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Kate Crump

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

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TITRE DE LA THÈSE / TITLE OF THESIS

Dr. V. Trudeau

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. T. Scheuhammer

Dr. T. Moon

Dr. D. Lean

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

The effects of methylmercury on the reproductive axis of goldfish (*Carassius auratus*)

by

Kate Crump

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Abstract

Methylmercury is both a potent neurotoxin and putative non-steroidal endocrine disruptor. Laboratory studies have provided evidence of reproductive impairment in many fish species after mercury exposure. Effects have been observed at every level of the reproductive axis including degeneration of neurons in the hypothalamus, decreased activity of gonadotrophs in the pituitary, and reduced steroidogenesis in the gonads. Despite the growing amount of research linking methylmercury exposure to reproductive impairment, the target tissues and mechanisms of action involved are still largely unknown. This thesis extends the body of evidence supporting the hypothesis that methylmercury is an endocrine disruptor in fish. To my knowledge, this is the first study to compare the effects of sub-chronic methylmercury exposure on adult goldfish at two different periods within the annual spawning cycle. The effects of endocrine disrupting toxins may differ depending on the season of exposure as goldfish exhibit seasonal fluctuations in gonad size, circulating sex steroids, and luteinizing hormone (LH). The present study demonstrates that ovarian steroidogenesis is impaired by methylmercury in both pre- and post-spawning fish. Analyses of pituitary and serum LH levels suggest that reductions in sex steroid levels are independent of alterations in LH. The possibility that reduced sex steroid levels in fish exposed to dietary methylmercury may be secondary to hypothalamic dysfunction remains to be addressed. Microarray analysis identified 59 genes differentially expressed in the hypothalamus after methylmercury accumulation. These candidate genes were categorized into discrete functional groups including apoptosis and stress response, calcium binding and transport, and protein synthesis, degradation, and transport. In addition, several neuroendocrine-related genes were differentially regulated including transcripts encoding peptides involved in stimulating the production and release of gonadotropin releasing hormone, secretion of LH, and

gonadal steroidogenesis. This thesis demonstrates changes in the endocrine system linked to methylmercury exposure and provides the first step towards identifying the mechanistic relationship between changes in reproductive biomarkers and gene expression.

Résumé

Le méthyle-mercure est une neurotoxine très puissante et présumée comme étant un perturbateur endocrinien. Des études en laboratoire ont démontré que l'exposition au mercure entraîne des déficiences dans le système reproducteur chez plusieurs espèces de poissons. Ces effets ont été observé à tous les niveaux de l'axe reproducteur incluant la dégénération de neurones dans l'hypothalamus, la baisse d'activité des gonadotropes dans la glande pituitaire ainsi que la réduction de la synthèse des stéroïdes dans les gonades. Malgré qu'il y ait de plus en plus d'études reliant l'exposition du méthyle-mercure à des déficiences du système reproducteur, les tissus cibles et les mécanismes d'action impliqués sont toujours inconnus. Cette thèse apporte plusieurs autres évidences supportant l'hypothèse que le méthyle-mercure est un perturbateur endocrinien chez les poissons. À ma connaissance, c'est la première étude qui compare les effets sub-chroniques d'une exposition au méthyle-mercure chez les poissons rouges adultes durant deux différentes périodes de leur cycle de reproduction. Les effets des toxines perturbatrices du système endocrinien peuvent changer selon la saison puisque le poisson rouge possède une variation saisonnière dans la taille de ses gonades, la circulation de ses stéroïdes sexuels et de l'hormone lutéinisante (HL). La synthèse des stéroïdes ovariens est perturbée par le méthyle-mercure avant et après la ponte chez le poisson. Des analyses de niveaux de l'HL dans la glande pituitaire et dans le sérum suggèrent que ces réductions de concentrations de stéroïdes sont indépendants des effets sur l'HL. La possibilité que la réduction des stéroïdes chez les poissons exposés au méthyle-mercure via leur alimentation puisse être causée en conséquence d'un mauvais fonctionnement de l'hypothalamus reste à être analyser. L'analyse de biopuces a permis d'identifier 59 gènes étant exprimés différemment dans l'hypothalamus après une accumulation de méthyle-mercure. Ces gènes ont été catégorisés par groupes fonctionnels incluant l'apoptose,

la réponse au stress, la liaison et le transport du calcium, ainsi que la synthèse, la dégradation et le transport des protéines. De plus, plusieurs gènes reliés au système neuroendocrien ont été modifié incluant les transcrits codant pour les peptides impliqués dans la production et le relâchement de l'hormone de libération des gonadotrophines (GnRH), la sécrétion de l'HL et de la synthèse des stéroïdes dans les gonades. Cette thèse démontre qu'il y a des changements du système endocrinien lorsqu'exposer au méthyle-mercure et fournie des pistes afin de mieux comprendre les mécanismes entre les changements des bio-marqueurs reliés à la reproduction et l'expression génique.

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Abbreviations

11-KT	11-ketotestosterone
3βHSD	3β-hydroxy-Δ_5-steroid dehydrogenase
5-HIAA	5-hydroxyindole acetic acid
5-HT	serotonin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA	analysis of variance
ATPase	adenosine triphosphatase
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
Cy3/5	cyanine 3/5
D2	dopamine receptor subtype D_2
DA	dopamine
DNA	deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
DORM-2	dogfish muscle standard reference material (NRC Canada)
E2	17β-estradiol
EDC	endocrine disrupting chemical
FDR	false discovery rate
FSH	follicle stimulating hormone
GABA	gamma-aminobutyric acid
GnRH	gonadotropin-releasing hormone
GO	gene ontology
GSI	gonadosomatic index
Hg	mercury
HPG	hypothalamic-pituitary-gonadal
hsp	heat shock protein
LH	luteinizing hormone
MAO	monoamine oxidase
MeHg	methylmercury
mRNA	messenger ribonucleic acid
MS222	3-aminobutyric acid ethyl ester
NE	norepinephrine
NMDA	N-methyl-D-aspartic acid
PCR	polymerase chain reaction
QPCR	real-time quantitative reverse transcriptase-PCR
RIA	radioimmunoassay
RIN	RNA integrity number
RNA	ribonucleic acid
T	testosterone

Thesis outline and rationale

Environmental mercury (Hg) is a concern for human and wildlife health due to its ability to bioaccumulate and its potent neurotoxicity. Mercury is relatively stable in the atmosphere and undergoes long-range transport which has made mercury a global issue. Through the methylation of inorganic mercury by microorganisms in aquatic sediments, methylmercury is present in nearly all aquatic species. Studies over the past three decades have provided evidence of reproductive impairment in fish after mercury exposure. The evidence includes reductions in gonad size, circulating reproductive steroids, fertility, and spawning success. Although many effects of methylmercury exposure have been studied, the mechanisms underlying these effects remain unclear. There also exists a disparity between studies examining the endocrine disrupting effects of methylmercury exposure and those examining the neurotoxic effects. Methylmercury is a well known neurotoxin and a putative endocrine disrupting chemical, however, the link between these two effects remains to be examined.

The following hypotheses were examined within this thesis:

1) Methylmercury, at environmentally relevant concentrations, is an endocrine disruptor in fish (Chapter 2)
2) Methylmercury exposure will produce a distinctive hypothalamic gene expression signature (Chapter 3)
3) Methylmercury exposure will alter the expression of genes involved in the neuroendocrine control of reproduction (Chapter 2 and 3)

This thesis is organized into four chapters. The first chapter presents a review of the current data demonstrating the effects of sub-lethal mercurial pollution on the reproductive axis of teleost fish and provides a framework for understanding the mechanisms underlying methylmercury-induced reproductive impairment. The second chapter evaluates the effects of environmentally relevant concentrations of methylmercury on the hypothalamic-pituitary-gonadal (HPG) reproductive axis in adult goldfish (*Carassius auratus*). Previous studies have demonstrated endocrine disrupting effects of methylmercury exposure in fish. There is, however, a lack of studies assessing sub-chronic environmentally relevant exposures in adult fish. A novel aspect of this study is the comparison between effects observed at two different stages of reproduction (pre- and post-spawning). Methylmercury contaminated diets were prepared using concentrations previously determined to be sub-lethal and similar to those present in some aquatic food webs (Hammerschmidt et al., 2002; Drevnick and Sandheinrich, 2003). Adult female goldfish were fed methylmercury contaminated diets for 28 days. The results of this study demonstrate that sub-chronic methylmercury exposure can reduce sex steroid production and gonadal development in pre-spawning goldfish. While in post-spawning goldfish, effects were observed at the level of the pituitary and the gonads with reduced circulating luteinizing hormone (LH) and sex steroid levels. In chapter 3, a cDNA microarray was used to profile gene expression in the neuroendocrine brain of pre- and post-spawning goldfish exposed to dietary methylmercury. Three groups of methylmercury exposed fish were compared to control fish using a goldfish brain enriched cDNA microarray (pre-spawning goldfish fed a high methylmercury diet, post-spawning goldfish fed a low methylmercury diet, and post-spawning goldfish fed a high methylmercury diet). Goldfish from two of the methylmercury exposed groups had reduced sex steroid levels as a result of methylmercury exposure. Differentially expressed genes regulated in two or more exposures were compiled into a consensus

hypothalamic gene profile for methylmercury exposure. These genes were organized based upon the biological process in which they are involved. The data suggested that methylmercury exposure may induce an oxidative stress response, alter calcium homeostasis, and increase protein synthesis in the hypothalamus. Furthermore, several genes involved in the neuroendocrine control of reproduction were differentially regulated by methylmercury exposure and may provide targets for future studies. A summary of the results and proposed mechanisms are discussed in the final chapter along with possible areas of future research. This thesis provides the first step in examining the mechanistic link between methylmercury-induced alterations in the central nervous system and reductions in reproductive endpoints.

CHAPTER 1

General Introduction

The presence of industrial and agricultural contaminants in the aquatic environment has been of increasing concern for human and wildlife health. Mercury is of particular concern due to the bioaccumulation and potent neurotoxicity of some of its forms. Atmospheric emissions of mercury are the result of anthropogenic activities (e.g., metal production and coal-fired generating stations) and natural geochemical processes (e.g., volcanic eruptions and forest fires). Based on lake sediment records from midcontinental North America, atmospheric deposition of mercury is estimated to have tripled over the past 140 years (Swain et al., 1992). Once in the atmosphere, mercury can exist for up to a year allowing for widespread distribution via long-range atmospheric transport. Mercury pollution is a ubiquitous problem with atmospheric deposition contaminating watersheds in areas far from anthropogenic or natural atmospheric point sources (Swain et al., 1992; Fitzgerald et al., 1998).

Several forms of mercury are present in the aquatic environment including elemental, ionic, and organic (e.g., methylmercury) (Morel et al., 1998). Methylmercury is one of the most toxic forms and bioaccumulates in fish primarily through dietary uptake (Spry and Wiener, 1991; Hall et al., 1997). The level of bioaccumulation is a function of age, species, and trophic transfer (Spry and Wiener, 1991). Preferential bioaccumulation of methylmercury over inorganic mercury is due to the greater assimilation efficiency (Pickhardt et al., 2006) and trophic transfer efficiency (Mason et al., 1996) of methylmercury. Inorganic mercury is found in piscivorous birds and mammals, but its presence is generally attributed to the ability of these species to convert methylmercury to the less toxic form and not to dietary uptake or bioaccumulation (Wiener et al., 2003). Specific bioaccumulation of methylmercury is evident as the average proportion increases

from approximately 5% methylmercury in the aqueous environment to 15% in phytoplankton, 30% in zooplankton, and over 90% in fish (Watras and Bloom, 1992). Total mercury levels in fresh waters of North America generally do not exceed the ng/L range (Weiner and Spry, 1996; Wiener et al., 2003). Methylmercury in surface water normally comprises 0.1 to 5% of the total mercury content, but in anoxic waters it can be one of the dominant mercury species (Wiener et al., 2003). Concentrations of methylmercury in aquatic organisms increase due to trophic transfer. Methylmercury concentrations in phytoplankton range from 0.003 to 0.005 μg/g wet weight (ww) (Watras and Bloom, 1992), while the methylmercury concentration range increases to 0.03 to 0.22 μg/g ww in lower trophic level fish such as bluegill (*Lepomis macrochirus*) and black crappie (*Pomoxis nigromaculatus*) (Sveinsdottir and Mason, 2005). In higher trophic level fish including the largemouth bass (*Micropterus salmoides*), methylmercury concentrations range from 0.10 to 1.0 μg/g ww (Sveinsdottir and Mason, 2005). Health Canada has established a human consumption guideline level of 0.5 μg/g ww total mercury in commercial marine and freshwater fish. Even in remote regions, methylmercury in fish sometimes exceeds the concentration deemed safe for human consumption (Fitzgerald et al., 1998). A study of 775 water bodies in the province of Québec reported maximum muscle mercury levels of 2.30, 4.90, and 5.60 μg/g ww for northern pike (*Esox lucius*), walleye (*Stizostedion vitreum*), and lake trout (*Salvelinus namaycush*) respectively (Laliberté, 2004). The adverse effects of inorganic mercury and methylmercury on humans is well documented (Castoldi et al., 2003; Aschner and Syversen, 2005), however relatively little is known about the effects on the health or condition of wild fish populations.

Methylmercury exposure can affect behaviour, biochemistry, growth, reproduction, development, and survival in fish (Sorensen, 1991; Weiner and Spry, 1996). Long-term

laboratory studies of fish exposed to dietary methylmercury have noted incoordination, diminished swimming activity, starvation, and mortality (Wiener et al., 2003). Industrial activities have resulted in highly contaminated local fish populations with mercury concentrations ranging from 8.4 to 24 μg/g ww in Minamata Bay, Japan (Kitamura, 1968) and 6.3 to 16 μg/g ww in Clay Lake, Canada (Lockhart et al., 1972). The symptoms of methylmercury intoxication in humans include impaired behaviour, brain lesions, and mortality (Takeuchi et al., 1962; Nierenberg et al., 1998). At lower concentrations, mercury may affect fish populations indirectly through impairment of physiological processes rather than the severe neurological impairment and death observed in extreme contamination incidents. Over the past three decades, studies using sub-lethal concentrations of mercurials have demonstrated negative impacts on reproduction in numerous freshwater fish species (reviewed in Kime, 1998). Weiner and Spry (1996) concluded that reduced reproductive success was the most plausible effect of mercury on wild fish populations at current exposure levels in aquatic ecosystems. However, the mechanistic effects of methylmercury on reproduction remain unclear. The goal of this chapter is to present a comprehensive review of data demonstrating the effects of sub-lethal mercurial pollution on the reproductive axis of teleost fish, from neuroendocrine control through to gonadal development and spawning. Effects of mercury exposure on the reproductive axis from relevant laboratory and wild fish studies are summarized in Table 1.1.

1.1 Disruption of Hypothalamic and Pituitary Function

1.1.1 The Hypothalamus

The inhibitory effects of pollutants on reproduction may be mediated via changes in regulation at the level of the hypothalamus and pituitary. Methylmercury is a known neurotoxin

Table 1.1 Summary of consensus effects of mercury on the reproductive axis in wild and laboratory exposed fish

Endpoint	# of species	Life stage	Route of exposure	Length of exposure[b]	Hg species	Hg concentration	References
Brain							
↓ Stimulatory neurotransmitter (5-HT)	2	Larval to juvenile, recrudescent	Water	Sub-chronic	Inorganic, organic	μg/L	Kirubagaran and Joy (1990), Tsai et al. (1995)
↑ Inhibitory neurotransmitter (DA)	2	Larval, recrudescent	Water	Short-term, sub-chronic	Inorganic, organic	μg/L	Kirubagaran and Joy (1990), Zhou et al. (1999)
↓ Degradation enzyme (MAO)	3	Juvenile, recrudescent to spawning	Water, diet	Sub-chronic	Inorganic, organic	μg/L, μg/g	Berntssen et al. (2003), Kirubagaran and Joy (1990), Ram and Sathyanesan (1985)
Pituitary							
Reduced and/or inactive gonadotrophs	2	Recrudescent to spawning	Water	Sub-chronic	Inorganic, organic	μg/L	Joy and Kirubagaran (1989), Ram and Joy (1988), Ram and Sathyanesan (1983, 1986b)
Testes							
Histopathological changes	3	Juvenile, postspawning to recrudescent	Water, diet, intraperitoneal	Sub-chronic, chronic	Organic	μg/L, ng/g	Friedmann et al. (1996), Wester (1991), Wester and Canton (1992)
Sperm necrosis	2	Juvenile	Water	Short-term, sub-chronic	Organic	μg/L	Liao et al. (2006), Wester (1991), Wester and Canton (1992)
↓ Spermatogenesis	2	Recrudescent to spawning	Water	Sub-chronic	Inorganic, organic	μg/L	Kirubagaran and Joy (1992), Ram and Joy (1988), Ram and Sathyanesan (1983, 1986b)
↓ GSI[a]	4	Juvenile, immature, recrudescent to spawning	Water, diet, environment	Sub-chronic, chronic	Organic	μg/L, ng/g, environmental	Friedmann et al. (1996), Kirubagaran and Joy (1992), Ram and Sathyanesan (1986b), Webb et al. (2006)
↓ Testosterone and 11-ketotestosterone synthesis	3	Juvenile to spawning, immature, postspawning to recrudescent	Diet, intraperitoneal, environment	Chronic	Organic	μg/g, ng/g, environmental	Arnold (2000), Drevnick and Sandheinrich (2003), Webb (2006)
Ovaries							
Histopathological changes	6	Juvenile to spawning, recrudescent to spawning, spawning, sexually mature, postspawning to recrudescent	Water, diet, intraperitoneal, environment	Short-term, sub-chronic, chronic	Inorganic, organic	μg/L, μg/g, ng/g, environmental	Adams et al. (1999), Arnold (2000), Dey and Bhattacharya (1989), Drevnick et al. (2006), Ram and Sathyanesan (1986a, 1986b), Victor et al. (1986)
↓ Oogenesis	2	Recrudescent to spawning, spawning	Water	Sub-chronic	Inorganic, organic	μg/L	Dey and Bhattacharya (1989), Kirubagaran and Joy (1988), Ram and Joy (1988), Ram and Sathyanesan (1983, 1986a, 1986b)
↓ GSI	4	Juvenile to spawning, immature, recrudescent to spawning, spawning	Water, diet, environment	Sub-chronic, chronic	Inorganic, organic	μg/L, μg/g, ng/g, environmental	Dey and Bhattacharya (1989), Drevnick and Sandheinrich (2003), Hammerschmidt et al. (2002), Kirubagaran and Joy (1988), Ram and Sathyanesan (1983, 1986a, 1986b), Webb et al. (2006)
↓ 17β-Estradiol synthesis	4	Juvenile to spawning, immature, postspawning to recrudescent, sexually mature	Diet, intraperitoneal, environment	Chronic	Organic	μg/L, ng/g, environmental	Adams et al. (1999), Arnold (2000), Drevnick and Sandheinrich (2003), Webb (2006)

[a] equivocal data

[b] short-term: <1–2 weeks; sub-chronic: <10% lifespan of the organism; chronic: a significant fraction of the organism's lifetime (>200 days)

The abbreviations are as follows: 5-HT, serotonin; DA, dopamine; MAO, monoamine oxidase; GSI, gonadosomatic index.

whose effects in fish after long-term dietary exposure are characterized by brain lesions, oxidative stress, and decreased activity (Berntssen et al., 2003). Various molecular targets within the nervous system have been implicated in methylmercury neurotoxicity including the cytoskeleton, axonal transport, neurotransmission parameters, protein synthesis, and the mitochondrial energy-generating systems (Castoldi et al., 2001). Although methylmercury readily reacts with any sulfhydryl group, it shows brain region specificity with neuronal loss specific to certain areas. Methylmercury exposure in Atlantic salmon (*Salmo salar*) caused widespread vacuolation with the most severe effects noted in the medulla and cerebellum, while the cerebrum and other regions were less affected (Berntssen et al., 2003). Furthermore, mitochondria from cortical astrocytes are more sensitive to methylmercury toxicity than those in cerebellar astrocytes (Morken et al., 2005). Thus it is important to examine the effects of mercury exposure on isolated brain regions. In addition, the endocrine system amplifies signals such that minor changes in neuronal function may cause major disturbances in reproduction (Kime, 1998). Consequently, mercury may affect reproduction via the hypothalamus at levels lower than those that induce general neuronal damage.

In teleosts, gonadotropin-releasing hormone (GnRH) neurons, originating in the ventral telencephalic-preoptic-anterior region of the hypothalamus, project into the anterior pituitary gland (Van Der Kraak et al., 1998). Gonadotropin-releasing hormone regulates the synthesis and release of the gonadotropins (LH and follicle stimulating hormone [FSH]) making it a crucial neuroendocrine mediator of reproductive function. Histological analysis of neurons in the hypothalamic preoptic nuclei of the murrel (*Channa punctatus*) has provided evidence for potential inhibitory effects of mercury on neurosecretion. The magnocellular isotocin and vasotocin-secreting neurons of the preoptic nuclei were smaller and inactive (characterized by reduced staining of neurosecretory material) in murrels treated for six months to 10 μg/L of

aqueous inorganic mercury (Ram and Joy, 1988). Many neurons also exhibited various degrees of degeneration, from apoptotic chromatin condensation to necrosis. Some of the affected neurons were uncharacterized and may have included GnRH cells although this was not proven. In rats, dietary methylmercury decreased GnRH content in the medial hypothalamus (Oliveira et al., 2006). The paucity of information on the effects of mercury on GnRH neurons in vertebrates may be a reflection of the difficulty in studying them, as they do not form a compact nucleus but rather a diffuse interconnected network (i.e., hypothalamus and telencephalon) specifically involved in reproductive control (Norris, 1997).

Genomic techniques (e.g., real-time quantitative polymerase chain reaction [QPCR] and microarray) allow for the simultaneous measurement of genes involved in multiple biochemical pathways. Assessment of methylmercury-induced changes in hypothalamic gene expression could provide mechanistic information relating methylmercury exposure to reproductive changes. Genetic responses to methylmercury exposure were assessed in whole brain of zebrafish (*Danio rerio*) using QPCR; no changes in transcript levels were measured for any of the 13 genes assayed (Gonzalez et al., 2005). None of the genes assessed were specifically involved in regulating reproduction and the results of the study were limited by the use of whole brain rather than isolated brain regions. A rainbow trout (*Oncorhynchus mykiss*) cDNA microarray was recently used to identify differences in liver gene expression patterns between wild caught cutthroat trout (*Oncorhynchus clarki*) from two lakes with significantly different mercury concentrations (0.016 and 0.054 μg/g ww) in their respective fish (Moran et al., 2007). Surprisingly, several reproductive neuroendocrine genes (GnRH-1, GnRH-2 and GnRH receptor) were expressed in the liver and were affected by mercury. A comprehensive assessment of gene

expression changes in the hypothalamus in relation to methylmercury-induced reproductive impairment in any species remains to be done.

1.1.2 Neurotransmission

Neurotransmitters (e.g., norepinephrine [NE], dopamine [DA], and serotonin [5-HT]) are released by presynaptic nerve cells into the synapse where they stimulate receptors on postsynaptic nerve cells (Brown, 1994). The release of hypothalamic and pituitary hormones is regulated by neurotransmitters and neurotransmitter release is, in turn, modulated by hormones. The effects of mercury on neurotransmission may be mediated by disruption of synthesis, storage, release, and/or reuptake by the presynaptic cell, changes in activity of the deactivating enzyme, or by mimicking/blocking the neurotransmitter at the synapse (Brown, 1994). Individual neurotransmitters may be stimulatory and/or inhibitory on reproduction and may modulate the effects of other neurotransmitter systems resulting in a multitude of effects from alterations in one neurotransmitter (Trudeau et al., 2000). Furthermore, as each neurotransmitter may have more than one type of receptor and these varied receptors may be on different types of cells, alterations in the level of a single neurotransmitter may affect multiple cells in different ways. In fish, mercury has been shown to affect the levels of several neurotransmitters involved in regulating the synthesis and release of GnRH and the gonadotropins.

