concentration in a time- and concentration-dependent fashion was identified. Short-term Cd exposure increased and long-term exposure decreased, sex steroid concentrations with more prominent effects at higher Cd concentrations. These apparent opposing effects are consistent with the possibility of two sites of Cd action within the steroidogenic pathway in fish, both an inhibitory site and a stimulatory site, as previously observed in mammals. Although both sites are probably different from the sites of Cd action in mammals, sex steroids production in fish is likely a sum of inhibitory and stimulatory Cd actions, as suggested for mammals.

Cd affected the HPI and stress response differently than previously reported in laboratory studies. Low concentrations of Cd decreased baseline plasma cortisol concentrations after sub-chronic exposure. However these persistent low concentrations of plasma cortisol were not accompanied by interrenal impairment or by alterations in
other metabolic parameters. Generally, previous studies have described two different patterns of cortisol modulation by Cd: increase of plasma cortisol concentrations accompanied by preserved or increased ACTH responsiveness; or decrease in plasma cortisol concentrations and decreased ACTH responsiveness. Our study suggests another pattern: decrease in plasma cortisol concentrations and preserved ACTH responsiveness. Rather than corroborating the popular hypothesis that low Cd concentrations stimulate interrenals until exhaustion, our study indicates that Cd may act through several mechanisms also, including acclimation to long-term low Cd exposure. However, our in vitro study demonstrated that, in fact, interrenals can be affected when higher Cd concentrations are used. Our study also increased our knowledge regarding the mechanisms of Cd action in interrenal cells. Previous studies have indicated that the major site(s) of Cd action within the interrenal steroidogenic pathway in fish was located after cAMP formation and before pregnonelone conversion. Our in vitro study indicates that one major site(s) of action of Cd on the cortisol synthesis pathway is likely prior to cytochrome P450 side chain cleavage.

The negative effects of Cd with time on both the reproductive and developmental axes and stress response in rainbow trout may indicate that even at the low levels observed in the environment, Cd may affect the survival of wildlife on the longer term. Fish in nature are probably exposed for rather prolonged periods to these low Cd concentrations potentializing its deleterious effects.

The observed results are particularly relevant in British Columbia, where the water quality guidelines to protect aquatic life for Cd are currently being developed by the Ministry of Environment. These guidelines, mainly based on scientific literature data,
are set to provide the benchmarks for the assessment of water quality in the environment. The current Canadian guideline and the interim BC standard guideline for protection of aquatic life, 0.017 \( \mu g/L \) Cd (Environment Canada 2005), are below the concentrations tested here and seem to be effective on protecting wildlife from Cd toxic effects. Our data indicate however, that concentrations below the current BC freshwater aquatic life protective site-specific water standard for contaminated sites (0.1 \( \mu g/L \) Cd at hardness \( \leq 30 \text{ mg/L} \) \( \text{CaCO}_3 \); BC Ministry of Water, Land and Air Protection 2001) may affect teleosts. It is important to note also, that the ‘Protocol 10 for Contaminates Sites’ (BC Ministry of Water, Land and Air Protection 2001), which establishes the above standard, gives specific guidelines for sites where hardness \( \geq 500 \text{ mg/L} \) \( \text{CaCO}_3 \). Since Cd toxicity increases with decreased water hardness and our results demonstrated abnormal effects in fish at Cd concentrations below 0.1 \( \mu g/L \) Cd (at hardness = 6.8 \( \text{mg/L} \) \( \text{CaCO}_3 \)), special guidelines perhaps should also be enforced for sites with hardness \( \leq 30 \text{ mg/L} \) \( \text{CaCO}_3 \).

Overall, our study demonstrated that Cd affects fish reproduction, development and organismal stress response, being time especially important for the observed effects on reproduction and development. A complex concentration-response relationship was evident and future studies are necessary to fully understand this important point. It is likely that the concentration-response relationship is one of the keys to predicting effects in the environment and setting appropriate water quality guidelines. In order to elucidate the mechanisms involved on the modulation of steroidogenesis in fish, the first steps of the steroid biosynthetic pathway should be investigated further. In addition, future acute \textit{in vivo} studies could help to understand the organismal stress response during long-term low-Cd concentration exposure. Investigations are also necessary to address the relative
importance of the different uptake routes to overall Cd toxicity and their importance to the susceptibility of different life stages of fish.

References