Mercury exposure decreased 5-HT concentrations in the brain of several fish species. Serotonin is synthesized from the amino acid tryptophan and activates the GnRH neurons to stimulate release of LH from the pituitary. In a laboratory study with Nile tilapia (*Oreochromis mossambicus*), 5-HT levels in the hypothalamus of males decreased significantly after six months with increasing concentrations of inorganic mercury from 15 to 30 μg/L (Tsai et al., 1995). No change in 5-HT levels was observed in the telencephalon or optic lobe. Serotonin levels also

decreased in whole brain of walking catfish (*Clarias batrachus*) exposed from 90 to 180 days to 40 μg/L methylmercury (Kirubagaran and Joy, 1990). A field study with mummichog (*Fundulus heteroclitus*), reported a decrease in 5-HT in the medulla but not the cerebellum of fish from a mercury contaminated site (Smith et al., 1995). The authors also noted a decrease in the 5-HT metabolite, 5-hydroxyindole acetic acid (5-HIAA), in contaminated fish suggesting the decrease in 5-HT may be due to reduced synthesis rather than increased degradation. These results agree with those of Tsuzuki (1982) who observed a decrease in 5-HT and 5-HIAA in the hypothalamus of rats exposed orally to 4.0 μg/g methylmercury. Further support in rats comes from the methylmercury-induced decrease in activity of tryptophan hydroxylase, the rate limiting biosynthetic enzyme in the 5-HT pathway (Tsuzuki, 1981). The inhibitory effects of mercury on the 5-HT pathway may differ based on developmental stage of fish. For example, methylmercury exposure (10 μg/L) increased 5-HT in heads of 7 and 14 day post-hatch mummichog larvae with no concurrent change in 5-HIAA (Zhou et al., 1999). Collectively these studies indicate that in sexually mature fish mercury exposure can decrease 5-HT levels.

Levels of the catecholamines, NE and DA, have also been measured after mercury exposure in fish. Dopamine is an inhibitory neurohormone in fish; it disrupts LH secretion by binding directly to D2 receptors on the gonadotrophs in the pituitary and by altering GnRH signaling (Van Der Kraak et al., 1998). Dopamine was significantly increased in brains of walking catfish after a 90 day exposure to 40 μg/L methylmercury (Kirubagaran and Joy, 1990). Levels of NE, a stimulatory monoamine synthesized from DA, were also increased significantly in whole brain of walking catfish after methylmercury exposure (Kirubagaran and Joy, 1990). Aqueous methylmercury elevated DA levels in heads of 7 and 14 day post hatch mummichog larvae without any change in its major metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) or

3-methoxy-4-hydroxyphenylacetic acid (Zhou et al., 1999). No significant differences in DA, nor NE or 5-HT, were observed in fathead minnows (*Pimephales promelas*) after short-term aqueous exposure however whole brain measurements were made which would have obscured any brain region specific changes (Grippo and Heath, 2003). In rats, methylmercury has also been shown to increase DA in the hypothalamus while decreasing the levels of DOPAC (Tsuzuki, 1982). Mercury concentrations in the brain of wild mink were negatively correlated with D2 receptor density and binding affinity suggesting a potential adaptive mechanism to counter the mercury-induced increase in DA (Basu et al., 2005). It is unknown whether this adaptive mechanism exists in fish. If mercury-exposed fish are unable to counter the increases in DA, LH release may be reduced and reproduction impaired.

As discussed above, LH release in fish is affected by several monoamine neurotransmitters (DA, NE, and 5-HT) suggesting that alterations in their rate of synthesis or degradation could have impacts on reproduction. The degradation of monoamines involves monoamine oxidase (MAO), which is an intraneuronal enzyme complex responsible for oxidative deamination (Norris, 1997). Inorganic mercury decreased MAO activity in murrel after aqueous exposure (Ram and Sathyanesan, 1985; Ram and Joy, 1988) and in Atlantic salmon after dietary exposure (100 μg/g) (Berntssen et al., 2003). Methylmercury induced a much more potent inhibitory effect on MAO activity than inorganic mercury in Atlantic salmon (Berntssen et al., 2003). Similarly, organic mercury was a stronger inhibitor of MAO than inorganic mercury in walking catfish (Kirubagaran and Joy, 1990). These results indicate that mercury in both its inorganic and organic forms is capable of impairing the monoaminergic systems known to be key modulators of hypothalamic-pituitary control of gonadotropic function, and thus reproduction.

However, the direct causal link between monoaminergic disruption and decreased reproduction has yet to be clearly established and is required for substantiation of this hypothesis.

1.1.3 The Pituitary

The gonadotropic hormones LH and FSH are released from the pituitary in fish and control the annual cycle of gonadal growth, ovulation in females, sperm release in males, and production of sex steroids in both sexes. Therefore disruption of gonadotropin secretion can have a major impact on fertility. The effects of mercury on the pituitary have therefore been limited to morphological analysis and measurements of pituitary derived hormone levels in plasma. Critically missing are studies on the direct effects of mercury on pituitary hormone release in vitro.

The morphological effects of mercurials on the pituitary gonadotroph in murrel and walking catfish have been studied by several groups. Histological analyses demonstrated that gonadotrophs were smaller, inactive, and fewer in number in both species of fish exposed to 10 to 50 μg/L methylmercury, inorganic mercury, or a mercurial fungicide for six months compared to controls (Ram and Sathyanesan, 1983; Ram and Sathyanesan, 1986b; Ram and Joy, 1988; Joy and Kirubagaran, 1989). The mercury-induced inhibition of gonadotropic activity was correlated with impaired gonadal development. It is not known if it is the decrease in sex steroid levels, and thus an alteration of gonadal feedback signals that is causing the inhibition of gonadotropic function. Conversely, if the action of mercurials is primarily directed at the hormone synthesis and release mechanisms of the pituitary, the resultant decrease in gonadotropin could lead to the inhibition in gonadal development. A thorough assessment of LH and FSH serum and mRNA levels would help to identify whether the pituitary is directly affected by methylmercury exposure. A recent study in ovariectomized rats observed a decrease in plasma LH after a three

day exposure to dietary methylmercury (Oliveira et al., 2006). Further studies are required in fish to determine the mechanism(s) of mercury-induced inactivation of the gonadotrophs. Nevertheless, evidence exists that exposure to mercurials upsets the fish HPG axis at some level.

1.2 Disruption of Male Reproductive Function

The cycle of spermatogenesis in which stem cells divide into mature spermatozoa is controlled by gonadotropic hormones (Gomez et al., 1999; Dziewulska and Domagala, 2003). Throughout this cycle, the testes increase in size which can be measured by an increase in the gonadosomatic index (GSI; gonad weight/body weight x100) and is usually maximal in the breeding season.

Disruption of testes function can be classified as either cytotoxicological or endocrine in origin (Kime, 1998). Cytotoxic effects are characterized by damage to cellular integrity or alterations in cell function, while endocrine effects involve the disruption of specific cells as a result of alterations in pituitary secretion or gonadal steroidogenesis. The following section describes the effects of mercury on the male gonad in terms of changes in testicular morphology and sperm development, changes in hormonal secretion, and direct effects on sperm.

1.2.1 Testicular morphology and spermatogenesis

Testicular growth (GSI) and spermatogenesis in murrel were arrested following exposure to inorganic mercury (10 μg/L) or a mercurial fungicide (200 μg/L) for six months compared to control fish (Ram and Sathyanesan, 1983; Ram and Sathyanesan, 1986b; Ram and Joy, 1988). Necrotic areas were visible within the testes of fish exposed to the mercurial fungicide suggesting direct cytotoxic effects (Ram and Sathyanesan, 1986b), however no obvious signs of degeneration were observed after inorganic mercury exposure (Ram and Sathyanesan, 1983).

Spermatogenesis was also inhibited and GSI decreased in male walking catfish after 180 days of exposure to organic and inorganic mercury at 30 to 50 μg/L (Kirubagaran and Joy, 1992). Similarly, guppies (*Poecilia reticulata*) (Wester, 1991; Wester and Canton, 1992) exposed to 5.6 μg/L and medaka (*Oryzias latipes*) (Liao et al., 2006) exposed to 40 μg/L methylmercury exhibited signs of testicular atrophy and arrested spermiation. Wester (1991, 1992) concluded that necrosis of the sperm was occurring during the process of spermatogenesis and proposed a mechanism involving the disruption of the mitotic spindle. Mercury has been shown to result in depolymerization of microtubules (Vogel et al., 1985; Miura et al., 1998) which form the mitotic spindle and this ability to interfere with the mitotic spindle has also been suggested as the mechanism behind mercury-induced genotoxicity in mammalian cells (Thier et al., 2003).

Disruption of testicular function has also been observed in environmentally relevant methylmercury exposures. Dietary exposure to methylmercury concentrations (0.1–1.0 μg/g) similar to those encountered by piscivorous fish in North America resulted in a significant decrease in GSI in juvenile male walleye with mean mercury concentrations of 0.25 and 2.37 μg/g ww (Friedmann et al., 1996). The normal morphology of the testes was significantly altered with the appearance of atrophy. Decreased spermatogenesis and atrophied seminiferous tubules were observed in male Nile tilapia after a six month exposure to methylmercury (Arnold, 2000). In this study, intraperitoneal implants released methylmercury gradually with final muscle concentrations ranging from 0.08 to 0.54 μg/g ww (assuming 80% water content) similar to levels reported for largemouth bass in the northeastern United States (Sveinsdottir and Mason, 2005). From these experiments, it can be concluded that both inorganic and organic mercury exposure via a variety of exposure routes can arrest gonadal development in male fish. This effect may be cytotoxic in nature as effects at the cell level include structural alterations in Leydig cells

(Ram and Sathyanesan, 1983; Ram and Sathyanesan, 1986b; Ram and Joy, 1988) and necrotic cell death (Wester, 1991; Wester and Canton, 1992). However, there are also implications of non-gonadal endocrine involvement as in several studies the inhibition of spermatogenesis was correlated with small and inactive gonadotrophs of the pituitary (Ram and Sathyanesan, 1983; Ram and Joy, 1988).

The data is inconclusive on the impacts of mercury exposure on testicular growth. In addition to the studies mentioned previously, there are several studies in which no change in GSI occurred. In wild caught northern pike there were no significant correlations between mercury content and GSI or gonadal sex steroid concentration (Friedmann et al., 1996); however the small sample size ($n = 7$) and high variability may have masked any subtle effects. Dietary methylmercury decreased sex steroid levels but had no effect on gonadal development in male fathead minnows after 250 days of exposure (Drevnick and Sandheinrich, 2003). Similarly, GSI was not affected in male tilapia exposed to methylmercury despite changes in testicular morphology and spermatogenic arrest (Arnold, 2000). This indicates that histology may provide a more sensitive indicator than GSI for assessing the effects of mercury on gonadal development.

Mechanisms underlying testicular atrophy resulting from mercury exposure are unknown but may involve alterations in mitotic activity (Wester, 1991; Thier et al., 2003). Alternatively, mercury exposure can reduce both the size and number of gonadotrophs (Joy and Kirubagaran, 1989). In goldfish, gonadotropin promotes the survival of gonadal cells during the late stage of spermatogenesis (Andreu-Vieyra and Habibi, 2001). Therefore, methylmercury-induced testicular atrophy may be due to the induction of apoptosis through alterations in pituitary-derived survival factors. Atrophy in developing testes could directly impact individual reproductive potential.

1.2.2 Testicular hormone production

Gonadotropic regulation of spermatogenesis and spermiogenesis in fish is mediated by androgens secreted by the interstitial cells (Yaron, 1995). Murrel testes undergoing spermiation contain active interstitial cells characterized by large rounded nuclei with prominent nucleoli. In contrast, inactive and atrophied interstitial cells were observed in murrels exposed to mercury (Ram and Sathyanesan, 1983; Ram and Sathyanesan, 1986b; Ram and Joy, 1988; Kirubagaran and Joy, 1988a). Interstitial cells were inactive and showed signs of degeneration including atrophy and pyknosis in the male walking catfish after mercury exposure (Kirubagaran and Joy, 1992). Exposure to methylmercury resulted in inflammation and fibrosis of the interstitium in male guppies (Wester, 1991; Wester and Canton, 1992). These cytotoxic effects imply potential adverse effects on the steroidogenic potential of the interstitial cells.

The biosynthesis of androgens and estrogen requires 3β-hydroxy-Δ_5-steroid dehydrogenase (3βHSD); a decrease in 3βHSD activity has been used to indicate decreased steroidogenic activity (Kime, 1998). Kirubagaran and Joy (1988a, 1988b) exposed male catfish to organic and inorganic mercury at 30 to 40 μg/L for six months and then measured 3βHSD activity, *ex vivo*, in sections of testes. The activity of 3βHSD was completely inhibited in testes from methylmercury exposed fish and to a lesser degree by inorganic mercury. The loss of enzyme activity may be a direct action of mercury on the testes or an indirect one at the hypothalamic-pituitary level through inhibition of gonadotropin secretion as 3βHSD is influenced by gonadotropin (Jones and Hsueh, 1982; Prasad et al., 1999). Furthermore, impaired steroidogenesis may have lead to the inhibition of gonadal growth and spermatogenesis reported in a similar experiment (Kirubagaran and Joy, 1992).

In male fish, androgens are responsible for gonadal development, secondary sexual characteristics, and behaviour (Kime, 1998). Testosterone (T) and/or 11-ketotestosterone (11-KT) secretion is high during gonadal recrudescence and declines prior to spermiation (Kime, 1993). Plasma T levels significantly decreased in male fathead minnows fed diets containing 0.87 to 3.93 μg/g methylmercury for 250 days (Drevnick and Sandheinrich, 2003). Decreases in plasma 11-KT were also observed in male Nile tilapia after a six month exposure to methylmercury with no concurrent decrease in 17β-estradiol (E2) (Arnold, 2000). These studies provide further evidence that mercury alters steroidogenesis in male fish. However, further studies are required to determine whether the effect is direct at the gonad level and/or indirect via disruption of the hypothalamus or pituitary.

In wild fish, the effects of mercury accumulation on androgens, particularly 11-KT, are unclear. Similar to laboratory studies, both plasma androgens (T and 11-KT) were negatively and significantly correlated with muscle mercury content in sexually immature white sturgeon (*Acipenser transmonatus*) (Webb et al., 2006). From this study a mercury concentration threshold associated with steroidogenic effects was estimated to be 0.2 μg/g ww (Scheuhammer et al., 2007). However, no significant alterations in 11-KT levels were observed in sexually mature common carp (*Cyprinus carpio*), brown bullhead (*Ameiurus nebulosus*), smallmouth bass (*Micropterus dolomieui*), or largemouth bass containing mercury concentrations greater than this threshold. Nor were any alterations in T levels correlated with mercury accumulation in recrudescent northern pike with mercury concentrations ranging from 0.28 to 0.64 μg/g ww (Friedmann et al., 1996). Although not statistically significant, T levels in prespawning largemouth bass from a high mercury lake (mean muscle concentration 5.42 μg Hg/g ww) were found to be over 50% lower than those of bass in a control lake (mean muscle concentration 0.30

μg Hg/g ww) (Friedmann et al., 2002). Conversely, a positive correlation between plasma 11-KT concentrations and mercury residues was observed in the male bass. To our knowledge, this is the only instance of increased steroidogenesis in male fish associated with methylmercury exposure. Increased steroidogenic enzyme activity and a concurrent increase in plasma steroid levels were observed in female fish exposed to inorganic mercury. Positive correlations were also observed between 11-KT and tissue mercury concentrations in female smallmouth bass and common carp (Baldigo et al., 2006). Field surveys are complicated by the presence of multiple contaminants in addition to methylmercury which may impact steroidogenesis. Although correlations exist between mercury concentrations and sex steroid levels, the effect of additional contaminants cannot be ruled out. This is particularly true for the largemouth bass study showing increased 11-KT as the levels of other contaminants were not measured in these fish. In addition to the confounding factor of multiple contaminants, the differing results may also be attributed to species differences and the state of gonadal development during which the sampling took place.

Cholesterol plays a key role in reproduction in teleosts serving as the lipid precursor for gonadal steroids (Kirubagaran and Joy, 1992). Lipids mobilized from the liver gradually increase in the gonads from the preparatory to spawning phase of the reproductive cycle. Testicular cholesterol levels decreased in male catfish exposed for 180 days to both organic and inorganic mercury (Kirubagaran and Joy, 1992), while hepatic cholesterol levels increased after mercury exposure in the freshwater murrel (Ram and Sathyanesan, 1987). Both studies suggest mercury exposure resulted in a decreased mobilization of lipids from the liver to gonads. Impaired lipid metabolism may be partially responsible for the inhibition of spermatogenic and steroidogenic activities in mercury treated fish.

1.2.3 Sperm morphology and motility

The morphology and motility of sperm are useful in assessing the total impact of mercurials on the male reproductive system as they are critical factors for fertilization success in teleosts. Instantaneous (5 s) and short term (24 h) exposure of sperm are representative of the effect of mercury on sperm released into a polluted aquatic environment (Van Look and Kime, 2003). Short-term exposure of milt to 1.0 μg/L inorganic mercury decreased the motility of African catfish (*Clarius gariepinus*) sperm (Rurangwa et al., 1998). A short-term aqueous exposure of mummichog sperm to 50 μg/L methylmercury resulted in decreased motility without any apparent morphological effects (Khan and Weis, 1987b; Khan and Weis, 1987c). Instantaneous exposure of goldfish sperm to inorganic mercury reduced curvilinear velocity at 1 μg/L, the percentage of motile sperm at 10 μg/L, and flagellar length at 100 μg/L (Van Look and Kime, 2003). The decreased motility of sperm and morphological changes in the flagellum reported in some species may be a result of mercury binding to protein sulfhydryl groups located on the membranes of the nucleus, midpiece and tail. Mercury can react with sulfhydryl groups on microtubules and inhibit microtubule assembly *in vitro* (Vogel et al., 1985) which may affect the microtubule sliding mechanism required to generate waves along the flagellum (Gagnon, 1995). Mercury may also interfere with the function of sperm mitochondria resulting in a decrease in energy production (Rao, 1989).

The success of fertilization events are likely to be reduced or completely inhibited by alterations in sperm motility and morphology. Fertilization success was decreased after short term ($\leq$ 40 min) exposure of rainbow trout (McIntyre, 1973; Billard and Roubaud, 1985) and mummichog (Khan and Weis, 1987b; Khan and Weis, 1987c) sperm to inorganic or methylmercury (1–1000 μg/L). However, it is now known that fertilization success is dependent

on the ratio of sperm:egg used in each test since high ratios will mask toxic effects by providing sufficient spermatozoa to overcome the spermiotoxic effects (Rurangwa et al., 1998). None of the above studies discuss the ratio used, and therefore may have underestimated the toxicity of inorganic and organic mercury.

Sperm can also be exposed to mercury in seminal fluids (i.e., *in vivo*) of contaminated fish. A 24 hour exposure in an extender buffer has been designed to mimic this type of exposure *in vitro*. Goldfish sperm, which exhibited no effects after instantaneous exposure, had decreased flagella length and motility after pre-incubation in an extender buffer with 100 μg/L inorganic mercury (Van Look and Kime, 2003). Attempts have been made to measure the effects of *in vivo* sperm exposure on fertilization success (Hammerschmidt et al., 2002). Thus far, the results have been inconclusive due to the inherent difficulty in assessing the effects on sperm in isolation from other reproductive impairments.

1.3 Disruption of Female Reproductive Function

Mercurial exposure can also affect reproductive endpoints in female fish. Mercury has been shown to affect ovarian morphology, delay oocyte development, and inhibit steroid hormone synthesis. As with the testes, it is difficult to determine whether mercury affects the ovaries directly or indirectly via alterations at the level of hypothalamus or pituitary, and whether the effects are the cause or result of changes in steroidogenesis. Unlike the male, female gamete production involves the synthesis and incorporation of a yolk protein which may transfer some of the maternal mercury burden to the embryo. The effects of mercury on the female reproductive system discussed in the next section are separated into changes at the level of ovarian morphology and egg development, steroid hormone and vitellogenin synthesis, fecundity and spawning, and, ultimately, egg and embryo survival.

1.3.1 Ovarian morphology and oogenesis

Oogenesis in teleosts involves the maturation from immature or previtellogenic oocytes through the accumulation of yolk vesicles during vitellogenesis and finally the formation of mature oocytes (Tyler and Sumpter, 1996). This cycle of oogenesis was significantly repressed in the freshwater murrel after exposure to inorganic mercury or mercurial fungicide (10–20 μg/L) with a concurrent decrease in GSI (Ram and Sathyanesan, 1983; Ram and Sathyanesan, 1986a; Ram and Sathyanesan, 1986b; Ram and Joy, 1988; Dey and Bhattacharya, 1989). After 180 days of exposure only immature oocytes completely devoid of vitellogenin were present while the control ovaries were fully developed with a large number of mature and maturing oocytes (Ram and Sathyanesan, 1983; Ram and Sathyanesan, 1986a; Ram and Sathyanesan, 1986b; Ram and Joy, 1988; Dey and Bhattacharya, 1989). Degenerative changes, namely nucleolar necrosis and atresia (follicular degeneration), were also observed in some of the fungicide and inorganic mercury treatment groups (Ram and Sathyanesan, 1986a; Ram and Sathyanesan, 1986b; Dey and Bhattacharya, 1989). Ovarian recrudescence was arrested with some atretic changes and a decrease in GSI of walking catfish exposed over six months to inorganic and organic mercury (Kirubagaran and Joy, 1988b). Atretic follicles were also more common in female Nile tilapia after exposure to methylmercury implants; however no change in GSI was observed (Arnold, 2000). Common spiny loach (*Lepidocephalichtyhs thermalis*) exposed to inorganic mercury for 10 days at 100 μg/L had extensive vacuolation in the oocortex, necrosis of the oolemma and hypertrophy of follicular cells leading to atresia of the oocytes after 20 days (Victor et al., 1986). In sexually mature largemouth bass the frequency of atretic oocytes was positively correlated with sediment mercury concentrations in a Tennessee reservoir system (Adams et al., 1999).

These degenerative changes (e.g., atresia, necrosis) are suggestive of cytotoxic actions on the ovary.

Apoptosis is the main trigger for follicle atresia in mammals (Hsueh et al., 1994) and is involved in normal teleost ovarian development and regression (Wood and Van Der Kraak, 2001). A significant increase in the number of apoptotic cells was measured in ovaries from fathead minnows exposed to dietary methylmercury as juveniles through to sexual maturity (Drevnick et al., 2006). Thus the induction of apoptosis in steroidogenic gonadal cells is one possible mechanism by which sex steroid levels are reduced in fish exposed to methylmercury. Apoptosis may be a result of oxidative stress (Ueda et al., 2002) which can be induced by methylmercury exposure (Berntssen et al., 2003). Apoptosis occurs spontaneously in ovaries and is regulated by factors that promote survival of ovarian follicles including FSH, LH, and E2 (Wood and van der Kraak, 2002). Therefore, an alternative mechanism may involve methylmercury-induced alterations in follicular survival factors resulting in apoptosis. Reduced serum levels of LH were observed in methylmercury-exposed goldfish and decreased serum E2 levels are correlated with mercury concentrations in wild caught fish and in laboratory fish studies. Furthermore, in the absence of degenerative changes, gonadal arrest is related to endocrine alterations resulting from a decrease in activity of pituitary gonadotrophs (Ram and Sathyanesan, 1983; Ram and Joy, 1988).

1.3.2 Steroidogenesis

In female fish, E2 is the major reproductive hormone and is primarily produced in the follicle of the developing oocyte (Kime, 1998). Luteinizing hormone stimulates the production of T which is then converted into E2 via the aromatase enzyme. One of the primary roles of E2 is to stimulate liver production of the yolk protein, vitellogenin, which is then incorporated into the

maturing oocyte to later serve as nourishment for the developing fish larvae. Similar to T, gonadal E2 secretion and subsequent vitellogenin production change with maturation of the oocytes and thus, plasma levels can be used to monitor the reproductive stage of fish.

In female Nile tilapia, E2 levels decreased over the summer months and remained low until November when there was a surge in hormone level to prepare for the new reproductive season (Arnold, 2000). This hormone surge was repressed in fish exposed to methylmercury which may suggest an interference with the production of stimulatory pituitary hormones or gonadal steroidogenesis. Methylmercury accumulation was also correlated with decreased E2 in fathead minnows exposed as juveniles through to sexual mature adults (Drevnick and Sandheinrich, 2003). In addition to estrogen, androgen (11-KT) levels were measured in laboratory exposures with female tilapia, however no significant changes were observed. In wild fish, sex steroid levels have been both negatively and positively correlated with total mercury concentrations. Similar to laboratory studies, plasma E2 concentrations in sexually mature female largemouth bass were negatively correlated with total mercury in muscle (Adams et al., 1999). Significant negative correlation between plasma E2 and liver mercury was also detected in sexually immature white sturgeon collected in the lower Columbia River (Webb et al., 2006). No significant correlations between mercury and E2 were measured in sexually mature female smallmouth bass and carp from the Hudson River; however, in the same fish 11-KT was significant positively correlated with total mercury (0.09–0.47 μg/g ww) (Baldigo et al., 2006). Taken together, these laboratory and field based studies suggest that methylmercury exposure, at concentrations currently found in the environment, can induce alterations in serum sex hormone levels.

The synthesis of steroid hormones involves a series of enzymatic reactions which may be sensitive to mercury exposure. Inorganic mercury has been shown to specifically bind to oocyte plasma membrane where it inhibits sodium-potassium-ATPase activity and alters steroidogenesis in murrel (Mondal et al., 1997). One of the initial steps in E2 production is the conversion of pregnenolone to progesterone via 3βHSD (Norris, 1997). Both *in vitro* and *in vivo* experiments demonstrated that mercury exposure results in a decrease in E2 and a build up of progesterone in the ovary related to an increase in 3βHSD activity (Mondal et al., 1997). Further along the steroidogenic pathway, aromatase catalyzes the conversion of androgens to estradiol. Using a rainbow trout microsome-based assay, methylmercury was shown to significantly reduce aromatase activity in both the ovary (IC_{50} 0.78 μM) and brain (IC_{50} 11 μM) (Hinfray et al., 2006). Therefore, alterations in steroidogenic enzyme activity may be one direct mechanism by which methylmercury exposure reduces serum E2. Further mechanistic studies are required to clarify the effects of mercury on the molecular regulation of steroidogenesis, including the expression and activity of steroidogenic enzymes *in vivo*. Whether the suppression of E2 is sufficient to induce apoptosis in ovarian follicular cells is unclear and it remains to be determined whether methylmercury exposure can alter other follicular survival factors.

1.3.3 Vitellogenesis

Estradiol stimulates the liver to synthesize the phospholipoglycoprotein yolk precursor vitellogenin in oviparous vertebrates (Ng and Idler, 1983). Altered expression of vitellogenin mRNA and its protein is one of the most commonly used biomarkers of aquatic contaminant exposure (Sumpter and Jobling, 1995); in males, vitellogenin is not normally expressed but can be induced by exposure to estrogenic chemicals. In control female catfish, serum vitellogenin levels doubled after E2 injection (Panigrahi et al., 1990). However, after injection of mercuric

chloride, serum vitellogenin levels decreased and did not respond to subsequent E2 injection. Vitellogenin synthesis was also inhibited in catfish after a 90 to 180 day exposure to inorganic and organic mercury as indicated by a decrease in ^{32}P-labelled phosphoprotein content in the liver and ovaries (Kirubagaran and Joy, 1995). A survey of freshwater species from various sites along the Hudson River provided equivocal data; vitellogenin levels are negatively correlated with mercury concentrations in female smallmouth bass and male common carp but not altered in either sex of largemouth bass or bullhead (Baldigo et al., 2006). This inhibition of vitellogenin synthesis upon exposure to inorganic or organic mercury may be a result of impaired steroidogenesis or alterations in estrogen signalling pathways.

Recent studies have demonstrated alterations in vitellogenin mRNA levels in livers from fish exposed to dietary methylmercury in lab-based and *in situ* exposures. Similar to the reduction in vitellogenin protein observed in catfish, vitellogenin mRNA was down-regulated by 2- to 4-fold in female fathead minnows exposed for 600 days to dietary methylmercury (3.93 μg/g) (Klaper et al., 2006). Conversely, some male fathead minnows had elevated vitellogenin levels but the results were highly variable and not statistically significant. Cutthroat trout from a high mercury lake (0.054 μg/g ww whole fish tissues) had 5-fold lower vitellogenin mRNA levels than fish sampled from a low mercury lake (0.016 μg/g ww whole fish tissues) based upon cDNA microarray analysis of liver RNA (Moran et al., 2007). Unfortunately, this study with trout did not consider gene expression in males and females separately making it difficult to make any specific conclusions regarding the impact of methylmercury exposure on vitellogenin expression. On the whole, there is significant evidence suggesting that methylmercury alters vitellogenesis in females with reductions measured in mRNA, protein and lipid levels after exposure. Increased

vitellogenin expression in males may be due to an estrogen-like activity of methylmercury as it has been shown to bind to and activate estrogen receptors (Martin et al., 2003).

1.3.4 Lipids

During the gonadal cycle, there is a reciprocal relationship between hepatic and gonadal lipids; the balance of which may be altered by pollutants such as mercury. In the peak of reproductive activity, hepatic lipid levels decrease with a concomitant increase in gonadal levels. Phospholipids are an important component in the structure of proliferating and developing germ cells and as reserve food in the form of yolk or as energy sources. Cholesterol, on the other hand, is the precursor for steroid hormones. Lipid content increased in the liver (and muscles) with a concurrent decrease in the ovaries of bronze featherback (*Notopterus notopterus*) exposed for 30 days to inorganic mercury (44–88 μg/L) (Verma and Tonk, 1983). Lipid levels (total lipids, phospholipids, and cholesterol) also increased in the liver of walking catfish with a simultaneous decrease in all forms of lipids in the ovaries after a 180 day exposure to inorganic and organic mercury (Kirubagaran and Joy, 1995). However, murrel exposed to inorganic mercury had a significant reduction of lipid and cholesterol in the liver and ovary (Ram and Sathyanesan, 1985). In summary, these results suggest a mercury induced change in lipid retention and/or inhibition of seasonal mobilization of lipids from liver to ovary during ovarian recrudescence. Lipid balance disturbance by mercury may result in other disruptions including impairment of vitellogenesis (implicated in arrest of oogenesis) and steroidogenesis.

1.3.5 Effects on ovulation and spawning

Mercury exposure can alter fecundity (number of eggs produced) and spawning of female fish. Fewer zebrafish eggs were produced after exposure to 1.0 μg/L of a mercurial fungicide

(Kihlstrom et al., 1971). Fertilization success was reduced in offspring (whole-body concentrations of 1.1 to 1.2 μg Hg/g ww) of mummichog fed methylmercury-contaminated diets (Matta et al., 2001). Recently two studies were performed with fathead minnows exposed to dietary methylmercury as juvenile fish through sexual maturation to determine the effects on reproduction (Hammerschmidt et al., 2002; Drevnick and Sandheinrich, 2003). In these studies, an overall decrease in spawning success was observed as a consequence of reduced gonadal growth correlated to a decrease in E2 levels, decreased fecundity, and increased number of days to spawning in fish with carcass mercury concentrations ranging from 0.57 to 3.68 μg/g ww. Days to spawning appears to be influenced by the female as contaminated females mated with control males took on average three days longer to spawn than control females mated with contaminated males (Hammerschmidt et al., 2002). In male fathead minnows with carcass mercury concentrations of 0.71 to 4.23 μg/g ww, spawning behaviour and spawning success were reduced after dietary methylmercury exposure as juveniles through to sexual maturity (Sandheinrich and Miller, 2006). As androgens are critical in regulating reproductive behaviour, methylmercury-induced impairment of reproduction may be due to alterations in sex steroids resulting in altered behaviour. A female-specific delay in spawning can be especially detrimental if sperm release is not similarly delayed. Delayed spawning can have significant effects on the offspring as it may disrupt the timing of feeding relative to seasonal food resources. If young fish are unable to find food as an indirect effect of mercury exposure, their growth may be affected leading to increased susceptibility to predation and reduced survival.

1.3.6 Eggs and embryos

Mercury in eggs originates primarily from maternal transfer and is predominantly (> 90%) in the methylated form (Hammerschmidt et al., 1999; Johnston et al., 2001; Latif et al.,

2001). Originally, it was believed that maternal transfer occurred by mobilization and transfer of stored mercury to the gonads during oogenesis (Niimi, 1983). However, the actual amount transferred is only 0.2 to 2.3% of the maternal burden (Niimi, 1983; Hammerschmidt et al., 1999; Johnston et al., 2001). In contrast, organochlorines are transferred to the eggs at 5.5% to 25.5% of the whole body burden. The diet of the maternal adult during oogenesis, rather than body burden, is now accepted as the principal source of methylmercury in eggs (Hammerschmidt and Sandheinrich, 2005). Therefore, the exposure of embryos to methylmercury depends on the levels of mercury in the prey of the adult during oogenesis, which can vary intra- and interannually.

Mercury exposure may diminish the reproductive capacity of maternal fish by reducing the hatching success of embryos. Hatching success of zebrafish embryos in unpolluted water was decreased after maternal exposure to 0.2 μg/L of a mercurial fungicide (Kihlstrom et al., 1971). Maternal exposure may also alter the sensitivity of eggs to mercury in the environment. Mummichog eggs from a polluted site and a control site were exposed to methylmercury prior to fertilization with untreated sperm (Khan and Weis, 1987a). Eggs from the polluted creek demonstrated an increased tolerance to methylmercury. Scanning electron microscopy revealed that the control eggs exposed to methylmercury had an increased incidence of spontaneous micropyle blockage resulting in reduced fertilization success (Khan and Weis, 1993). These studies indicate that pre-exposure of eggs to mercury in maternal fish can decrease hatching success in the lab while multi-generational exposure in wild populations may convey increased resistance to mercury pollution.

In addition to maternal transfer, mercury bioaccumulation in eggs and embryos can occur via bioconcentration of mercury from the water. Upon spawning, eggs are released into the aquatic environment where they may be exposed to additional methylmercury. Mercury was

initially bound primarily to the chorion of Chinook salmon (*Oncorhynchus tshawytscha*) eggs, and over a five day exposure period partitioned into the yolk mass (Hammock et al., 2003). Bioconcentration factors averaging 2025 were measured after a 16 day exposure of medaka embryos to inorganic mercury (10–30 μg/L) (Heisinger and Green, 1975). Fathead minnow embryos quickly accumulated methylmercury to a final bioconcentration factor of 70 after 96 hours (Devlin, 2006). In these embryos, protein synthesis was initially stimulated followed by a significant reduction after longer exposure to 25 μg/L methylmercury. Hatching success was decreased in embryos exposed to 15 μg/L with no hatching occurring at higher concentrations. Short term exposure (1–5 days) of mummichog embryos to higher concentrations of inorganic (40 μg/L) or methylmercury (30 μg/L) decreased survival while a sub-chronic (32 day) exposure to 10 μg/L decreased hatching success (Sharp and Neff, 1980; Sharp and Neff, 1982). Embryos of the mummichog exposed to 5 μg/L methylmercury demonstrated no change in their morphology, yet exhibited impairment in the ability of larvae to capture prey (Zhou et al., 2001). Similarly Atlantic croaker (*Micropogonias undulatus*) larvae exposed to maternally derived methylmercury (50–100 μg/L) had impaired survival skills as measured by the responsiveness to a vibratory startle stimulus (Alvarez et al., 2007). Together these studies clearly show that at μg/L concentrations, aqueous mercury can quickly accumulate in eggs and have negative impacts on hatching success, embryonic development, and larval behaviour. These effects are significant however their ecological relevance are limited as mercury concentrations in natural habitats are in the ng/L range and diet, not water, is the primary route of mercury exposure for fish in the environment.

Environmentally relevant concentrations of waterborne methylmercury were used to examine the effects on embryonic and larval stages of walleye (Latif et al., 2001). Hatching

success significantly declined with increasing waterborne methylmercury (0.1–7.8 ng/L), while the methylmercury concentration in the eggs did not have any significant effect. In the same way, parental exposure to dietary methylmercury had no significant effect on development, hatching success or larval survival in fathead minnows (Hammerschmidt et al., 2002) and mummichog (Matta et al., 2001). The effect of waterborne methylmercury is likely due to bioconcentration during development as the larvae contained 2- to 21-fold higher methylmercury concentrations than the unfertilized eggs. Therefore, independent of maternal transfer, methylmercury in aqueous environments at current concentrations has the potential to negatively impact embryonic development of wild fish populations.

1.4 Conclusions

This review has collated data from both laboratory and field studies supporting the hypothesis that mercury in the aquatic environment impacts the reproductive health of fish. The evidence presented suggests that the inhibitory effects of mercurials on reproduction may occur at multiple sites within the reproductive axis. A variety of endpoints have been measured but the majority of studies have focused at the level of the gonads measuring alterations in GSI, gametogenesis, steroidogenesis, and vitellogenesis. There are some notable variations in response to mercury exposure which may be attributed to one or more of the following factors: species, life stage (e.g., larval, juvenile, adult), state of gonadal development (e.g., recrudescent, spawning, regressed), route of exposure, length of exposure, mercurial species, and mercury concentration. For example, spawning success was reduced in fathead minnows exposed as juveniles but not as sexually mature adults (Hammerschmidt et al., 2002) suggesting that some life stages or stages of gonadal development may be more sensitive to methylmercury exposure. Species differences are likely as negative correlations were significant between vitellogenin levels and mercury content

in smallmouth bass and carp but not in bullhead or largemouth bass collected from the same locations and with comparable mercury burdens (Baldigo et al., 2006). Further, dietary methylmercury exposure stimulated somatic growth in juvenile fathead minnows while a similar exposure in juvenile walleye significantly decreased growth (Friedmann et al., 1996). Nevertheless, across all studies there appears to be a trend for reduced gonadal development, impaired gametogenesis and decreased sex steroid levels. The consensus effects of mercury exposure on the reproductive axis in fish are listed in Table 1.1. These alterations are associated with altered vitellogenesis and reduced spawning success suggesting that methylmercury-induced reproductive impairment may have negative impacts on wild fish populations.

There is some evidence that fish and other wildlife species may develop an adaptation to mercury. Mercury exposure can affect reproduction by reducing fecundity which may result in more offspring produced by fish which are, or whose gametes are, more resistant. This possible scenario could result in more mercury-resistant individuals. Studies with mummichog from both a clean reference site and a mercury contaminated site provides evidence for this occurring in wild fish populations (Khan and Weis, 1987b). Gametes and embryos of contaminated mummichogs exhibited tolerance to methylmercury and, as a result, were relatively unaffected by concentrations that had severe effects on gametes and embryos from the reference site. The effects however in mummichog are stage specific with contaminated adults exhibiting reduced growth and condition (Weis et al., 2001). Receptor binding studies in wild mink have shown correlations between neurotransmitter receptor densities and mercury concentrations in the brain which the authors suggest may represent adaptive mechanisms to maintain normal neurotransmission (Basu et al., 2005). Adaptation may occur in wild populations however, the long-term effects and ecological significance remain unknown.

Despite the large number of studies indicating the harmful effects of mercurials on reproduction, there still remains a paucity of studies examining the specific mechanism and site of action. Methylmercury is both a putative endocrine disrupting chemical and a neurotoxicant (Kime, 1998; Zhou et al., 1999; Hammerschmidt et al., 2002; Aschner et al., 2007). In mammalian models, research has primarily focused on the underlying mechanisms of methylmercury-induced neurotoxicity while potential endocrine disrupting effects are limited to developmental alterations in the fetus. Conversely, the majority of fish studies have followed the classical endocrine disruptor approach of measuring reproductive impairment through fecundity, spawning success and alterations in the HPG axis. Studies in fish have identified impacts of methylmercury on reproduction but in order to understand the underlying mechanisms of these effects it is critical to look at alterations in neuroendocrine control as the central nervous system is the primary target for methylmercury action (Clarkson and Magos, 2006).

Several physiological and cellular mechanisms have been proposed in this review. From the physiological perspective, reproductive impairment may be due to alterations in any of the factors involved in regulating the HPG axis. First, changes in neurotransmitter levels or other parameters of neurotransmission could affect the release of GnRH and/or LH from the hypothalamus and pituitary. Second, inhibition of GnRH or LH synthesis and release would cause a cascade effect resulting in decreased gonadal hormone production and subsequent decreases in vitellogenesis, gonadal growth, and GSI. Third, alterations in gonadal steroidogenesis would affect gamete production and pituitary hormone synthesis and release via positive feedback mechanisms. Last, altered lipid balance may lead to disruption of normal processes including steroidogenesis and vitellogenesis. Cellular mechanisms have also been proposed which may result in general toxicity and/or specific impairment of endocrine processes. The cytoskeleton is a target for methylmercury and disruption of cytoskeletal components could

impair the normal mitotic division which occurs during gamete development. Apoptosis has also been proposed as a mechanism by which methylmercury impairs the steroidogenic capacity of gonads. The reproductive axis is an integrated system, however many of the studies reviewed here examined only a single parameter of the axis. Further, data implicating the direct involvement of any of these mechanisms in reproductive impairment is still lacking. These studies provide evidence of reproductive inhibition but do not clarify whether the primary site of action is the gonad itself or whether it is due to inhibition of pituitary secretion or altered neurotransmission in the hypothalamus or other brain regions. Additionally, changes at the level of the transcriptome or proteome associated with reproductive dysfunction remain to be determined.

In summary, there appears to be sufficient evidence from laboratory studies to link exposure to mercury with reproductive impairment in many fish species. It is now necessary to determine the primary site of action and mechanisms involved in endocrine disruption at environmentally relevant exposures to methylmercury. Linking reproductive impairment with an ecologically relevant impact on real fish populations is a further challenge. Fish are a large food source for both humans and wildlife and the effects of mercury exposure described in this review on fish may similarly occur in these piscivorous vertebrates. Therefore, mercury pollution is a concern for both fish populations and their consumers as it can act both indirectly to decrease the food supply and directly by concentrating in the food chain itself to affect top predators such as humans.

CHAPTER 2

Methylmercury-induced disruption of reproductive hormones in adult female goldfish (*Carassius auratus*)

2.1 Introduction

Contaminants in the environment are a concern due to increasing levels and potential impacts on human and wildlife health. Of particular concern are those contaminants capable of disrupting normal function of the endocrine system. These substances are generally known as endocrine disrupting chemicals (EDC) based upon their "ability to disrupt the synthesis, secretion, transport, binding, action or elimination of hormones in an organism, or its progeny, that is responsible for the maintenance of homeostasis, reproduction, development or behaviour of an organism" (Canadian Environmental Protection Act 1999). Chemicals classified as endocrine disruptors include persistent organohalogens, pesticides, and heavy metals (i.e., cadmium, lead, mercury). Methylmercury is a putative endocrine disruptor and a potent neurotoxin found in aquatic species worldwide. Mercury is emitted into the environment through anthropogenic and natural processes and converted to methylmercury by organisms in aquatic sediments. Once in the aquatic environment, methylmercury bioaccumulates and concentrates in food chains such that fish can have methylmercury concentrations 10^6 to 10^7 times higher than the surrounding water (Kim and Burggraaf, 1999; Bowles et al., 2001).

Bioaccumulation of methylmercury in fish occurs predominantly through dietary uptake (Hall et al., 1997). Methylmercury enters through the gut, binds to red blood cells, and is transported throughout the body; it readily crosses biological membranes, including the blood-brain barrier, and binds to proteins and free amino acids (Weiner and Spry, 1996; Wiener et al., 2003). The distribution of methylmercury within fish is highly efficient occurring in a matter of hours to days (Leaner and Mason, 2004). Conversely, the elimination of methylmercury is very

slow occurring in the order of months to years (Lockhart et al., 1972; McKim et al., 1976; Trudel and Rasmussen, 1997; Clarkson, 2002; Liao et al., 2006). Consequently methylmercury content typically increases with age and/or size of fish within a given population (Phillips and Buhler, 1978).

Accumulation of methylmercury can affect a wide range of physiological processes in fish from growth and development to reproduction (Sorensen, 1991; Weiner and Spry, 1996). A systematic analysis of studies assessing sub-lethal endpoints (growth, reproduction, development, behaviour) of mercury exposure determined a protective (i.e., unlikely to cause adverse effects in fish) threshold of 0.2 μg Hg/g ww for juvenile and adult fish (Beckvar et al., 2005). Muscle mercury concentrations in freshwater piscivorous fish from Québec have been reported as high as 5.6 μg Hg/g ww for lake trout (Laliberté, 2004). However, mean mercury concentrations in muscle of freshwater fish from North America normally range from 0.01 to 1.2 μg/g ww with a nationwide mean concentration of 0.10 μg/g ww in the United States (Schmitt and Brumbaugh, 1990; Fisk et al., 2003; Kamman et al., 2005). At current levels of methylmercury in the environment, reduced reproductive success has been suggested as the most plausible effect of mercury on wild fish populations (Weiner and Spry, 1996).

Studies in fish have assessed a variety of reproductive endpoints and demonstrated that methylmercury can affect the reproductive health of several species. Impairments in gonadal development, steroidogenesis, spawning behaviour, and spawning success have been observed after environmentally-relevant dietary methylmercury exposures in laboratory studies with walleye, tilapia, and fathead minnows (Friedmann et al., 1996; Arnold, 2000; Hammerschmidt et al., 2002; Drevnick and Sandheinrich, 2003; Drevnick et al., 2006; Sandheinrich and Miller, 2006). Some risk assessment studies of freshwater ecosystems have also demonstrated negative

correlations between mercury content and reproductive biomarkers (e.g., GSI, E2, T, 11-KT, vitellogenin) in largemouth bass, smallmouth bass, common carp, and white sturgeon (Adams et al., 1999; Baldigo et al., 2006; Webb et al., 2006). However, there exists some disparity in the effects observed in wild fish species. For example, a study of largemouth bass and bluegill sunfish from a large river-reservoir system observed a positive correlation between follicular atresia and sediment mercury while only largemouth bass demonstrated a negative correlation between muscle mercury concentration and E2 (Adams et al., 1999). Studies with wild fish are confounded by the natural fluctuations in population demographics as well as the presence of other contaminants which may impact the reproductive axis. Based upon laboratory studies using chronic exposures initiated in juvenile fish, impairment of reproductive health has been observed repeatedly in fish with tissue mercury concentrations above the 0.2 μg/g ww protective threshold. Whether these effects occur in wild fish populations is less clear. Further, there are limited studies assessing the impacts of ecologically relevant methylmercury exposure on adult fish.

From 1983 to 1998, mercury exposures with adult fish were predominantly aqueous using mercury concentrations at least 10^1 to 10^3 times higher than those found in the environment. These studies have demonstrated negative effects of mercury at each level of the reproductive axis, however the relevance to wild populations may be limited as diet is the primary route of exposure for fish. More recently two laboratory studies have examined the effects of environmentally relevant concentrations of methylmercury on adult fish. In her Ph.D. thesis, Arnold (2000) outlines an intraperitoneal implant exposure in adult tilapia resulting in methylmercury accumulation of 0.12 to 1.4 μg/g ww. Sexually regressed tilapia were exposed for approximately 32 weeks through gonadal recrudescence. A second study exposed sexually mature fathead minnows to dietary methylmercury for 20 weeks with resultant whole body

concentrations ranging from 0.20 to 2.24 μg Hg/g ww (Hammerschmidt et al., 2002). Impacts on steroidogenesis were measured in tilapia with significant reductions in E2 (in females) and 11-KT (in males) (Arnold, 2000). Histological changes in the gonads, including an increase in atretic follicles, were correlated with mercury exposure in tilapia but were not examined in fathead minnows. Gonadal development (GSI) was unaltered after methylmercury exposure in both tilapia and fathead minnows. Thus, there is insufficient data to support the hypothesis that at environmental concentrations methylmercury is an endocrine disruptor in adult fish.

The principal objective of this study was to evaluate the effects of methylmercury on the reproductive axis in adult goldfish. Methylmercury contaminated diets were prepared using concentrations previously determined to be sub-lethal and similar to those present in some aquatic food webs (Hammerschmidt et al., 2002; Drevnick and Sandheinrich, 2003). Adult female goldfish were fed one of three or four dietary concentrations of methylmercury for 28 days. Effects at the level of the gonad and pituitary were assessed through measurement of GSI, serum sex steroids (T, E2), pituitary LH content, and serum LH. To examine the significance of reproductive stage on methylmercury-induced alterations in reproductive biomarkers, the dietary exposure was replicated in pre- and post-spawning goldfish.

2.2 Materials and Methods

2.2.1 Methylmercury-Contaminated Diets

Contaminated diets were prepared by mixing floating trout pellets (Martin Mills Inc, Ontario, Canada) with 95% ethanol containing dissolved methylmercury (II) chloride (CH_3HgCl; Sigma Aldrich, Oakville, Ontario, Canada) at nominal concentrations of 0.8 μg/g ww (low), 4.0 μg/g ww (medium) and 8.0 μg/g ww (high). The control diet was prepared by mixing food with

ethanol only. Ethanol was evaporated from the mixtures in acid-cleaned, glass beakers in a fume hood over a period of one week. Prepared diets were stored in the dark at -20°C in 50 mL tubes. Samples of each diet were analyzed for total mercury.

2.2.2 Experimental design and sample collection

2.2.2.1 Pre-spawning methylmercury exposure (March 14 – April 13, 2007)

Common female adult goldfish were purchased from Aleong's International Inc. (Mississauga, Ontario, Canada) in February 2007. Fish were acclimatized over several weeks to 18°C under a natural photoperiod and fed with standard floating trout pellets (Martin Mills Inc). In March, 292 female fish were separated into groups of 13 to 15 individuals and placed in 70 L flow through tanks. Females were identified by the absence of tubercles on the leading ray of the pectoral fin. Fish were fed once daily for four weeks *ad libitum* (~0.9% body mass per day) one of four diets, three of which were contaminated with methylmercury (i.e., low, medium, high methylmercury). The control and three contaminated methylmercury concentrations are comparable to those previously used and identified as sub-lethal in chronic fathead minnow exposures (Hammerschmidt et al., 2002; Drevnick and Sandheinrich, 2003). Each tank was randomly assigned one of the four diets to yield five tanks per treatment.

After the 28 day exposure period, goldfish were anaesthetized using 3-aminobutyric acid ethyl ester (MS222). Fish were weighed and a blood sample was collected from the caudal vein using a 25 gauge needle attached to a 1 mL syringe. Serum was collected by centrifugation (7500 rpm at 4°C for 15 min) and stored at -20°C until analysis of sex hormones and LH. Fish were euthanized by spinal cord transection. Gonads were dissected from the fish, weighed and examined to confirm sex. Brain tissues and pituitaries were immediately removed, frozen on dry ice and stored at -80°C for subsequent RNA isolation or LH analysis. Disposable biopsy punches

(8 mm; Robbins Instruments, Chatham, New Jersey, USA) were used to collect a muscle sample, which were frozen at -80°C until lyophilization and analysis of total mercury.

2.2.2.2 Post-spawning methylmercury exposure (May 29 – June 25, 2006)

Common female adult goldfish were purchased from Aleong's International Inc. in April 2006 and allowed to acclimatize over several weeks to 18°C under a natural photoperiod. In May 2006, 158 female fish were separated into groups of 13 individuals and placed in 70 L flow through tanks. Fish were fed once daily for 28 days *ad libitum* (~0.8% body mass per day) one of three diets, two of which were contaminated with methylmercury (i.e., low and high methylmercury). Each tank was randomly assigned one of the three diets to yield four tanks per treatment.

After the 28 day exposure period, goldfish were anaesthetized using MS222, weighed, a blood sample collected and serum separated as above. Fish were euthanized by spinal cord transection. Gonads were dissected from the fish, weighed and the sex confirmed. Brain tissues and pituitaries were immediately removed, frozen on dry ice and stored at -80°C until required for RNA isolation, LH analysis, or lyophilization and analysis of total mercury. Skeletal muscle biopsy punches were frozen at -80°C until lyophilization and analysis of total mercury. Three whole fish from each treatment group were frozen at -20°C until homogenization, lyophilization and analysis of total mercury.

2.2.3 Mercury Determination

Lyophilized brain regions (hypothalamus, cerebellum and telencephalon; ~2 mg) and pituitaries (~0.2 mg), sub-samples (<30 mg) of lyophilized whole fish, muscle biopsies, and diets were analyzed for total mercury by cold-vapour atomic absorption spectroscopy. Total mercury

measurements were obtained using a SP-3D Total Hg Analyzer (Nippon Instruments Corporation, Tokyo, Japan) set for solid analysis and the measurement range was adjusted according to the methylmercury exposure level. The detection limit for the instrument was 0.01 ng Hg. The accuracy of mercury determination for each analytical batch was determined by analyses of (i) certified reference material (DORM-2; National Research Council of Canada), (ii) replicate sub-samples of homogenized fish, muscle biopsies, and diets, and (iii) procedural blanks and calibration standards. Fish tissues were only analyzed for total mercury because the method is straightforward and previous studies have shown that >90% of mercury in fish tissue is in the methylmercury form (Bloom, 1992; Gonzalez et al., 2005).

2.2.4 Reproductive Biomarkers

Steroid hormones were extracted from 200 μL of serum and measured in duplicate by radioimmunoassay (RIA) following the protocol by McMaster et al. (1992). The limit of detection for each assay was determined using two standard deviations of the binding achieved with the zero hormone standard. Hormone levels below the detection limit of the assay were assigned the detection limit, 0.09 ng/mL for T and 0.08 ng/mL for E2, of the RIA.

Gonadal development of goldfish was used as an additional reproductive biomarker of methylmercury exposure. Gonads from individual fish were weighed and expressed as GSI. This measure represents the percentage of total body weight contributed by the gonads.

2.2.5 LH Radioimmunoassay

Pituitary and serum LH concentrations were determined by double-antibody RIA using rabbit anti-carp LH antibody and I^{125}-labeled LH as described previously (Zhao et al., 2006). Individual pituitaries were disrupted via sonication in 6 mL diluent (0.5% bovine serum albumin

in barbitone buffer) and diluted another 10-fold in diluent prior to analysis. Diluted pituitary samples (10 μL) were assayed in triplicate. Pre-spawning serum samples (10 μL) were assayed in triplicate and post-spawning serum samples (50 μL) were assayed in duplicate.

2.2.6 Statistical Analyses

SPSS 15.0 for Windows software was used for all statistical analyses. A two-way analysis of variance (ANOVA) was used to test whether dietary methylmercury concentration and exposure period were significant factors in muscle mercury concentration or GSI. The effects of tissue type and dietary methylmercury concentration on mercury accumulation were also assessed using a two-way ANOVA. When significance was detected, Bonferroni post-hoc tests were used to determine the simple main effects of each factor. Within tissue types, a one-way ANOVA was used to determine significance of mercury accumulation. Somatic growth, fork length, and steroid levels were assessed for differences between methylmercury treatments using a one-way ANOVA. Dunnet's post-hoc tests were used for planned comparisons between control and treatments to determine differences. Linear regression models were used to assess relationships between sex hormone levels and gonadal development. When necessary, statistical analyses were conducted using transformed data to achieve normality and homoscedasticity. For sex steroid data which did not meet the normality assumptions of parametric tests, the Kruskal-Wallis test was used followed by the Mann-Whitney U test for post-hoc comparisons between control and methylmercury treatment groups.

2.3 Results

2.3.1 Mercury: dietary exposure and tissue accumulation

Actual mercury concentrations were measured in control and methylmercury contaminated diets. Nominal concentrations were 0.8 μg/g ww (low), 4.0 μg/g ww (medium) and 8.0 μg/g ww (high). A mean water content of 6.5% was measured for the floating trout pellets. Actual measured mean concentrations (μg/g ww) ± 1 SE of total mercury in the pre-spawning diets were 0.035 ± 0.004 (control), 0.69 ± 0.04 (low), 4.48 ± 0.45 (medium) and 7.78 ± 0.30 (high). Mean concentrations of total mercury in post-spawning diets were 0.022 ± 0.000 (control), 0.83 ± 0.04 (low) and 8.21 ± 0.74 (high). The low diet approximates the concentrations found in the diet of invertivorous and piscivorous fish in North America (Watras and Bloom, 1992; Hall et al., 1998; Paterson et al., 1998).

Total mercury concentrations were determined in the skeletal muscle after both exposures and in whole fish, pituitary, and select brain regions after the post-spawning exposure. Tissue concentrations are expressed on a wet weight basis (Table 2.1). A mean water content of 79% was measured for all tissues and used to convert concentrations from initial dry weight measurements. Mean muscle mercury concentrations were negligible in fish fed the control diet and did not differ between pre- and post-spawning exposures ($p = 0.143$). Exposure was a significant factor in muscle mercury concentrations from fish fed the low ($p < 0.001$) and high diets ($p \leq 0.001$). In the pre-spawning exposure, muscle mercury concentrations increased by approximately 10-fold between fish fed the control and low diet and between fish fed the low and high diet. Goldfish fed the medium diet accumulated a mean mercury concentration of 0.949 μg/g, approximately half the concentration measured in the high diet exposure group. A similar 10-fold increase in mercury accumulation was seen for the post-spawning exposure with the low

Table 2.1 Total mercury (Hg) in muscle of pre-spawning female goldfish exposed to dietary methylmercury and muscle, carcass, and discrete brain regions of post-spawning female goldfish exposed to dietary methylmercury[a]

	Total Hg (μg/g ww[b])						
	Muscle		Whole Fish	Hypothalamus	Telencephalon	Cerebellum	Pituitary
Diet	pre-spawning n = 10	post-spawning n = 8	post-spawning n = 3	post-spawning n = 5	post-spawning n = 5-6	post-spawning n = 5	post-spawning n = 5-6
Control	0.020 (0.002)	0.024 (0.002)	0.008 (0.001)	0.011 (0.000)	0.012 (0.001)	0.009 (0.001)	0.019 (0.002)
Low	0.210 (0.008)*	0.135 (0.018)*	0.107 (0.009)*	0.097 (0.011)*	0.102 (0.012)*	0.103 (0.008)*	0.117 (0.015)*
Medium	0.949 (0.074)*						
High	2.037 (0.096)*	1.373 (0.136)*	1.026 (0.057)*	1.635 (0.176)*	1.784 (0.198)*	1.307 (0.191)*	1.397 (0.068)*

Data were statistically evaluated by means of one-way ANOVA followed by Dunnet's post-hoc tests (* $p < 0.001$).

[a] mean (standard error)

[b] based upon 79% H_20 content

Table 2.2 Results of exposure to dietary methylmercury in pre- and post-spawning female goldfish[a]

	Mean ww (g)		Length (cm)		GSI (%)		Mortality (%)	
Diet	pre-spawning n = 56-63	post-spawning n = 18-20	pre-spawning n = 56-63	post-spawning n = 18-20	pre-spawning n = 32-42	post-spawning n = 17-18	pre-spawning	post-spawning
Control	21.8 (0.8)	25.1 (1.3)	9.9 (0.1)	10.2 (0.2)	7.2 (0.8)	4.9 (1.0)	5	0
Low	23.8 (1.2)	26.5 (1.5)	10.0 (0.2)	10.3 (0.2)	5.8 (0.7)	3.6 (0.7)	12	0
Medium	22.5 (0.7)		9.9 (0.1)		6.0 (0.7)		3	
High	21.2 (0.6)	23.2 (0.9)	9.9 (0.1)	10.0 (0.1)	3.2 (0.4)*	6.5 (1.0)	8	0

Data were statistically evaluated using one-way ANOVA followed by Dunnet's post-hoc tests (* $p \leq 0.001$).

[a] mean (standard error)

and high diets resulting in muscle mercury concentrations of 0.107 μg/g and 1.026 μg/g, respectively.

In brain regions from the control fish, the average mercury concentration ranged from 0.009 μg/g in the cerebellum to 0.011μg/g in the telencephalon. After 28 days of post-spawning exposure to methylmercury, mercury concentrations were similar between each of the discrete brain regions (i.e., hypothalamus, cerebellum, and telencephalon). In control fish, pituitaries had mercury concentrations comparable to muscle ($p = 0.100$) and higher than the three brain regions (hypothalamus, $p = 0.046$; cerebellum, $p = 0.003$; telencephalon, $p = 0.152$). No significant differences in mercury concentration were detected between tissues from fish fed the low methylmercury diet. Likewise, fish fed the high diet had similar concentrations of mercury in hypothalamus, telencephalon, cerebellum, pituitary, and muscle samples.

2.3.2 Survival and growth

The concentrations of dietary methylmercury used in these exposures were not lethal to either pre- or post-spawning adult female goldfish. Some mortality occurred in the pre-spawning exposure; however it was unrelated to dietary methylmercury and is typical of what occurs in the goldfish colony at some times of the year. The rates ranged from 3% (2/73) for fish fed the medium methylmercury diet to 12% (9/73) for fish fed the low methylmercury diet. In the post-spawning exposure, no mortality was observed in any of the methylmercury diet groups over the 28 day period.

Somatic growth of goldfish was not affected by methylmercury exposure. Mean somatic weight significantly increased by approximately 15% in the pre-spawning exposure ($p < 0.001$) and 19% in the post-spawning exposure ($p \leq 0.001$) but did not differ among treatments. Mean somatic weight ranged from 20.3 g (control) to 22.4 g (low methylmercury) in the pre-spawning

exposure ($p = 0.225$) and from 22.2 g (high methylmercury) to 23.5 g (low methylmercury) in the post-spawning exposure ($p = 0.409$). Mean fork length of fish did not vary among treatments, averaging 10 cm in all treatments for both exposures (pre-spawning: $p = 0.797$; post-spawning: $p = 0.605$).

2.3.3 Gonadosomatic index (GSI)

Goldfish undergo an annual cycle of gonadal growth reaching a maximum GSI just prior to spawning in May (Table 2.2). In pre-spawning goldfish, ovaries are increasing in mass. Female goldfish sampled on March 14, 2007, prior to initiation of the pre-spawning exposure, had mean GSI of 4.7 ± 1.1%. After 28 days (April 11, 2007), control fish sampled had a mean GSI of 7.2 ± 0.8%. In post-spawning goldfish undergoing regression the average GSI was lower at 4.9 ± 1.0%, although not significantly ($p = 0.088$). Dietary methylmercury exposure reduced gonadal development in pre-spawning female goldfish but had no effect on post-spawning fish. In the pre-spawning exposure, GSI was slightly reduced in fish exposed to the low and medium methylmercury diets and significantly reduced by over 2-fold in fish fed the high methylmercury diet in comparison to control ($p < 0.001$). This reduction is specific to the ovaries as no corresponding change in somatic weight was observed. In the post-spawning exposure, no significant differences in gonad weight or GSI were observed between any of the three treatment groups ($p = 0.159$).

2.3.4 Sex steroids: testosterone (T) and 17β-estradiol (E2)

Methylmercury suppressed levels of T and E2 in pre-spawning female goldfish (Figure 2.1) exposed to the high methylmercury diet. Fish fed the control diet had mean T concentrations 1.5- and 2.7-fold greater than those fed the medium and high diets. Significant suppression of T

levels occurred in fish fed the high diet ($p = 0.014$). Similarly, control fish had mean E2 concentrations that were 2.2- and 7.0-fold greater than those fed the medium and high methylmercury diets, respectively. Differences in E2 concentrations between fish fed the control diet and those fed the high methylmercury diet were statistically significant ($p = 0.006$).

Sex steroid levels in post-spawning goldfish were also reduced after methylmercury exposure. In contrast to the pre-spawning exposure, reduced steroid levels were measured in the low methylmercury exposure group while no reductions or increases were detected with the high methylmercury exposure. Testosterone levels were reduced 6.4-fold in comparison to controls ($p < 0.001$) and E2 levels by 2.1-fold, although not significantly ($p = 0.076$).

Testosterone and E2 were positively related to GSI in fish from all dietary exposures and reproductive stages (Figure 2.2). In pre-spawning fish, T ($r^2 = 0.787$) and E2 ($r^2 = 0.722$) were significantly correlated with GSI ($p < 0.001$). Similarly in post-spawning fish significant positive linear relationships ($p < 0.001$) were measured across treatment groups between the sex steroid hormones (T: $r^2 = 0.558$; E2: $r^2 = 0.657$) and GSI.

2.3.5 Luteinizing Hormone (LH)

Methylmercury had no affect on serum LH concentration or pituitary LH content in pre-spawning goldfish (Figure 2.3). Pituitary LH content was similarly unaltered in post-spawning goldfish exposed to dietary methylmercury exposure. Low methylmercury exposure resulted in suppressed levels of serum LH in post-spawning fish. Fish fed the control diet had mean serum LH levels that were 3.9-fold greater than those measured in fish fed the low methylmercury diet ($p < 0.001$).

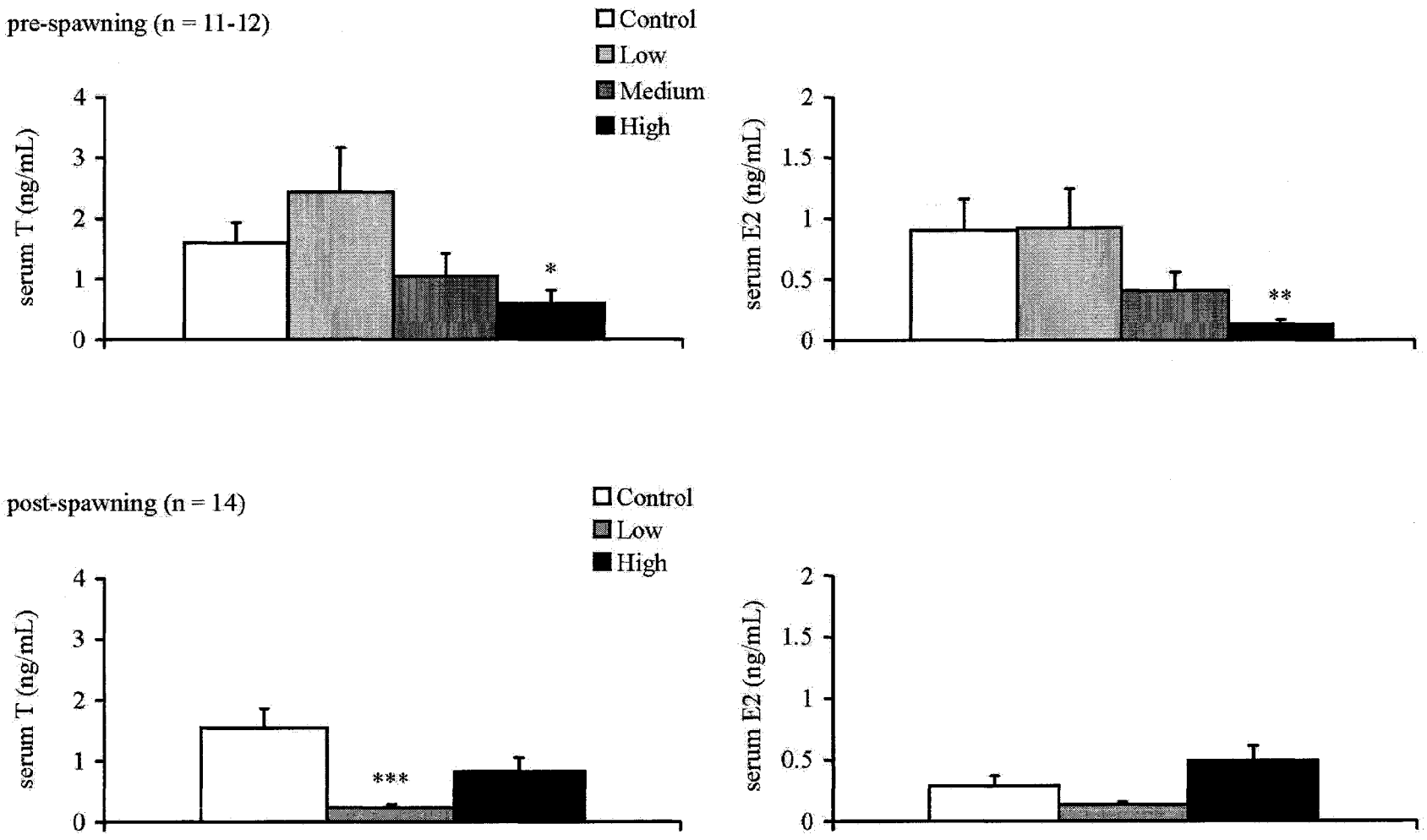

Figure 2.1 Serum testosterone (T, left) and 17β-estradiol (E2, right) levels measured by radioimmunoassay in pre- and post-spawning female goldfish fed diets with different levels of methylmercury (MeHg) for 28 days. Means (+ 1 SE) are presented and asterisks indicate significant differences between control and MeHg treated fish. Statistical significance was assessed using the nonparametric Kruskal-Wallis test followed by Mann Whitney U for post-hoc comparison (* $p < 0.05$, ** $p < 0.01$, *** $p<0.001$).

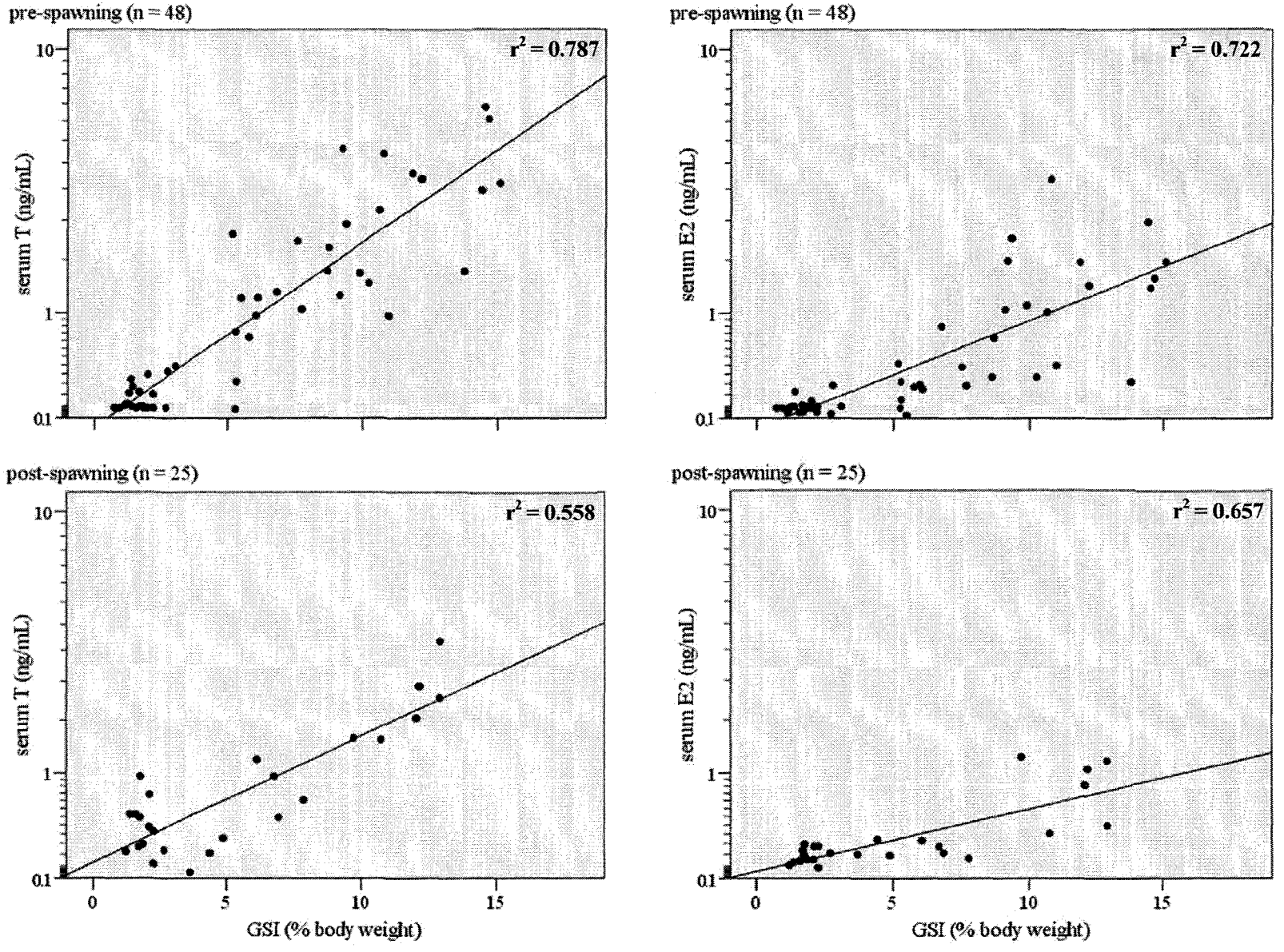

Figure 2.2 Linear relationships between serum testosterone (T, left) and 17β-estradiol (E2, right) and the gonadosomatic index (GSI) of pre- and post-spawning female goldfish fed diets with different concentrations of methylmercury.

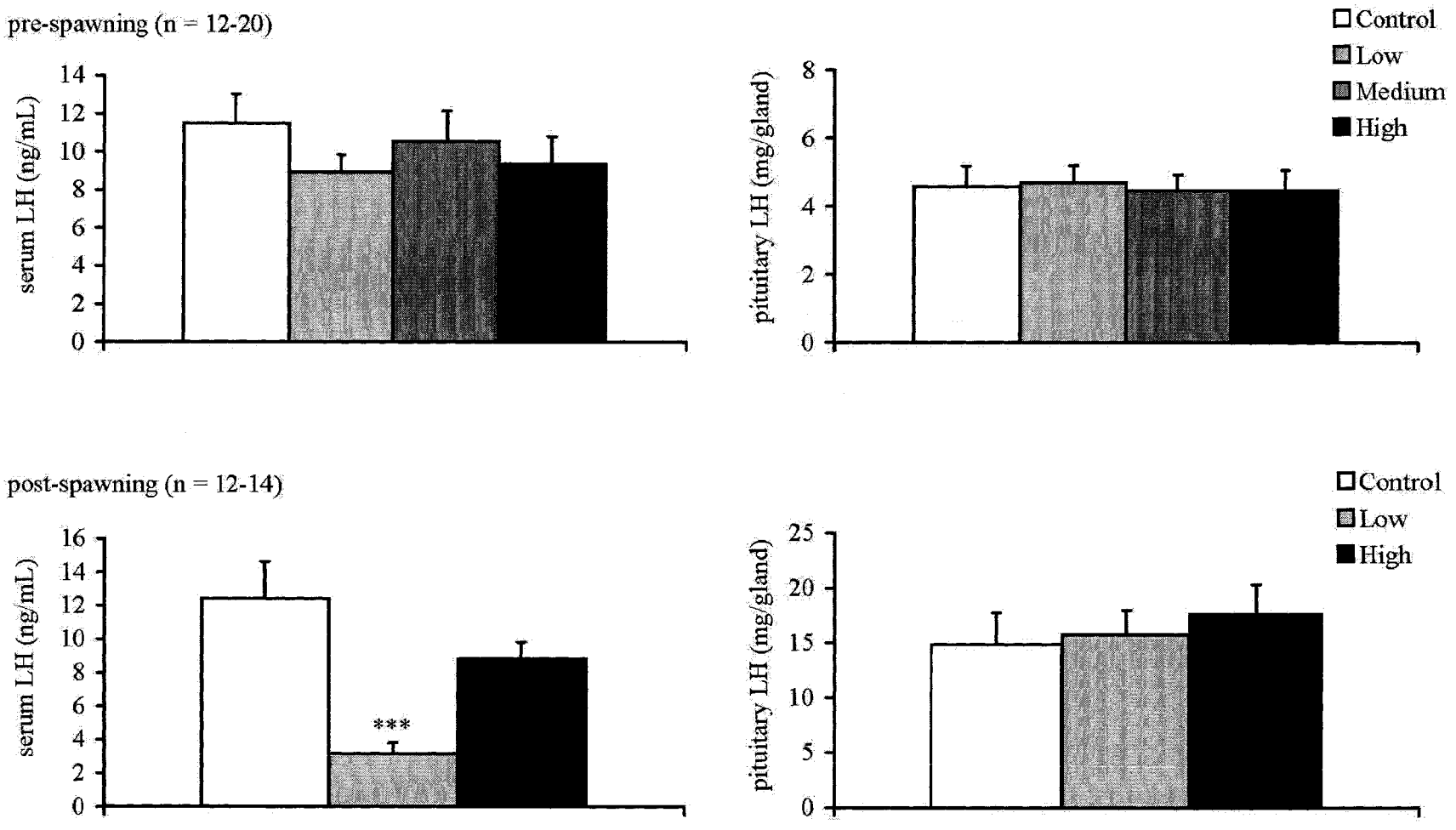

Figure 2.3 Serum luteinizing hormone (LH, left) levels and pituitary LH content (right) measured by radioimmunoassay in pre- and post-spawning female goldfish fed diets with different levels of methylmercury (MeHg) for 28 days. Means (+ 1 SE) are presented and asterisks indicate significant differences between control and MeHg treated fish. Statistical significance was assessed using one-way ANOVA followed by Dunnet's post-hoc tests (*** $p < 0.001$).

2.4 Discussion

The results of the present study demonstrate that methylmercury exposure can affect multiple sites within the pituitary-gonadal axis of adult fish including gonadal development, ovarian steroidogenesis and pituitary LH secretion. The sub-chronic (28 day) exposure to environmentally-relevant concentrations of methylmercury was sufficient to significantly impair the reproductive axis in female adult goldfish. This length of exposure may have ecological importance as methylmercury levels can fluctuate seasonally such that for short periods within the year fish may be exposed to elevated concentrations of methylmercury in the food web. A novel finding of these experiments is that the reproductive stage of the fish is a significant factor affecting sensitivity of the goldfish reproductive axis to methylmercury. The response to methylmercury exposure measured at both the level of the pituitary and the gonad varied with the period of the spawning cycle during which the adult fish were exposed. The differential effect of methylmercury exposure in pre- and post-spawning fish indicates that there may be stages of gonadal development which are more sensitive to impairment by methylmercury. Moreover, the absence of a clear dose-response relationship in the post-spawning exposure demonstrates that threshold limits determined in chronic exposures may not be predictive of steroidogenic impairment in fish exposed as adults. In general, the results are consistent with other sub-chronic and chronic laboratory fish studies showing impairment of steroidogenesis in fish after dietary exposure to methylmercury (Arnold, 2000; Drevnick and Sandheinrich, 2003).

The methylmercury exposures used in the present study resulted in mercury accumulation levels comparable to those found in the environment. In the wild, carp (similar species to goldfish) have whole body mercury concentrations ranging from 0.03 to 0.20 μg Hg/g ww (Schmitt et al., 2005; Hinck et al., 2006) similar to fish fed the controls and low methylmercury

diets in the present study. The mercury concentration in the medium treatment group was comparable to the mercury concentrations (0.63 μg Hg/g ww) found in liver tissue from carp living downstream from an industrialized area in the Ebro River, Spain (Lavado et al., 2006). In heavily polluted sites downstream from inactive chloralkali plants, omnivorous fish such as northern hogsucker (*Hypentelium nigricans*) and rock bass (*Ambloplites rupestris*) have muscle mercury concentrations that range from 1.2 to 2.5 μg Hg/g ww (Hildebrand et al., 1980) comparable to our high treatment group. Mercury concentrations in the medium and high exposure groups are also similar to concentrations measured in higher trophic level fish such as walleye, northern pike, and lake trout; mean mercury concentrations in these species sampled from various lakes throughout Québec ranged from 1.00 to 2.55 μg/g ww (Laliberté, 2004).

Dietary exposure to methylmercury for 28 days resulted in comparable levels of mercury bioaccumulation in muscle, pituitary, and the three brain regions tested. Feeding studies have shown that fish readily accumulate dietary methylmercury with varying degrees (30-80%) of assimilation (Rodgers and Beamish, 1982; Houck and Cech, 2004; Amlund et al., 2007). In goldfish, whole fish mercury concentrations were approximately 77% of the muscle concentrations in fish fed the methylmercury contaminated diets. This is similar to the average of 63% observed in a survey of three freshwater species (Hickey et al., 2005). Accumulation of mercury is rapid and equilibrium is rarely if ever reached as mercury is a non-essential metal, readily binds to sulfhydryl groups of proteins, and is excreted slowly. Mercury concentrations in the brain and skeletal muscle of zebrafish increased throughout a 63 day exposure to dietary methylmercury and the accumulated mercury existed almost exclusively in the methylated form (Gonzalez et al., 2005). Atlantic cod fed a dietary concentration of methylmercury, comparable to the low diet used in the present study, exhibited a continuous increase in blood and muscle

mercury concentrations over a three month exposure period (Amlund et al., 2007). Assimilated methylmercury undergoes a dynamic internal redistribution between tissues and organs usually from the visceral organs to the skeletal muscle. In control fish, mean muscle mercury concentrations were three fold higher than those measured in whole fish. As these fish had been chronically exposed to very low levels of dietary methylmercury, the increased muscle concentration may be due to the redistribution and storage of methylmercury in fish muscle as cysteine-thiol complexes (Harris et al., 2003).

Methylmercury exposure in adult goldfish reduced gonadal function and development as determined by decreased circulating sex steroid levels and GSI. Testosterone levels were comparable between pre- and post-spawning control fish and significantly reduced by methylmercury exposure in fish at both stages of reproduction. Estradiol levels were lower in post-spawning control females and reduced by methylmercury exposure in fish at both stages, however this effect was only statistically significant in pre-spawning goldfish. Goldfish with small ovaries in the present study also had low levels of sex hormones. Sex steroid levels were positively correlated to GSI in pre- and post-spawning females. Pre-spawning goldfish fed the high diet and post-spawning fish fed the low diet generally had the smallest gonads and lowest levels of sex steroids, suggesting that methylmercury reduced the capacity of ovaries to produce sex hormones. Gonadosomatic index was decreased and correlated positively with E2 levels in female fathead minnows chronically exposed to dietary methylmercury (Drevnick and Sandheinrich, 2003). Sex steroids were also significantly suppressed in fathead minnows and negatively correlated with concentrations of total mercury in carcasses (Drevnick and Sandheinrich, 2003). In the present study, GSI was significantly reduced in pre-spawning goldfish exposed to high dietary methylmercury resulting in gonadal indices comparable to those in early-recrudescing fish, suggesting an impairment of normal gonadal maturation. The effect of

methylmercury on gonadal development appears to vary between species and life stage of exposure as no correlation existed between female GSI and total mercury in carcasses of fathead minnows exposed to methylmercury as sexually mature adults (Hammerschmidt et al., 2002). Nor was any reduction in GSI noted for adult tilapia exposed to intraperitoneal methylmercury despite an increase in atretic follicles (Arnold, 2000). When gonadal development is inhibited, the reproductive potential of fish may be affected as spawning success and GSI were negatively correlated with whole body mercury concentrations in fathead minnows exposed as juveniles through to sexual maturity (Hammerschmidt et al., 2002).

The mechanism(s) by which methylmercury exposure impairs gonadal development and sex steroid production are still unclear. Changes in steroid hormone levels may be due to changes in steroid metabolism or biosynthesis or as a secondary effect to other dysfunctions within the HPG axis. One mechanism that has been proposed attributes the decrease in circulating sex steroids to methylmercury-induced apoptosis in ovarian follicles (Drevnick et al., 2006). In support of this hypothesis, a significant increase in the number of apoptotic cells was measured in ovaries from fathead minnows chronically exposed to dietary methylmercury (Drevnick et al., 2006). As apoptosis is involved in normal teleost ovarian development and regression (Wood and Van Der Kraak, 2001), premature induction of apoptosis may impede normal gonadal development. In fathead minnows, a significant relationship was observed between increased ovarian follicular apoptosis and reduced GSI (Drevnick et al., 2006). Follicular apoptosis was not assessed in pre- or post-spawning goldfish, but GSI was significantly reduced in pre-spawning fish. The lower GSI could be a result of methylmercury-induced apoptosis or may simply be due to reduced serum E2 levels as GSI has a significant positive relationship with E2.

Effects of methylmercury exposure on pituitary LH production and release were assessed through the quantification of pituitary LH content and serum LH. Pituitary LH was not affected

by methylmercury accumulation at either stage of reproduction suggesting that altered LH production cannot explain the reduction in sex steroid levels. In catfish, pituitary content was assessed via immunocytochemical analysis of gonadotropin cells after a 180 day exposure to aqueous methylmercury (Joy and Kirubagaran, 1989). Based upon analysis of gonadotropin (LH) staining patterns, the authors indicated that there were no signs of active hormone synthesis and release. The concentration of methylmercury to which these fish were exposed was 10^5 to 10^6 times higher than levels found in natural waters (Beyer et al., 1996) and as no tissue residues were measured it is difficult to compare to studies with other routes of exposure. Although pituitary content was unaltered, release of LH was significantly reduced in post-spawning goldfish fed the low methylmercury diet while no effect on LH release was observed in pre-spawning goldfish. The differential response to methylmercury exposure in pre- and post-spawning goldfish may suggest that different mechanisms underlie the effects at the two different exposure periods. In ovariectomized rats plasma LH levels were reduced after dietary methylmercury exposure (Oliveira et al., 2006). This effect was not seen in rats treated with E2 suggesting a protective effect of estrogen. The higher levels of E2 in pre-spawning fish as compared to post-spawning fish may be one explanation for the lack of pituitary effect in pre-spawning goldfish. Further studies are required to determine the role of the pituitary in methylmercury-induced steroidogenic impairment at different stages within the spawning cycle.

Threshold levels of mercury accumulation associated with endocrine modulation have been estimated at 0.2 μg/g for immature white sturgeon and pre-vitellogenic largemouth bass and at 0.8 μg/g for fathead minnows and northern pike (Scheuhammer et al., 2007). In pre-spawning goldfish, GSI and sex steroid levels were reduced in fish with a mean mercury accumulation of 0.95 μg/g and the reductions were statistically significant in fish with 2.04 μg Hg/g. Fish with

mean muscle concentration of 0.21 μg Hg/g had sex steroid levels comparable to control fish (0.02 μg Hg/g). The data correspond with the threshold for steroidogenic effects measured in adult fathead minnows and northern pike. In contrast, sex steroid levels in post-spawning goldfish were significantly reduced in fish with mean mercury accumulation of 0.135 μg/g, while in fish with mercury concentrations ten times higher, no change in sex steroid levels were measured. The differing response between post-spawning goldfish fed the low and high methylmercury diets may be due to differing efficiencies of protective mechanisms. The absence of a direct correlation between mercury accumulation and severity of effects has also been detected in rats (Falnoga et al., 1993; Oliveira et al., 2006).

Laboratory studies demonstrating methylmercury-induced steroidogenic impairment can generally be categorized as sub-chronic, spanning a period of months in adult fish, or chronic, spanning multiple life stages starting with juvenile fish. Assessing the effects of short-term or sub-chronic methylmercury exposure on fish physiology is important as environmental methylmercury undergoes seasonal fluctuations both in fresh and salt water ecosystems. Seasonal fluctuations in ocean mercury were observed in a survey of squid (*Loligo forbesi*) from UK waters (Pierce et al., 2007). Muscle mercury concentrations were over 2.5-fold higher in squid collected in the spring (March/April) compared to those collected in the fall (November). A seasonal trend was also demonstrated in zooplankton sampled from boreal Shield lakes: concentrations were lowest in spring (late May to early June), followed by a peak in mid-summer (July), and decreased in late summer (late August to mid-September) (Garcia et al., 2007). Mean methylmercury concentrations in zooplankton increased by 48% to 82%. Increased methylmercury in the aquatic food web may also result in seasonal variability of mercury concentrations in fish. Largemouth bass sampled in Oregon had liver mercury concentrations

which were over 2-fold higher in summer (June) than in spring (April) and ovarian mercury concentrations which were highest in the fall (November) (Foster et al., 2000). In summary, dietary levels of methylmercury in the environment can be significantly elevated for short periods of time (i.e., 4-8 weeks) which may result in short-term increases in fish tissue mercury concentrations. As this study has demonstrated exposure to elevated methylmercury concentrations for 4 weeks is sufficient to negatively impact the reproductive axis in adult goldfish. As the majority of teleosts have distinct gonadal cycles with ovulation limited to particular times of year, exposure to elevated methylmercury during specific sensitive periods within the spawning cycle may have detrimental effects on the reproductive success of fish.

Mercury contamination is a problem in many areas of the world with mercury burdens in fish sampled from Canadian Arctic lakes (Lockhart et al., 2005), Florida estuaries (Kannan et al., 1998), and European rivers (Raldúa et al., 2007) exceeding the 0.8 μg/g threshold to inhibit steroidogenesis. Whether these mercury burdens are sufficient to alter steroid hormone levels in wild fish is unclear, as are the potential population effects resulting from reduced steroid levels. Results have been equivocal in wild caught freshwater fish with negative correlations measured between mercury content and sex steroid levels in a subset of species from various locations. Further, there is a lack of studies directly linking methylmercury exposure and adverse effects on wild fish populations. Studies in wild fish are confounded by the natural fluctuations in population demographics as well as the synergistic effects of natural and anthropogenic endocrine active contaminants. For example, polychlorinated biphenyls and methylmercury have been shown to have synergistic and antagonistic interactions at the level of intracellular calcium regulation that may lead to alterations in cellular function, including changes in dopamine regulation (Bemis and Seegal, 1999; Bemis and Seegal, 2000). Thus, mixtures of organic contaminants and methylmercury may have synergistic effects on fish populations.

2.5 Conclusion

In summary, the present results extend the body of evidence supporting the hypothesis of methylmercury as an endocrine disruptor in fish. To my knowledge, this is the first study to compare the effects of sub-chronic methylmercury exposure on adult goldfish at two different periods within the annual spawning cycle. Ovarian steroidogenesis appears to be sensitive to methylmercury-induced impairment in both pre- and post-spawning fish. The results suggest that reduced steroid levels are independent of alterations in pituitary LH synthesis and release. The possibility that reduced steroid levels in fish exposed to dietary methylmercury may occur secondary to hypothalamic dysfunction remains to be addressed. The significance of impaired steroidogenesis and gonadal development on the reproductive potential of goldfish was not examined. However, reductions in T and E2 in female fathead minnows has been shown to be predictive of fecundity in fish exposed to chemicals that affect the HPG axis through inhibition of steroidogenesis (Ankley et al., 2008). This does suggest that further studies are warranted to assess the relationship between methylmercury exposure, reproductive biomarkers, and reproductive success in wild fish populations.

CHAPTER 3

Gene expression profiling in the hypothalamus of female goldfish (*Carassius auratus*) after sub-chronic dietary methylmercury exposure

3.1 Introduction

Methylmercury exposure can affect biochemistry, development, growth, behaviour, reproduction, and survival in fish (Sorensen, 1991; Weiner and Spry, 1996). In juvenile fish, chronic exposure to environmentally relevant concentrations of methylmercury increased gonadal apoptosis, impaired steroidogenesis, and reduced reproductive success (Hammerschmidt et al., 2002; Drevnick and Sandheinrich, 2003; Drevnick et al., 2006; Sandheinrich and Miller, 2006). Sub-chronic exposures in adult fish using ecologically relevant methylmercury exposures show a general trend for reduced sex steroid levels, however these studies are few in number and the effects on gonadal morphology and development have been inconsistent (Fynn-Aikins et al., 1998; Arnold, 2000; Hammerschmidt et al., 2002). Similar impairments of steroidogenesis have been correlated with mercury accumulation in some wild fish studies (Adams et al., 1999; Webb et al., 2006). Despite the increasing evidence to suggest methylmercury is endocrine disrupting, few studies have addressed the potential mechanisms underlying the suppressive effects of methylmercury on fish reproduction.

The toxicity of methylmercury arises from its high affinity for sulfhydryl groups. The non-specific binding of methylmercury to sulfhydryl-containing proteins results in the disruption of numerous cellular components and systems including the brain (Hughes, 1957; Ballatori, 2002). The mechanisms associated with methylmercury neurotoxicity have not been clearly defined and the current mammalian literature suggests that no single mechanism can explain the range of effects observed (Aschner and Syversen, 2005). *In vivo* and *in vitro* studies have demonstrated a number of methylmercury-induced cellular responses including disruption of

protein synthesis and microtubule assembly (Cheung and Verity, 1985; Miura et al., 1998; Hunter and Brown, 2000), generation of reactive oxygen species (Sarafian and Verity, 1991), induction of apoptosis (Wilke et al., 2003), disruption of calcium homeostasis (Denny and Atchison, 1996; Sirois and Atchison, 2000), altered protein phosphorylation (Yagame et al., 1994; Moretto et al., 2005), cell cycle inhibition (Ponce et al., 1994), and neurotransmitter imbalance (Fitsanakis and Aschner, 2005). Microtubules are structural components of the cytoskeleton and form the mitotic spindle required for mitosis. Methylmercury can depolymerize microtubules and this depolymerization may contribute to mercury-induced genotoxicity in mammalian cells (Thier et al., 2003) and sperm necrosis in guppies (Wester, 1991; Wester and Canton, 1992). Oxidative stress induces many intracellular events including apoptosis (Ueda et al., 2002). In fish, methylmercury at lower concentrations induces protective redox defences, while at higher concentrations anti-oxidant enzymes may be overcome resulting in lipid peroxidative injury (Berntssen et al., 2003). In addition to producing brain pathologies, oxidative stress may be one mechanism by which methylmercury exposure increases apoptosis in ovarian follicles and reduces serum steroid hormone levels (Drevnick and Sandheinrich, 2003; Drevnick et al., 2006). Studies are required to determine whether any of these mechanisms occur in the neuroendocrine tissues and contribute to methylmercury-induced impairment of the reproductive axis in fish exposed via diet to environmentally relevant concentrations.

The introduction of transcriptomics technologies has made it feasible to simultaneously assess the impacts of contaminant exposure on multiple biochemical pathways. Most toxicological responses involve changes in gene expression, thus profiling the transcriptome may improve our ability to identify toxic mechanisms. One of the challenges in applying microarray technology to aquatic toxicology is demonstrating a link between gene expression patterns and specific toxic modes of action. Arrays have been used to assess the effects of dietary

methylmercury on fish in several recent studies. A 200 gene cDNA macroarray and QPCR were used to examine gene expression in the liver of fathead minnows chronically exposed to environmentally relevant doses of dietary methylmercury (Klaper et al., 2006). Genes associated with endocrine disruption were altered, notably vitellogenin was decreased in females. However, less than 2% of the genes identified as differentially regulated in this study were statistically significant. These gene expression changes were related to declines in reproduction and reproductive biomarkers (i.e., E2, T, and GSI) observed previously (Drevnick and Sandheinrich, 2003). Hepatic gene expression changes associated with methylmercury accumulation were also assessed in wild-caught lake trout using a cDNA microarray comprising 147 stress-responsive genes (Moran et al., 2007). The results of this study further suggest that methylmercury may play a role in disrupting metabolic and reproductive pathways in fish. Interestingly, several neuroendocrine genes involved in the functioning of the HPG axis were up-regulated in liver tissue of fish with elevated mercury concentrations (0.048 μg/g ww). However, physiological effects associated with the gene expression changes observed were not tested nor were males and females treated separately. The expression levels of 13 genes in zebrafish were assessed using QPCR after exposure to dietary methylmercury (Gonzalez et al., 2005). In the liver, significant changes were measured for genes involved in mitochondrial metabolism, oxidative stress, and apoptosis. Whole brains were also examined, however no changes were observed after 7, 21, or 63 days of dietary exposure. The results from this study were limited by the small number of genes examined and the use of whole brain rather than individual brain regions for analysis. Further, the study did not connect the changes in gene expression with any specific physiological response. On the whole, gene expression studies in methylmercury exposed fish have focussed on the peripheral tissues yet it is well known that the central nervous system is the primary target for

methylmercury action (Clarkson and Magos, 2006). In addition, these studies have been limited to a small number of genes (≤ 200). Thus, in order to gain a better understanding of the mechanisms underlying methylmercury-induced impairment of the HPG axis, it is critical to look at alterations in neuroendocrine tissues using large-scale transcriptomics techniques.

In contrast, mammalian studies have primarily examined the mechanisms underlying methylmercury-induced neurotoxicity with limited focus on endocrine disrupting effects. The effects of methylmercury on the mammalian nervous system have been assessed in a few microarray studies. In one study, differential expression of genes in the cerebellum of rats was assessed following exposure to methylmercury *in utero* and via lactation (Padhi et al., 2008). Of the over 14,000 sequences spotted on the cDNA microarray, no genes in females and only nine genes in males were identified as differentially expressed. Cultures of rat adrenal medulla tumour-derived cells (PC12) were also used to assess the impacts of methylmercury exposure using microarray technology. The use of cell culture reduced the inherent variability of outbred rat strains. PC12 cells challenged with methylmercury demonstrated significant alterations in the expression of genes associated with oxidative stress, DNA damage repair, cell cycling, and apoptosis (Wilke et al., 2003).

In the present study, the effects of sub-chronic dietary methylmercury exposure were assessed in the hypothalamus of female goldfish using a brain enriched cDNA goldfish-carp microarray. The hypothalamus was examined as this region of the brain plays a significant role in the control of reproduction. Serum sex hormone levels were previously measured in these fish (Chapter 2) and significant reductions were observed after methylmercury exposure. In order to more accurately characterize the differential gene expression associated with methylmercury exposure, microarray experiments were carried out with hypothalamic RNA from fish exposed to both low and high methylmercury diets. In addition, genes associated with neuroendocrine

control of reproduction may vary with reproductive stage. Thus, gene expression was also examined in goldfish exposed during two separate reproductive stages (pre- and post-spawning). Ultimately, a consensus list of genes differentially expressed in the hypothalamus after methylmercury accumulation and independent of reproductive stage was generated. The goal of this research was to identify genes differentially expressed after ecologically relevant exposure to dietary methylmercury and provide an initial examination of potential neuroendocrine changes that may be linked to methylmercury-induced changes in reproductive biomarkers.

3.2 Materials and Methods

3.2.1 Experimental design and methylmercury exposure

Complete exposure conditions can be found in Chapter 2. Briefly, female adult goldfish were exposed for 28 days to dietary methylmercury. Four contaminated diets were used in the pre-spawning exposure ranging from 0.035 μg Hg/g ww in the control diet to 7.78 μg Hg/g ww in the high methylmercury diet. Similar dietary concentrations of mercury were used the post-spawning exposure: 0.022 μg Hg/g ww (control diet), 0.83 μg Hg/g ww (low methylmercury diet), and 8.21 μg Hg/g ww (high methylmercury diet). Food was supplied *ad libitum* once daily between 1600 hr and 1700 hr. After the 28 day exposure period, goldfish were anaesthetized using MS222 and euthanized by spinal cord transection. Hypothalami were pooled (two fish per pool), frozen on dry ice and stored at -80°C until required for RNA isolation.

3.2.2 RNA extraction, quantification and integrity assessment

Pooled hypothalami were thoroughly homogenized before RNA extraction with stainless steel beads (5 mm) in a MM301 Mixer Mill (Retsch, Pennsylvania, USA). Total RNA was extracted using Qiagen's RNeasy Mini Kit (Ontario, Canada) according to the manufacturer's

specifications including on-column DNase treatment. RNA was eluted twice in 30 µL of RNase-free water and stored at -80°C until further processing.

The concentration of RNA was measured with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Delaware, USA). RNA quality and integrity was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA) using the RNA 6000 Nano LabChip® kit (Agilent) according to the manufacturer's instructions. Agilent has developed the RNA integrity number (RIN), a tool which assists in estimating the integrity of total RNA samples (Schroeder et al., 2006). The RIN software algorithm allows for the classification of eukaryotic total RNA, based on a numbering system from 1 to 10, with 10 being the most intact (Mueller et al., 2004). RNA isolated from hypothalami was of high quality and had mean RIN values of 8.3 for pre- and post-spawning fish.

3.2.3 Microarray hybridization and analysis

The complete details describing the production, hybridization, and scanning of the goldfish brain enriched cDNA microarray are found in Martyniuk et al. (2006). Microarrays were prepared by spotting a collection of approximately 1100 goldfish cDNA sequences, previously isolated via directed cloning or suppression subtractive hybridization PCR and enriched for brain specificity. The goldfish cDNA sequences were combined with approximately 7000 carp cDNA sequences and spotted onto glass slides in duplicate at the University of Liverpool Microarray Facility, United Kingdom (Gracey et al., 2004).

Pools of hypothalamic total RNA from pre-spawning goldfish fed the high methylmercury diet (n = 6), post-spawning goldfish fed the low methylmercury diet (n = 6), and post-spawning goldfish fed the high methylmercury diet (n = 8) were used in the hybridization experiments. Total RNA isolated from hypothalamic tissue from control fish (pre-spawning n =

12; post-spawning n = 16) was combined for each reproductive stage into a reference control RNA pool. In all, a total of 20 microarray hybridizations were performed. The Genisphere Array 900MPX cDNA microarray labelling kit (Genisphere, Pennsylvania, USA) was used for all microarray hybridizations. This kit allows for indirect labelling of RNA using Cyanine 3 (Cy3) and 5 (Cy5) fluorescent dyes. The complete hybridization protocol can be found at (http://www.genisphere.com/pdf/Array900MPX_20July2007.pdf). For each microarray, 2 μg total RNA from the appropriate control reference pool was labelled with Cy5 and 2 μg total RNA from a treated pool was labelled with Cy3. The 2x formamide-based hybridization buffer was used in the prehybridization step and the 2x enhanced hybridization buffer was used for the cDNA probe and the signal amplification (3DNA) hybridization steps.

Microarrays were scanned at 543 and 645 nm using the ScanArray 5000XL system (Perkin Elmer, Massachusetts, USA). Images were analyzed using QuantArray (Perkin Elmer) for spot identification and quantification of the raw Cy3 and Cy5 signal intensity values. At this step, any erroneous spots were flagged for exclusion from further analysis.

As previously reported and validated by Martyniuk et al. (2006), microarrays were normalized using an intensity-dependent Lowess normalization (Yang et al., 2002). The significance analysis of microarray method was used to calculate the significance of gene expression changes (Tusher et al., 2001). This technique assigns a score to each gene based upon its change in gene expression and the standard deviation of repeated measurements for that gene. Repeated permutations of the data determine whether the expression of a specific gene was significantly different between test groups. The criterion for significance was a false discovery rate (FDR) of less than 5%. From the FDR filtered gene lists, only genes showing changes greater than 1.3-fold were further considered. This cut-off value was set based upon published

microarray profiling studies showing that the magnitude of gene expression changes is typically in the range of 30-50% for brain tissue (Bosetti et al., 2005; Newton et al., 2005; Martyniuk et al., 2006; Marlatt et al., 2008). Gene ontology (GO) analysis was performed for differentially expressed genes using Blast2GO (Conesa et al., 2005).

3.3 Results

Differential hypothalamic gene expression profiles were determined in female goldfish exposed to dietary methylmercury. Regulated cDNA sequences were assigned gene identities based upon sequence similarity to published sequences. The goldfish/carp microarray contains a total of 8089 cDNA fragments, however the actual number of genes represented on the array is less as some genes are encoded by multiple cDNA fragments. In the case of genes with redundant cDNA sequences identified as differentially regulated by the microarray, the largest fold change was assigned to that gene. In the few instances where the direction of regulation differed between redundant cDNA sequences, the gene was excluded from further analysis. One limitation to the analysis is the absence of sequence information for approximately 12% of the cDNA fragments spotted on the microarray. In addition, a number of sequences on the microarray have not yet been annotated. In pre-spawning goldfish, a total of 657 sequences showed a significant change in expression following methylmercury exposure. Of these, 284 were identified as unique genes with homology to previously described genes (Table 3.1). Analysis of the gene expression profile from post-spawning goldfish fed the low methylmercury diet demonstrated differential expression of 140 sequences, 49 of which were identified as unique genes with homology to previously described genes (Table 3.2). Differential expression in the hypothalami of post-spawning goldfish fed the high methylmercury diet was observed for 42 sequences; of these, 35 were identified as unique genes with homology to previously described genes (Table 3.3). Figure

3.1 contains a Venn diagram illustrating the overlap of unique genes similarly regulated in the three exposures. A total of 20 genes were regulated in the same direction by methylmercury in at least two of the exposures. Overall, the majority of regulated genes from goldfish fed the high methylmercury diet were up-regulated, while a higher percentage was down-regulated in fish fed the low methylmercury diet.

In order to interpret the nature of the data comprising a common response to methylmercury, a consensus list was generated of 59 unique genes whose expression level changed significantly in two or more of the methylmercury exposures (Table 3.4). Genes with fold change values less than the 1.3-fold threshold were included when the same gene was up- or down-regulated by more than the threshold value in another exposure. Of the common response genes, those related to endocrine function were grouped together and the remaining genes were organized based upon on their GO annotation for biological process. The endocrine related genes (i.e., activin βA, activin βB, isotocin, glutamate receptor 4, homer protein homolog 1, and secretogranin II [SgII]) encode peptides involved in stimulating the production and release of GnRH, secretion of LH, and gonadal steroidogenesis. Five broad GO themes were identified including apoptosis and stress response, calcium binding and transport, cytoskeleton organization, metabolism, and protein synthesis, degradation, and transport. Some of these processes, namely stress response, calcium homeostasis, and protein processing are known to be involved in methylmercury-induced neurotoxicity. Three genes related to protein synthesis and degradation were up-regulated in all three microarray experiments; 40S ribosomal protein S4 was increased 1.23 to 1.55-fold, 60S ribosomal protein L23 was increased 1.24 to 1.62-fold, and a ubiquitin protein ligase EDD1 was increased 1.31 to 1.52-fold. Both ependymin precursor I and

Table 3.1 List of 284 putative genes identified by microarray analysis in pre-spawning female goldfish after 28 days of high dietary methylmercury exposure (FDR < 5%, fold change ≥ ±1.30)

Gene Name	Fold change
16S rRNA, mitochondrial	-1.49
39S ribosomal protein L44, mitochondrial precursor	1.35
3-ketodihydrosphingosine reductase precursor	1.41
†40S ribosomal protein S15a	1.60
40S ribosomal protein S15	1.50
40S ribosomal protein S24	1.43
†40S ribosomal protein S4	1.55
40S ribosomal protein S5	1.31
40S ribosomal protein SA	1.37
60S ribosomal protein L11	1.45
60S ribosomal protein L13	1.43
†60S ribosomal protein L23	1.62
60S ribosomal protein L3	1.37
60S ribosomal protein L38	1.46
†60S ribosomal protein L7	1.97
60S ribosomal protein L8	1.69
Acidic leucine-rich nuclear phosphoprotein 32 family	1.37
†Actin-related protein 2/3 complex subunit 1B	1.44
Actin-related protein 2/3 complex subunit 4	1.38
†Activin beta A	-1.32
Acyl carrier protein, mitochondrial precursor	1.36
Adenlyate cyclase activating polypeptide 1b	-1.47
Adenylate kinase	1.34
ADP/ATP translocase 1	1.35
Aldolase A	1.77
AMPA	1.34
†Antizyme inhibitor 1	1.49
Apolipoprotein A-I	1.41
Aspartate aminotransferase, mitochondrial precursor	1.52
ATPase, H+ transporting, V1 subunit F	-1.47
ATP-dependent Clp protease ATP-binding subunit ClpX-like,	-1.35
BAG-family molecular chaperone regulator-1	1.53
Basigin-2	1.33
Beta-2-microglobulin	1.32
Beta-globin	-1.35
†Cadmium-Substituted Calcium-Binding Parvalbumin B	1.42
Calcipressin-1	-1.30
Calcium-binding protein p22	1.31

†Calmodulin	1.56
Carboxypeptidase E precursor	1.53
Cathepsin L preproprotein	1.33
Caytaxin	1.46
CCAAT/enhancer binding protein beta	1.31
Cell division protein kinase 10	1.50
Coatomer epsilon subunit	1.31
Cofilin-2	1.32
†Complement control protein factor I-B	1.51
Connexin 43	1.32
COP9 signalosome complex subunit 4	1.45
Core histone macro-H2A.1	1.34
†Creatine kinase brain	-1.41
Creatine kinase M2-CK	1.61
Creatine kinase M3-CK	2.04
CTD small phosphatase-like protein	1.37
C-type lectin	1.73
cysteine-rich with EGF-like domains 1	1.53
Cytochrome P450 2K1	1.33
Death effector domain-containing protein	1.43
DJ-1 protein	1.77
†DNA-binding protein inhibitor ID-1	1.52
DnaJ homolog subfamily A member 2	1.54
Dolichol-phosphate mannosyltransferase subunit 3	1.32
Dual specificity testis-specific protein kinase 2	1.33
Elastase-1 precursor	1.61
Electromotor neuron-associated protein 2	1.38
†Enolase, alpha	1.70
Enolase, beta	1.55
†Ependymin-I	-1.51
†Ependymin-II	-1.66
Ephrin type-A receptor 7 precursor	1.43
Equilibrative nucleoside transporter 1	1.35
†Eukaryotic translation initiation factor 1A, X-chromosomal	1.30
Eukaryotic translation initiation factor 4 gamma 1	1.45
Eukaryotic translation initiation factor 4E-1A-binding	1.42
Fast skeletal myosin light chain 3	1.98
Fatty acid-binding protein, intestinal	1.40
F-box only protein 32	1.34
Ferritin, lower subunit	1.53
Fibroblast growth factor 12	-1.31
FK506 binding protein 5	2.07

Gamma-aminobutyric acid receptor associated protein	1.51
Gamma-aminobutyric acid receptor-associated protein-like 2	1.32
Gastrula zinc finger protein XLCGF49.1	1.88
GATA-binding factor 5B	-1.44
Glucagon receptor precursor	-1.30
†Glutamate receptor 4	1.33
Glutamine synthetase	1.36
†Glutathione S-transferase A	1.50
†GPI transamidase component PIG-S	1.30
G-protein coupled receptor family C group 5 member C	1.50
Growth hormone	-1.42
GTP-binding nuclear protein Ran	-1.40
GTP-binding protein Di-Ras1	1.48
Guanidinoacetate N-methyltransferase	1.56
Guanine nucleotide-binding protein beta subunit 2-like 1	1.36
Heat shock protein HSC70-1, constitutive	1.46
Heat shock protein HSC70-2, constitutive	1.62
Heat shock protein Hsp70	1.35
†Heat shock protein 90-beta	1.57
Hemoglobin beta chain	-1.37
Heparan sulfate N-deacetylase/N-sulfotransferase	-1.32
†Hepatic leukemia factor	1.43
Heterogeneous nuclear ribonucleoprotein A/B	1.40
Heterogeneous nuclear ribonucleoprotein G	-1.30
Heterogeneous nuclear ribonucleoprotein L	1.33
High mobility group protein 4	1.42
†Histone H2B	1.53
Histone H3.3	-1.31
†Homer protein homolog 1	1.31
Hypothetical 26.3 kDa protein in RAD4-CHD1 intergenic	1.33
Hypothetical 34.0 kDa Trp-Asp repeats containing protein	2.18
Hypothetical 36.2 kDa protein in HAM1-PEM2 intergenic	1.33
Hypothetical protein C11D3.06	1.48
Hypothetical protein C8orf13	1.93
Hypothetical protein LOC406278	-1.33
Hypothetical protein LOC406771	1.36
hypothetical protein LOC431765	1.42
Hypothetical protein LOC445131	1.66
Hypothetical protein LOC449808	1.31
Hypothetical protein LOC550358	1.72
Hypothetical protein LOC550515	1.38
†Hypoxanthine-guanine phosphoribosyltransferase	1.50

Interferon-related developmental regulator 1	1.48
Interleukin enhancer-binding factor 3	1.49
Invariant chain like protein 1	1.91
Invariant chain like protein 2	1.66
†Isocitrate dehydrogenase [NADP], mitochondrial precursor	1.44
†Isotocin	-1.66
Kell antigen	-1.47
Kinectin 1	1.41
†Lactate dehydrogenase-A	1.31
Lactoylglutathione lyase	1.36
Liprin-alpha 1	1.34
Lupus La protein	1.38
Lysosomal-associated transmembrane protein 4A	1.37
Malate dehydrogenase	1.59
Malate dehydrogenase, mitochondrial precursor	1.39
†Malignant T cell amplified sequence 1	1.42
†Membrane-spanning 4-domains subfamily A member 4A	1.33
MHC class II alpha chain	1.33
†Mid1 interacting protein 1	1.53
Mitochondrial import receptor subunit TOM20 homolog	1.82
Mitochondrial prohibitin complex protein 2	1.35
Myeloid cell leukemia sequence 1-like	1.49
Myosin regulatory light chain	1.53
Myosin-18A	1.37
Myosin-9	1.64
†Myristoylated alanine-rich C kinase substrate 1	1.65
NADH-ubiquinone oxidoreductase B17 subunit	1.52
Nascent polypeptide-associated complex alpha subunit	1.79
NDRG4 protein	1.32
Nebulette	1.63
†Nedd4 family-interacting protein 2	1.38
Negative elongation factor E	1.55
Neuroserpin precursor	1.71
Nitric oxide synthase 1	-1.34
NRH dehydrogenase [quinone] 2	1.38
Nuclear receptor ROR-alpha	1.49
nucleolin 3	1.34
Pecanex-like protein 3	1.37
Peptidyl-prolyl cis-trans isomerase	1.37
Phosphatidylethanolamine-binding protein	1.49
Phospholipid hydroperoxide glutathione peroxidase,	1.34
Phosphomannomutase 1	1.41

Plasma membrane calcium-transporting ATPase 2	1.40
Pleiotrophic factor-alpha-2 precursor	1.32
PREDICTED: hypothetical protein XP_378730	-1.37
PREDICTED: hypothetical protein XP_683413	1.36
PREDICTED: hypothetical protein XP_687183	1.69
PREDICTED: hypothetical protein XP_692252	1.52
PREDICTED: hypothetical protein XP_692513	1.42
PREDICTED: hypothetical protein XP_699081	1.51
PREDICTED: hypothetical protein XP_703067 isoform 2	1.36
PREDICTED: RIKEN cDNA 1700015M15	-1.37
PREDICTED: similar to AF1q	1.35
PREDICTED: similar to Aminopeptidase puromycin sensitive	1.42
PREDICTED: similar to CG6282-PA, isoform A	1.45
PREDICTED: similar to HSPC300, partial	1.52
PREDICTED: similar to intracellular protein transport like	1.68
PREDICTED: similar to KIAA0819 protein	1.66
PREDICTED: similar to M protein repeat containing protein	1.43
PREDICTED: similar to microtubule-associated protein tau isoform 4	1.30
PREDICTED: similar to mKIAA0308 protein	1.34
PREDICTED: similar to mKIAA1822 protein	1.42
PREDICTED: similar to mKIAA4075 protein isoform 1	1.30
PREDICTED: similar to OSJNBa0011F23.1	1.30
PREDICTED: similar to peroxidasin	1.30
PREDICTED: similar to PGC-1-related estrogen receptor alpha	1.49
PREDICTED: similar to potassium channel, subfamily V, member 2	1.54
PREDICTED: similar to putative protein (5I806)	1.41
PREDICTED: similar to Ras and Rab interactor 1	1.46
PREDICTED: similar to RecQ protein-like	2.16
†PREDICTED: similar to ReO_6	-1.57
PREDICTED: similar to RNA-binding protein 10	1.33
PREDICTED: similar to rRNA intron-encoded homing endonuclease	1.41
PREDICTED: similar to SPRY domain-containing SOCS box protein SSB-1	1.33
PREDICTED: similar to testis-specific SSeCKS, partial	1.42
PREDICTED: similar to Zinc finger protein 647	1.34
PREDICTED: similar to zn-finger, CCHC type and RNA-directed DNA	1.44
Probable glutamate receptor precursor	1.31
Probable histone acetyltransferase MYST1	1.43
Probable ubiquitin carboxyl-terminal hydrolase FAF-X	1.36
Programmed cell death protein 6	1.31
Proteasome subunit alpha type 7	1.32
Proteasome subunit beta type 5 precursor	1.33
Proteasome subunit beta type 7 precursor	-1.36

†Protein 4.1	1.69
Protein phosphatase 2A, regulatory subunit B	1.39
Protein phosphatase 2C beta isoform	1.36
Protein translation factor SUI1 homolog GC20	1.30
Protocadherin gamma C5 precursor	1.38
Putative manganese superoxide dismutase	1.36
Pyruvate dehydrogenase E1 component alpha subunit, somatic	1.36
†RAB5B, member RAS oncogene family	1.38
†Ras-related C3 botulinum toxin substrate 2	1.42
Ras-related protein Rap-2c	1.41
Receptor-type tyrosine-protein phosphatase zeta precursor	1.31
Regulating synaptic membrane exocytosis protein 1	-1.33
†Reticulon 4-M	1.83
†Reticulon 4-N	1.50
Retinol-binding protein II, cellular	1.36
Ribokinase	1.34
Ribonuclease inhibitor	1.91
RING finger protein 28	1.32
RNA-binding protein 5	1.52
S-adenosylhomocysteine hydrolase-like 1	-1.50
Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	1.41
†Secretogranin II	-1.31
†Selenoprotein T precursor	1.43
Selenoprotein W	1.35
Septin-7	1.38
Serine/arginine repetitive matrix 1	1.45
Serine/threonine protein phosphatase 2A, catalytic	1.52
SET protein (Phosphatase 2A inhibitor I2PP2A)	1.59
Small ubiquitin-related modifier 2 precursor	1.39
S-methyl-5-thioadenosine phosphorylase	1.32
†Sodium/potassium-transporting ATPase alpha-1 chain precursor	1.76
Sodium/potassium-transporting ATPase beta-233 chain	1.39
Spectrin alpha chain, brain	1.51
S-phase kinase-associated protein 1A	1.30
Splicing factor 3B subunit 1	1.31
Splicing factor, arginine/serine-rich 3	1.43
Splicing factor, arginine/serine-rich 5	1.39
Stress-70 protein, mitochondrial precursor	1.41
Structural maintenance of chromosome 3	1.60
Synaptotagmin-11	1.78
TATA-box binding protein -like 1	1.58
Testin	1.32

THO complex 2	1.31
†Thioredoxin	1.44
Tmeff1 protein	1.31
Transcription elongation factor B polypeptide 1	1.39
Transcription factor AP-2 alpha	1.72
Transcription factor BTF3	1.33
Transmembrane protein 16B	1.32
Trifunctional enzyme beta subunit, mitochondrial precursor	1.81
Triple functional domain protein	1.36
Tropomyosin 1 alpha chain	1.33
Troponin C, skeletal muscle	1.80
Troponin T2, cardiac muscle isoforms	1.56
Troponin T3b, fast skeletal muscle isoforms	1.44
Tubulin alpha-6 chain	1.70
Tubulin-specific chaperone B	1.32
Tumor-associated calcium signal transducer 2 precursor	1.39
Ubiquinol-cytochrome c reductase complex 7.2 kDa protein	1.74
†Ubiquitin	1.36
†Ubiquitin-conjugating enzyme E2-17 kDa 10/12	1.67
Ubiquitin-conjugating enzyme E2 J2	1.41
Ubiquitin-conjugating enzyme E2 variant 1	-1.36
Ubiquitin-like protein FUBI	1.57
†Ubiquitin-protein ligase EDD1	1.52
UBX domain-containing protein 4	1.48
UPF0172 protein C14orf122	1.57
UPF0279 protein C14orf129 homolog	1.33
Vacuolar ATP synthase 16 kDa proteolipid subunit	1.46
Vacuolar ATP synthase subunit H	-1.48
†Vacuolar protein sorting 33B	1.45
Voltage-dependent anion-selective channel protein 1	1.32
Voltage-dependent anion-selective channel protein 2	1.39
Wilms tumor 1-associating protein	1.90
ZGC:91894	1.40
Zinc finger protein 271	1.36
Zinc finger protein 330	1.33
Zinc finger protein 364	1.40

† indicates genes listed in consensus gene list (Table 3.4)

Not listed are sequences that showed no nucleotide or amino acid similarity to sequences in GenBank (http://www.ncbi.nih.gov/GenBank). Gene names are from GenBank.

Table 3.2 List of 49 putative genes identified by microarray analysis in post-spawning female goldfish after 28 days of low dietary methylmercury exposure (FDR < 5%, fold change ≥ ±1.30)

Gene Name	Fold change
†40S ribosomal protein S15a	1.32
†60S ribosomal protein L23	1.31
†Antizyme inhibitor 1	1.33
†Apolipoprotein Eb	-1.41
Atp2a1 protein ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	-1.35
†Beta-actin	-1.32
Beta-tubulin	-1.32
†Calmodulin	-1.37
†Creatine kinase, brain	-1.32
Deoxyhypusine hydroxylase/monooxygenase	1.42
Flotillin-1	1.34
†Ependymin-I	-1.30
†Ependymin-II	-1.30
†Glyceraldehyde 3-phosphate dehydrogenase	-1.35
†Granulin-1	1.36
Granulin-3	1.38
†Heat shock protein 90-beta	-1.35
†Hypothetical 18K protein - goldfish mitochondrion	-1.36
†Hypothetical protein LOC393122	1.46
Hypothetical protein LOC405882	-1.63
Hypothetical protein LOC406278	-1.30
Hypothetical protein LOC554127	-1.41
†Hypoxanthine-guanine phosphoribosyltransferase	1.35
Immunoglobulin light chain	1.33
Interferon gamma inducible protein 30	-1.30
†Membrane-spanning 4-domains subfamily A member 4A	1.34
Midkine b	-1.34
Na+/K+ ATPase alpha subunit isoform 6	-1.35
†Nedd4 family-interacting protein 2	1.35
Pituitary adenylate cyclase activating polypeptide type 1 receptor precursor	-1.33
PREDICTED: hypothetical protein	-1.45
PREDICTED: hypothetical protein	-1.55
†PREDICTED: hypothetical protein	-1.30
PREDICTED: putative nucleic acid binding protein	-1.39
†PREDICTED: similar to 6-phosphofructokinase, liver type	-1.34
PREDICTED: similar to Solute carrier family 35, member E4	-1.31
PREDICTED: similar to testis enhanced gene transcript	-1.33
†Protein 4.1	1.47

Putative reverse transcriptase	-1.35
†RAB5B, member RAS oncogene family	1.41
†Reticulon 4-M	1.37
†Reticulon 4-N	1.31
†Secretogranin II	-1.36
Secretogranin V	-1.43
†Selenoprotein T precursor	1.32
Small nuclear ribonucleoprotein polypeptides B and B1	-1.30
Ubiquitin-conjugating enzyme E2 D2	-1.33
†Ubiquitin-protein ligase EDD1	1.31
†Vacuolar protein sorting 33B	1.45

† indicates genes listed in consensus gene list (Table 3.4)

Not listed are sequences that showed no nucleotide or amino acid similarity to sequences in GenBank (http://www.ncbi.nih.gov/GenBank). Gene names are from GenBank.

Table 3.3 List of 35 putative genes identified by microarray analysis in post-spawning female goldfish after 28 days of high dietary methylmercury exposure (FDR < 5%, fold change ≥ ±1.30)

Gene Name	Fold change
28S ribosomal protein S18a, mitochondrial precursor	1.39
†Activin beta B	-1.34
ADP-ribosylation factor-interacting protein 2	1.32
Anserinase	-1.39
†Apolipoprotein Eb	1.50
†Beta actin	1.31
†Calretinin	1.39
CDP-diacylglycerol--inositol 3-phosphatidyltransferase	1.38
COP9 signalosome complex subunit 3	1.38
DnaJ homolog subfamily B member 5	1.57
†Ependymin-I	1.34
Eukaryotic translation initiation factor 5	1.33
†Fast skeletal myosin light chain 1a	1.37
†Guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-3	1.56
Hemoglobin alpha	-1.36
High affinity nerve growth factor receptor precursor	1.33
†Hypothetical 18K protein - goldfish mitochondrion	-1.31
†Hypothetical protein LOC393122	1.31
Hypothetical protein LOC497146	1.35
Immunoglobulin heavy chain	1.38
†Isotocin	-1.51
Lipoprotein lipase precursor	1.33
Microsomal triglyceride transfer protein	-1.31
Neural cell adhesion molecule 1, 140 kDa isoform precursor	1.48
Nucleoplasmin-like protein NO29	1.52
PREDICTED: myelin basic protein isoform 1	-1.46
PREDICTED: myelin basic protein isoform 2	-1.31
†PREDICTED: myelin basic protein isoform 3	-1.45
PREDICTED: similar to Cleavage and polyadenylation specificity	1.39
PREDICTED: similar to SSB3 protein	-1.36
PREDICTED: similar to titin isoform N2-B	1.52
PREDICTED: similar to ubiquitin specific protease 34	1.34
Preprorenoguanylin	1.35
Smox protein	-1.39
†Ubiquitin-protein ligase EDD1	1.38

† indicates genes listed in consensus gene list (Table 3.4)

Not listed are sequences that showed no nucleotide or amino acid similarity to sequences in GenBank (http://www.ncbi.nih.gov/GenBank). Gene names are from GenBank.

Table 3.4 *Differentially expressed genes in female goldfish hypothalamus from microarray analysis (59 genes). All genes listed were identified as differentially expressed in at least two different methylmercury exposures (FDR <5%).*

Gene name and associated gene ontology		Fold change[a]		
		Pre-spawning	Post-spawning	
GO process	additional GO process	high MeHg	low MeHg	high MeHg
Endocrine related				
Activin beta A	cell proliferation	-1.32	(-1.20)	
Activin beta B	growth		(-1.21)	-1.34
Glutamate receptor 4	synaptic transmission	1.33		(1.24)
Homer protein homolog 1	glutamate receptor signaling	1.31		(1.19)
Isotocin	neuropeptide signaling pathway	-1.66		-1.51
Secretogranin II		-1.31	-1.36	
Apoptosis and stress response				
Glutathione S-transferase A		1.50	(-1.27)	(1.21)
Heat shock protein 90-beta	protein folding	1.57	-1.35	(-1.24)
Nedd4 family-interacting protein 2	regulation of NF-kappaB cascade	1.38	1.35	
Selenoprotein T precursor	cell redox homeostasis	1.43	1.32	
Thioredoxin	cell redox homeostasis	1.44	(1.25)	
Calcium binding and transport				
Cadmium-Substituted Calcium-Binding Parvalbumin B		1.42		(1.29)
Calmodulin	cell cycle	1.56	-1.37	
Calretinin		(1.29)		1.39
Ependymin-I	cell-matrix adhesion	-1.51	-1.30	1.34
Ependymin-II	cell-matrix adhesion	-1.66	-1.30	(1.27)
Cytoskeleton organization				
Actin-related protein 2/3 complex subunit 1B	cell motility	1.44		(1.26)
Beta actin	sarcomere organization		-1.32	1.31
Mid1 interacting protein 1	regulation of microtubule depolymerization	1.53		(1.26)
Protein 4.1	regulation of cell shape	1.69	1.47	
Metabolism				
Antizyme inhibitor 1	polyamine metabolic process	1.49	1.33	
Creatine kinase brain	arginine & proline metabolic process	-1.41	-1.32	
Enolase 1, (alpha)		1.70	(-1.22)	
Glyceraldehyde 3-phosphate dehydrogenase			-1.35	(-1.26)
Hypoxanthine-guanine phosphoribosyltransferase		1.50	1.35	
Isocitrate dehydrogenase [NADP], mitochondrial precursor	glutathione metabolic process	1.44	(-1.20)	
Lactate dehydrogenase-A		1.31	(-1.20)	
PREDICTED: similar to 6-phosphofructokinase, liver type			-1.34	(-1.29)

Protein synthesis, degradation, and transport					
	40S ribosomal protein S15a	translation	1.60	1.32	
	40S ribosomal protein S4	translation	1.55	(1.29)	(1.23)
	60S ribosomal protein L23	translation	1.62	1.31	(1.24)
	60S ribosomal protein L7	translation	1.97	(1.29)	
	Complement control protein factor I-B	proteolysis	1.51		(1.21)
	Eukaryotic translation initiation factor 1A	regulation of translational initiation	1.30		(1.26)
	Ubiquitin	protein modification process	1.36		(1.21)
	Ubiquitin-conjugating enzyme E2-17 kDa 10/12		1.67	(1.25)	
	Ubiquitin-protein ligase EDD1	ubiquitination	1.52	1.31	1.38
	Hypothetical protein LOC393122	proteolysis		1.46	1.31
	RAB5B, member RAS oncogene family	protein transport	1.38	1.41	
	Vacuolar protein sorting 33B	protein transport	1.45	1.45	
Other					
	Apolipoprotein Eb	lipid transport		-1.41	1.50
	DNA-binding protein inhibitor ID-1	regulation of transcription	1.52	(1.28)	
	Fast skeletal myosin light chain 1a	muscle development		(1.25)	1.37
	GPI transamidase component PIG-S		1.30		(1.26)
	Granulin-1		(1.28)	1.36	
	Guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-3	cell proliferation	(1.29)		1.56
	Hepatic leukemia factor	regulation of transcription	1.43		(1.23)
	Histone H2B	nucleosome assembly	1.53	(1.28)	
	Hypothetical 18K protein - goldfish mitochondrion			-1.36	-1.31
	Malignant T cell amplified sequence 1	regulation of cell cycle	1.42	(1.25)	
	Membrane-spanning 4-domains subfamily A member 4A		1.33	1.34	
	Myristoylated alanine-rich C kinase substrate 1	biological process	1.65		(1.23)
	PREDICTED: hypothetical protein	regulation of Rho protein signal transduction		-1.30	(-1.26)
	PREDICTED: myelin basic protein isoform 3	myelination		(-1.28)	-1.45
	PREDICTED: similar to ReO_6	RNA-dependent DNA replication	-1.57	(-1.20)	
	Ras-related C3 botulinum toxin substrate 2	small GTPase mediated signal transduction	1.42	(1.23)	
	Reticulon 4-M		1.83	1.37	
	Reticulon 4-N		1.50	1.31	
	Sodium/potassium-transporting ATPase alpha-1 chain precursor	regulation of cellular pH	1.76		(1.27)

[a]fold change values in brackets are less than the ± 1.3 fold change cutoff but are included for comparison with the other exposures

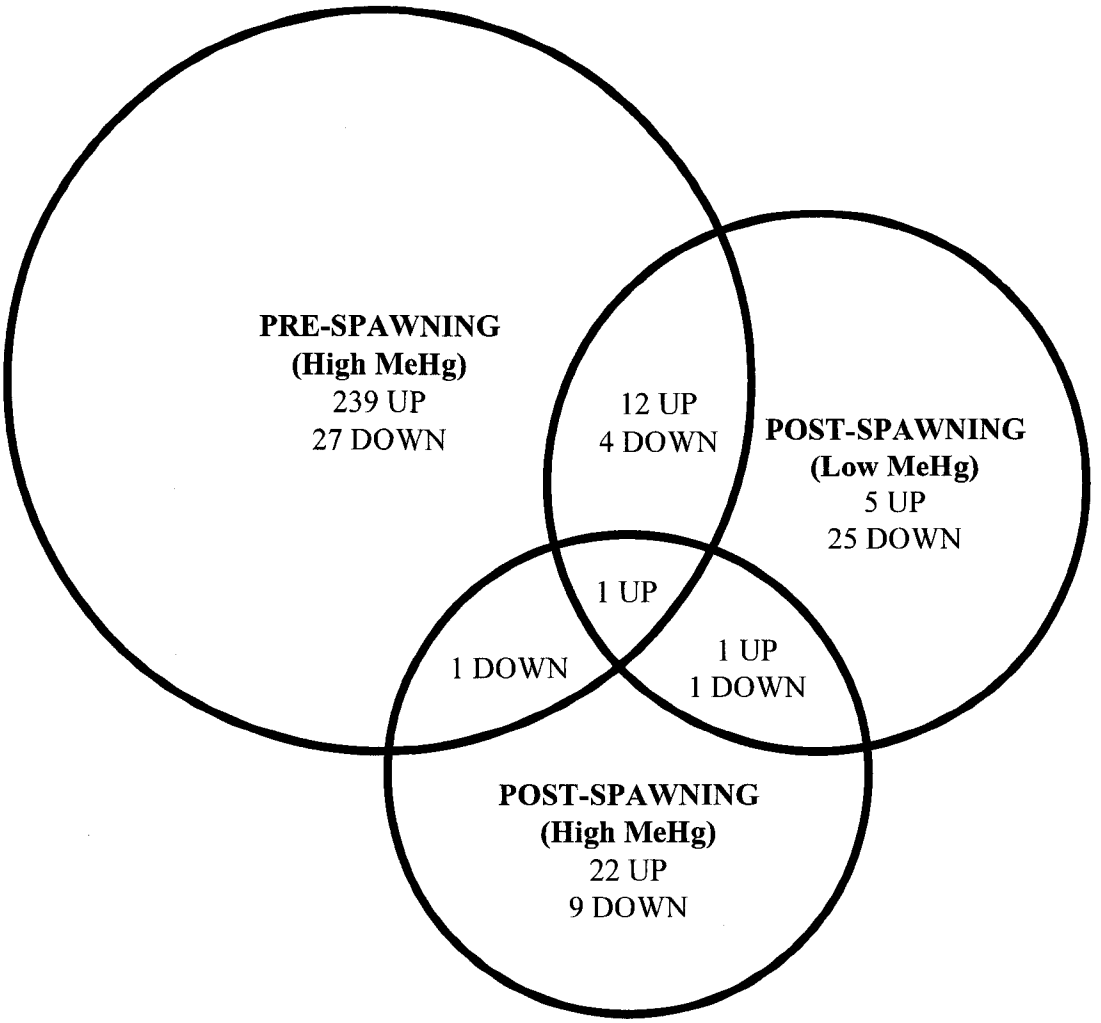

Figure 3.1 Venn diagram showing numbers of significant genes (FDR < 5%) for each methylmercury exposure. One gene is significantly up-regulated in all three exposures compared to controls.

ependymin precursor II were differentially regulated in all three exposures as were the stress response related genes heat shock protein (hsp) 90β and glutathione S-transferase A.

3.4 Discussion

The results from this study present a common gene expression pattern in the hypothalamus of female goldfish exposed to sub-chronic dietary methylmercury. The exposures have previously been shown to suppress the production of sex hormones, reduce LH release, and decrease GSI (Chapter 2). To my knowledge, this is the first study in fish to use a microarray approach to identify differentially regulated genes in neuroendocrine tissue after methylmercury accumulation. The data presented provides a starting point from which to explore a variety of mechanistic hypotheses ranging from general metal toxicity through to specific disruption of neuroendocrine control of reproduction. Based upon the analysis performed in the present study, the data suggest that exposure to environmentally-relevant levels of methylmercury modulates the expression of genes related to endocrine function, apoptosis and stress response, calcium binding and transport, cytoskeleton organization, metabolism, and protein synthesis, degradation, and transport. Many of the common differentially expressed genes are associated with known cellular responses to methylmercury toxicity and some are suggestive of potential alterations in neuroendocrine processes.

3.4.1 Endocrine related

Several genes related to neuroendocrine function were identified by the microarray as down-regulated in methylmercury exposed goldfish including isotocin, the activin subunits, and secretogranin II. High methylmercury exposure decreased transcription of isotocin, an abundant protein found in the brain of teleosts and homologous to mammalian oxytocin (Acher et al.,

1962; Venkatesh and Brenner, 1995). Evidence from fish suggests that isotocin plays a central role in neurohypophysial function and can modulate sexual behaviour (Goodson and Bass, 2000; Saito et al., 2004; Thompson and Walton, 2004). A study in killifish indicates that isotocin may stimulate spawning (Pickford and Strecker, 1977). The mammalian homologue oxytocin has been shown to stimulate GnRH mediated pituitary LH release (Rettori et al., 1997; Caligioni et al., 2007) and increase ovarian E2 secretion (Pitzel et al., 1990; Maas et al., 1992). Thus, alterations in circulating isotocin could have impacts at multiple levels of the reproductive axis.

Both activin subunits, βA and βB, were down-regulated in the hypothalamus of pre- and post-spawning goldfish after methylmercury exposure. Activins are members of the transforming growth factor β superfamily and are implicated in a wide range of physiological and developmental processes including the secretion and production of hypothalamic GnRH (Calogero et al., 1998; MacConell et al., 1999), the release of pituitary oxytocin (Sawchenko et al., 1988), and the synthesis of gonadal steroids (Hsueh et al., 1987; Rombauts et al., 1996). Activin subunits and receptors are expressed in a variety of tissues including the gonads and brain (Ge et al., 1997; Tada et al., 1998; Garg et al., 1999). Studies in goldfish have demonstrated that activins regulate pituitary gonadotropin biosynthesis via alterations in expression of FSHβ and LHβ genes (Yam et al., 1999). Also down-regulated by methylmercury exposure was SgII, the precursor for the bioactive peptide secretoneurin which is expressed at all levels of the goldfish reproductive axis (Samia et al., 2004). The localization of SgII and its regulation by reproductive hormones is suggestive of a role in reproduction (Samia et al., 2004). Secretoneurin may be involved in regulating LH release as goldfish exposed to a synthetic secretoneurin demonstrated a rapid release of LH (Zhao et al., 2006). Gene expression of the activin subunits and SgII are sensitive to GABA levels (Blazquez et al., 1998; Martyniuk et al., 2007) and, as

such, altered expression of these neuropeptides may be indicative of altered neurotransmission in methylmercury-exposed fish. Mammalian studies have demonstrated that mercury is capable of affecting the GABAergic system by altering the properties of various GABA receptors (Fitsanakis and Aschner, 2005) and mercury-induced alterations in neurotransmitter systems have been demonstrated in several fish species (Ram and Sathyanesan, 1985; Ram and Joy, 1988; Kirubagaran and Joy, 1990; Berntssen et al., 2003).

Altered neurotransmission was also suggested by increases in transcripts associated with the glutamate receptor signalling pathway. Glutamate receptor 4 and homer protein homolog 1 were up-regulated by high methylmercury exposure in pre- and post-spawning goldfish. Additionally, a probably glutamate receptor precursor was up-regulated in pre-spawning goldfish. Glutamate receptor 4 is an ionotropic AMPA receptor subunit expressed predominantly in the cerebellum of rats (Sato et al., 1993). Homer proteins are postsynaptic scaffolding proteins which function both as scaffolds for multi-protein complexes and mediators of postsynaptic metabotropic glutamate receptor signalling (Duncan et al., 2005). Glutamate neurotransmitter systems stimulate LH secretion by enhancing GnRH release through the activation of NMDA and AMPA-type glutamate receptors (Trudeau, 1997; Trudeau et al., 2000). Mammalian studies have demonstrated that methylmercury increases the extracellular concentrations of glutamate which may initiate or accelerate processes of excitotoxic neurodegeneration (Aschner et al., 2000). NMDA receptors have also been specifically implicated in methylmercury-induced neuronal damage (Miyamoto et al., 2001). Thus alterations in neurotransmission may be one result of methylmercury exposure in goldfish. On the whole, the data suggest that methylmercury accumulation in goldfish hypothalamic tissue alters the expression of genes related to several pathways involved in regulating reproduction at the neuroendocrine level. The altered expression of these genes may be associated with changes observed at other levels of the reproductive axis.

3.4.2 Apoptosis and stress response

Differential expression of genes associated with an oxidative stress response and apoptosis was observed in pre- and post-spawning goldfish exposed to methylmercury. Significant evidence exists linking oxidative stress and heavy metal toxicity (Kasprzak, 2002). Methylmercury has high affinity for the sulfhydryl groups in redox-sensitive proteins and this binding could negatively impact redox signalling and control pathways (Jones et al., 2004). Two of the genes commonly up-regulated in goldfish after methylmercury exposure are selenoproteins, namely thioredoxin and selenoprotein T. In addition, two other selenoproteins (phospholipid hydroperoxide glutathione peroxidase and selenoprotein W; Table 3.1) were up-regulated in pre-spawning goldfish. Selenoprotein W has also been implicated as a molecular target of methylmercury in human neuronal cells (Kim et al., 2005). Similar increases in selenoproteins were observed in flounder exposed to cadmium (Sheader et al., 2006; Williams et al., 2006). Selenoproteins are proposed to have a thioredoxin-like fold suggesting redox function (Dikiy et al., 2007), however the potential of these proteins to protect against oxidative stress has not been established.

Methylmercury exposure also resulted in the differential expression of hsp90β, nedd4 family interacting protein 2, and glutathione S-transferase A. The hsp response may represent a protective measure as these chaperone proteins have the ability to suppress apoptosis (Ravagnan et al., 2001). In addition to hsp90β, several isoforms of hsp70 were up-regulated in pre-spawning goldfish (i.e., hsp70-1 constitutive, hsp70-2 constitutive, and hsp70; Table 3.1). Both hsp70 and 90 were up-regulated in liver tissue of fathead minnows chronically exposed to dietary methylmercury (Klaper et al., 2006). Increased levels of chaperones could aid in the recovery of damaged neurons via refolding of denatured proteins and activation of anti-apoptotic mechanism

(Giffard et al., 2004). Further suggestive of an apoptotic response, the nedd4 family interacting protein 2 is involved in the positive regulation of the anti-apoptotic NF-κB cascade. This may also be associated with the heat shock protein response, as methylmercury activates hsp70 and the NF-κB-mediated protective pathway in response to oxidative stress in mammalian immune cells (Fremont et al., 2006).

Oxidative stress has been observed in fish chronically exposed to dietary methylmercury at levels comparable to the high diets used is the present study (Berntssen et al., 2003). Although several genes associated with oxidative stress and apoptosis were regulated by methylmercury exposure, no significant genomic changes were observed across the multiple exposures for any of the antioxidant enzymes (e.g., superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase). Manganese superoxide dismutase was up-regulated in pre-spawning goldfish fed the high methylmercury diet but not in post-spawning goldfish. This enzyme is also up-regulated in the brain tissue of mice exposed to mercuric chloride (Kumagai et al., 1997). In summary, the results give some indication that oxidative stress may be induced in pre-spawning goldfish exposed to methylmercury but this does not appear to be a significant response at the time of sampling. Similarly in liver tissue of fathead minnows chronically exposed to dietary methylmercury, there was some indication of reactive oxygen species generation with the induction of glutathione reductase in females and superoxide dismutase in males but overall no significant genomic changes associated with oxidative stress were identified (Klaper et al., 2006).

3.4.3 Calcium binding and transport

The list of commonly regulated genes also implicates calcium homeostasis as a cellular response altered by methylmercury exposure in goldfish hypothalamic tissue. The gene encoding calmodulin was differentially expressed upon methylmercury exposure. Pre-spawning goldfish

had significantly up-regulated levels while post-spawning fish had reduced levels. Calmodulin is a calcium-binding protein which has been proposed to regulate the action of increased intracellular calcium in neuronal and endocrine cells (Hanson and Schulman, 1992). Calmodulin has been implicated in signalling events mediating hypothalamic factor induced pituitary hormone secretion in goldfish (Chang et al., 1996; Jobin et al., 1996). Calmodulin gene expression is inhibited by calcium-dependent mechanisms and dopamine while cAMP- and protein kinase C-dependent pathways can induce expression (Huo et al., 2004). Mercury inhibits calmodulin-dependent calcium-ATPase in the rhesus monkey (*Macaca mulatta*) brain which may result in reduced calcium ion efflux at synapse leading to impaired neuronal function (Vig et al., 1989). In addition to calmodulin, calretinin and parvalbumin are also involved in calcium binding and transport and their transcript levels were altered in goldfish hypothalamus exposed to methylmercury. Disruption of calcium ion homeostasis has previously been associated with methylmercury neurotoxicity. Intracellular calcium levels increase in mammalian neuron-like cells after methylmercury exposure *in vitro* (Hare et al., 1993). In goldfish, disruption of calcium homeostasis has been suggested in bone cells where methylmercury exposure induced hypercalcemia (Suzuki et al., 2004). Thus, methylmercury-induced disruption of calcium transport or alterations in the level of calcium may occur in goldfish and could be responsible for some of the observed gene expression changes.

3.4.4 Protein synthesis, degradation, and transport

The present study shows the induction of protein-synthesis related genes encoding ribosomal proteins. An induction of similar genes was seen in the liver of cadmium exposed flounder (Williams et al., 2006). This induction may be indicative of repair to damaged protein biosynthetic machinery or an increased requirement for protein synthesis of protective proteins

(e.g., chaperones and antioxidant enzymes). Alterations in protein synthesis is one of the characteristic effects of methylmercury exposure (Atchison and Hare, 1994). Protein synthesis stimulation and inhibition has been reported in methylmercury-exposed animals suggesting that various factors including dose may impact the effects of methylmercury exposure (Yoshino et al., 1966; Sarafian and Verity, 1990). The induction seen in the present study may also be associated with a response to oxidative stress. Studies of oxidative stressors have observed alterations in numerous ribosomal genes and heat shock proteins (Thorpe et al., 2004; Hook et al., 2006). Genes involved in protein transport were also up-regulated in methylmercury exposed fish. Protein ubiquitination and proteolysis are the primary mechanisms of protein degradation. An overall induction of proteosomal and ubiquitin-conjugating gene transcripts was observed in cadmium-treated flounder (Williams et al., 2006). Similarly, methylmercury exposure induced expression of genes involved in protein ubiquitination and proteolysis. The induction of these genes suggests increased protein damage in response to methylmercury.

3.5 Conclusions

The microarray analysis presented in this study provides a general overview of genes differentially regulated in the hypothalami of goldfish exposed to environmentally relevant dietary levels of methylmercury independent of reproductive stage. Although no single biological process appears to be principally affected by methylmercury accumulation, the data does suggest the involvement of several key processes. The main cellular processes affected by methylmercury exposure include apoptosis and response to oxidative stress, calcium ion binding and transport, and protein synthesis, degradation and transport. These cellular responses are all characteristic of methylmercury toxicity in other systems (Atchison and Hare, 1994; Faustman et al., 2002).

In addition to genes associated with previously defined mechanisms of toxicity, methylmercury exposure also altered the expression of genes associated with regulation of the reproductive axis. Methylmercury has been proposed as a nonsteroidal endocrine disruptor based upon studies indicating negative impacts of exposure on reproductive endpoints such as sex steroid levels (Arnold, 2000; Drevnick and Sandheinrich, 2003) and vitellogenesis (Panigrahi et al., 1990; Kirubagaran and Joy, 1995). Additionally, mercury has been shown to mimic the effects of estrogens in stimulating cell growth and regulating gene transcription (Garcia-Morales et al., 1994; Martin et al., 2003). The data from this study provide a strong foundation from which to explore the mechanistic relationship between gene expression and the endocrine disrupting effects of methylmercury. Genes encoding peptides involved in stimulating the production and release of GnRH, secretion of LH, and gonadal steroidogenesis were differentially regulated. Although speculative, disruption in the expression of these proteins by methylmercury may have consequences for reproductive neuroendocrine function. The data however, do not identify a clear mechanistic relationship between hypothalamic gene expression and the reductions in sex steroid levels and GSI.

Genomics techniques have been applied to environmental toxicology in an attempt to define patterns of gene expression specific to toxicological effects associated with chemical exposure. Gene expression represents the physiological state of an organism at the time of sampling and thus alterations in gene expression may be indicative of toxicity and could be used to identify biomarkers of effect and exposure. Although the present study and others provide some clues as to processes that are being affected by methylmercury exposure, it remains a challenge to conclusively define causal relationships between gene expression and physiological changes. Further investigation is required to fully characterize the neurotoxic effects of

methylmercury and the relationship between these effects and the alterations in sex steroid levels and GSI.

CHAPTER 4

General Discussion

4.1 Thesis summary

This thesis examined the effects of methylmercury on the reproductive axis of fish focusing on gonadal development and steroidogenesis, pituitary LH synthesis and release, and hypothalamic gene expression. Environmentally relevant concentrations of methylmercury (Figure 4.1) were used in this thesis duplicating exposure levels used in previous fish studies (Hammerschmidt et al., 2002; Drevnick and Sandheinrich, 2003; Drevnick et al., 2006; Klaper et al., 2006). The overall objective was to quantify physiological and molecular changes associated with methylmercury exposure in order to gain insight into the mechanism(s) by which methylmercury alters reproduction. A prior study examined the link between decreased sex steroid production and hepatic mRNA expression in methylmercury exposed fathead minnows (Klaper et al., 2006). The data were difficult to interpret due to high variability but the authors did identify reduced vitellogenin transcript levels as an endocrine-related change associated with methylmercury exposure in female fish. Reduced vitellogenin levels have been correlated with methylmercury exposure in several fish species (Panigrahi et al., 1990; Kirubagaran and Joy, 1995; Baldigo et al., 2006). As vitellogenesis is a well known estrogen-mediated process in teleosts (Bentley, 1998) and fathead minnows with reduced vitellogenin expression also had methylmercury-induced decreases in E2 levels, the reduction in vitellogenin transcript levels is likely due to reduced estrogen levels. This does not, however, provide any further insight into the mechanisms whereby methylmercury impairs steroidogenesis. Thus, this thesis is the first fish methylmercury exposure study to measure physiological effects at both the gonads and the

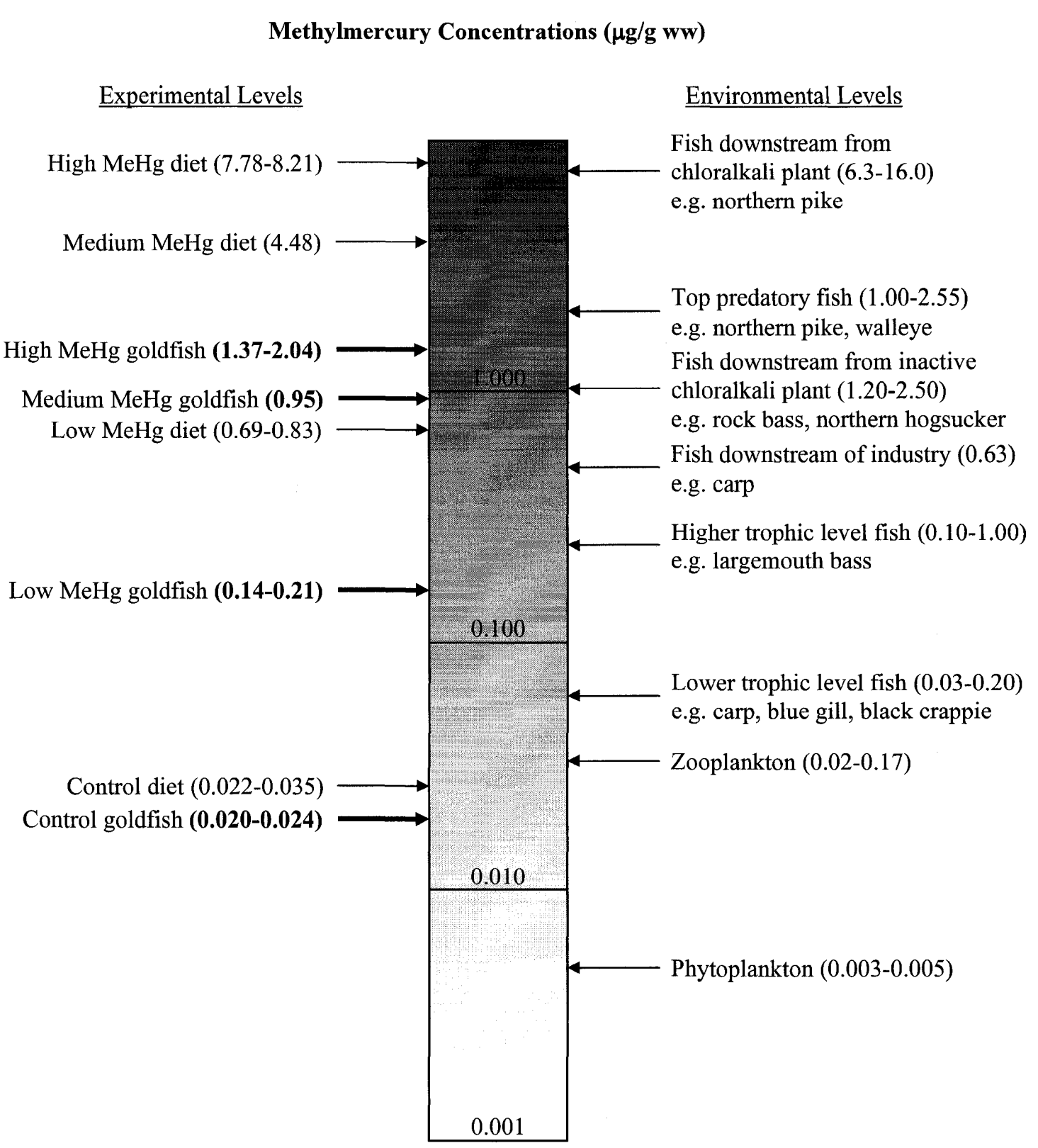

Figure 4.1 Schematic of environmental methylmercury (MeHg) concentrations. All concentrations are given on a wet weight (ww) basis, where applicable. Concentrations of methylmercury used in this study (left) are shown in the diagram for comparison with environmental concentrations (right). Literature values adapted from Hildebrand et al. (1980), Hinck et al. (2006), Laliberté (2004), Lavado et al. (2006), Lockhart et al. (1972), Schmitt et al. (2005), Sveinsdottir and Mason (2005), Watras and Bloom (1992).

pituitary and demonstrate changes in hypothalamic expression of genes encoding proteins involved in the neuroendocrine control of reproduction.

4.2 Methylmercury is an endocrine disruptor in fish

This thesis provides further evidence to support the hypothesis of methylmercury as an endocrine disrupting chemical based upon its ability to disrupt the synthesis of hormones in fish responsible for reproduction and development (i.e., T, E2, and LH). Sex steroid levels and GSI were reduced in methylmercury exposed pre- and post-spawning goldfish which corroborates effects seen in previous studies (Figure 4.2, Chapter 2). Despite repeated studies demonstrating impairment of gonadal development and steroidogenesis, few studies have provided any suggestions as to the mechanisms underlying these effects. One hypothesis tested in this thesis was that altered gonadal steroidogenesis was secondary to methylmercury-induced impairments of basal pituitary LH synthesis and release.

In teleosts, LH secretion is regulated by numerous factors including GnRH, hypothalamic monoamines, and gonadal steroids. Secreted LH stimulates gonadal growth and steroid production. Thus, reductions in circulating LH could impair sex steroid levels and gonadal development. Impairment of LH synthesis and release has been observed in fish exposed to a suite of EDCs. Atlantic croaker exposed to a polychlorinated biphenyl mixture during the early-recrudescence phase of the gonadal cycle exhibited impairment of LH secretion and gonadal growth (Khan et al., 2001). Basal LH pituitary content and serum LH levels were measured in this thesis (Figure 4.2, Chapter 2). The data suggest that basal LH release may be affected by methylmercury exposure but that alterations in pituitary function are unlikely to be the primary cause of reduced sex steroid levels. Stimulated LH release was also examined in pre- and post-spawning goldfish challenged with a GnRH analog (data not shown); however the small sample

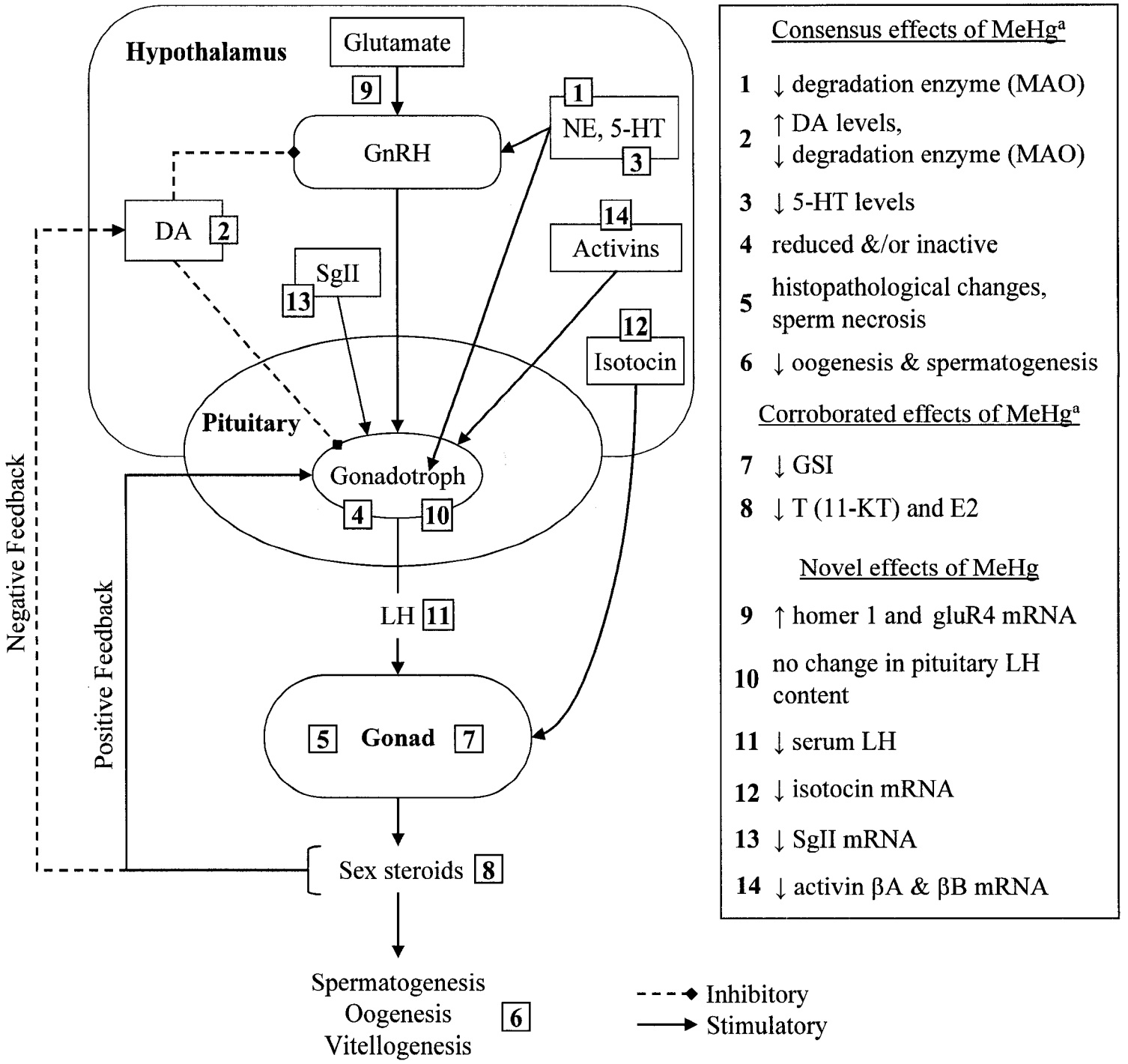

Figure 4.2 The hypothalamic-pituitary-gonadal (HPG) axis in fish showing several neuroendocrine pathways involved in the control of reproduction (adapted from Trudeau, 1997). Numbers indicate aspects of the HPG axis affected by methylmercury (MeHg) exposure. The abbreviations are as follows: GnRH, gonadotrophin-releasing hormone; DA, dopamine; 5-HT, serotonin; NE, norepinephrine; LH, luteinizing hormone; MAO, monoamine oxidase; GSI, gonadosomatic index; T, testosterone; 11-KT, 11-ketotestosterone; E2, 17β-estradiol; gluR4, glutamate receptor 4; SgII, secretogranin II.

[a] consensus effects of mercury on HPG axis summarized in Table 1.1

size and high variability rendered the data inconclusive. Although basal LH release is not significantly affected by methylmercury exposure, alterations in the response to GnRH stimulation remain to be fully examined. In future studies, I would investigate more closely the effects of methylmercury exposure on pituitary function and specifically GnRH stimulated LH release at clearly defined stages of reproduction (i.e., regressed, early recrudescence, and mature spawning). Alternatively, the methylmercury-induced reduction in sex steroid levels and GSI may be due to LH-independent mechanisms. For example, isotocin is a putative reproductive neuropeptide whose circulating levels have been shown to vary seasonally in female three spined sicklebacks (*Gasterostreus aculeatus*) with maximal levels occurring during the reproductive period. Isotocin can stimulate T release at the level of the testis *in vitro* in rainbow trout (Rodriguez and Specker, 1991). Additionally, mammalian oxytocin (a homologue of teleost isotocin) has a stimulatory effect on sperm release in the African catfish (Viveiros et al., 2003). Overall, methylmercury exposure has been shown to impair indicators of reproductive health in fish (Figure 4.2) but further studies are required to determine the dependent nature of these effects on hypothalamic-pituitary function at different stages of the reproductive cycle.

4.3 Gene expression signature of methylmercury exposure

Microarrays are useful tools for generating hypotheses related to modes of toxic action. Through the assessment of global gene expression changes it is possible to identify specific biochemical pathways or cellular responses altered upon chemical exposure. Individual toxicants can produce distinctive gene expression signatures linked to toxic mechanism of action rather than chemical structure (Bartosiewicz et al., 2001; Hamadeh et al., 2001; Hook et al., 2006). The results from this thesis demonstrate that there were 20 statistically significant differentially regulated genes having similar responses in at least two of the methylmercury exposures. This

suggests that methylmercury accumulation may be a more significant factor in regulating those genes than reproductive stage or dose. Transcriptome responses to contaminant exposure have been shown to vary with tissue type sampled (Volz et al., 2005). As no other microarray experiments have been performed on brain tissue of fish exposed to methylmercury it is difficult to make any statement as to whether the pattern of differential gene expression determined in this thesis is representative of a distinct gene expression signature for methylmercury. The consensus gene list (Table 3.4) provides a starting point for assessing the relationship between gene expression and toxic mechanism of action for methylmercury in fish. It has been suggested that gene expression changes are more strongly influenced by contaminant exposure than by age or sex differences in individual fish (Hook et al., 2006) but this has yet to be shown in response to methylmercury exposure. There is some overlap between the consensus gene list and genes regulated in fish liver after methylmercury exposure (e.g., heat shock proteins, isocitrate dehydrogenase, apolipoprotein, ependymin) (Klaper et al., 2006). However, macroarray analysis of fathead minnows exposed to methylmercury observed little overlap between genes differentially regulated in females and males. As microarray platforms are increasingly applied to aquatic toxicology, more genomic data will become available allowing for a continuation of this work to evaluate unique gene expression patterns specific to methylmercury exposure.

The microarray data presented in this thesis is specifically focused on the consensus transcripts altered by methylmercury exposure. The objective was to determine a subset of hypothalamic genes which are differentially regulated by methylmercury exposure. This analysis provides some insight into the general toxic mechanism of action suggesting that methylmercury exposure disrupts calcium ion homeostasis, increases protein synthesis and may initiate an oxidative stress response. These cellular responses have, to some extent, previously been observed in mammalian models (Cheung and Verity, 1985; Sarafian and Verity, 1991; Denny

and Atchison, 1996; Sirois and Atchison, 2000). The consensus list of genes involved in neuroendocrine processes may indicate molecular alterations at the level of the hypothalamus which could impact the reproductive axis in methylmercury exposed fish. This is the first study to suggest a possible link between methylmercury-induced hypothalamic gene expression and reproductive impairment. This analysis, however, does not consider effects specifically associated with altered sex steroid levels nor does it consider changes which are specific to pre- or post-spawning goldfish. Future investigations could include microarray analysis for transcriptome responses specific to reproductive stage and/or the physiological responses observed.

4.4 Establishing a link between gene expression and endocrine disruption

Based upon studies presented in this thesis, I propose a mechanistic link between methylmercury-induced changes in genes encoding neuroendocrine factors, LH release, and steroidogenesis. Figure 4.2 is a visual depiction of the novel effects of methylmercury on the HPG axis demonstrated in this thesis along with the effects previously observed in fish studies. In addition, changes in endpoints measured in this thesis which corroborate results of prior studies are also shown. This schematic demonstrates how methylmercury exposure has been shown affect all levels of the HPG axis. Whether the hypothalamic-pituitary effects observed are responsible for the observed alterations in reproductive biomarkers (e.g., T, E2, and GSI) has not yet been demonstrated. Goldfish reproduce annually with levels of serum LH and sex steroids reaching a maximum just prior to spawning (Trudeau et al., 1991). Basal LH levels were reduced by methylmercury in post-spawning goldfish but not pre-spawning fish suggesting that methylmercury-induced alterations may have different effects at different reproductive stages. In rats, estrogen has been shown to be protective against methylmercury-induced alterations in

serum LH levels (Oliveira et al., 2006). Post-spawning goldfish with lower sex steroid levels may be more sensitive to endocrine disruption by methylmercury. In post-spawning fish, reduced LH release may be due to alterations in stimulatory or inhibitory neurotransmission (e.g., monoamine signalling). These alterations may occur alone or in concert with reduced levels of activins and secretoneurin due to decreased expression of subunit and precursor mRNA. Reduced sex steroid levels and GSI in pre-spawning goldfish may be due to anterior pituitary-independent effects such as the reduced transcription of isotocin. Alterations in pituitary production and release of FSH has not been examined, however this hormone plays a role in gonadal development and vitellogenesis (Van Der Kraak et al., 1998; Trudeau et al., 2000). Altered neurotransmitters involved in regulating FSH may also play a role in methylmercury-induced endocrine disruption. Interestingly, transcript levels for monoamine oxidase were up-regulated in pre-spawning goldfish fed the high methylmercury diet and post-spawning fish fed the low diet which correlates with reduced sex steroid levels (data not shown). The significance of these alterations in transcript levels is unclear; however it suggests alterations in the monoamine neurotransmitter system which have been observed in other fish species (Figure 4.2). How these alterations relate to the reductions in sex steroid levels remain to be examined. In order to gain a better understanding of the relationship between hypothalamic gene expression changes and methylmercury-induced endocrine disruption, the following avenues of study are suggested: 1) examine the effects of methylmercury on gonadal steroidogenesis and development at clearly defined stages of reproduction (i.e., regressed, early recrudescence, and mature spawning); 2) assess temporal alterations in gene expression through a time course exposure study; 3) perform a GnRH challenge experiment at each of the defined stages of reproduction to assess in detail the effects of methylmercury on LH production and release by the anterior pituitary.

4.4 Concluding remarks

Methylmercury has become a significant concern for both fisheries management agencies due to the potential economic impacts of reduced fish population health and human health agencies over the potential health effects. Despite the large number of methylmercury studies demonstrating neurotoxicity and indicating potential endocrine disruption, it remains a challenge to understand the mechanistic effects of methylmercury on physiological processes such as reproduction. My thesis contributes to this research area by demonstrating that methylmercury can negatively impact the reproductive axis of fish in a dose and seasonally-dependent manner. It also provides the first hypothalamic gene expression data for fish providing a strong foundation for understanding the toxic mode of action for methylmercury and for examining the mechanistic link between alterations in neuroendocrine factors and reproductive biomarkers.

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