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Effects of Fibrate Drugs on Two Fish Species

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Human Pharmaceuticals in the Environment:

Effects of Fibrate Drugs on Two Fish Species

by

Caroline Mimeault

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies of the University of Ottawa in partial fulfillment of the requirements for the Chemical and Environmental Toxicology Ph.D. degree in the Ottawa-Carleton Institute of Biology.

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“Ne jamais perdre l’objectif de vue”.

Denise Desrochers, *depuis ma tendre enfance.*

EXAMINERS

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ABSTRACT

Human pharmaceuticals have been reported in the aquatic environment but information pertaining to their effect(s) in aquatic non-target species was scarce when this project was initiated. The two main objectives of this research were to determine if these new emerging contaminants are available to aquatic species and to assess if exposure can lead to unfavourable effects in two fish species, the goldfish (*Carassius auratus*) and the rainbow trout (*Oncorhynchus mykiss*).

Most studies were done with the fibrate drug gemfibrozil (GEM), although the other fibrates bezafibrate (BEZA), clofibrate (CLO) and fenofibrate (FENO) were used for specific experiments. Waterborne GEM exposure resulted in bioconcentration factors up to 500 in goldfish using LC-MS/MS estimates. Results provided strong evidence that environmental concentrations of GEM are not indicative of plasme concentration in fish. Tissue distribution for the three peroxisome proliferator-activated receptors (PPARs) subtypes were established in goldfish and rainbow trout by estimating mRNA levels using reverse-transcriptase PCR (RT-PCR). The monitoring of PPARα mRNA was found not to be a good indicator of GEM exposure as mRNA levels were not modified. Interestingly PPARβ mRNA appears to be modulated in comparable ways in the two species and PPARγ was affected but only in rainbow trout. Identified potentially harmful effects resulting from exposure to fibrate drugs include 1) a challenged hepatic antioxidant defense system in goldfish exposed to an environmentally relevant concentration of GEM determined using spectrophotometric assays and 2) an impaired cortisol response in rainbow trout exposed to high concentrations of CLO and FENO determined through *in-vitro* head kidney cell stimulation. A mechanistic approach to understand the effects of fibrate drugs allowed identifying potential target steps

of corticosteroidogenesis, hence contributing to the understanding of basic endocrinology. This thesis is an important contribution to the emerging field of the effects of pharmaceuticals in the aquatic environment as it provides evidence that environmental concentrations significantly underestimate *in-vivo* drug levels and that exposure to human drugs may challenge or impair essential physiological functions in fish. Future research should focus on assessing effects over longer periods of time and at lower concentrations.

RÉSUMÉ

La présence de produits pharmaceutiques dans l'environnement aquatique est maintenant un fait établi mais peu d'information à propos de leurs effets chez les espèces non-ciblées était toutefois disponible au début de ce projet. Les deux objectifs principaux de cette recherche étaient de déterminer si les espèces aquatiques peuvent assimiler ces nouveaux contaminants et d'évaluer si les produits pharmaceutiques peuvent causer des effets défavorables chez deux espèces de poissons, le poisson rouge (*Carassius auratus*) et la truite arc-en-ciel (*Oncorhynchus mykiss*).

La majorité des études ont été menées avec le fibrate gemfibrozil (GEM), mais d'autres fibrates tels que le bezafibrate (BEZA), le clofibrate (CLO) et le fenofibrate (FENO) ont aussi été utilisés pour certaines expériences. Des facteurs de bioconcentration atteignant 500 ont été démontrés dans le plasma de poissons rouges exposés au GEM dissout dans l'eau. Ces résultats prouvent que les concentrations environnementales du GEM ne sont pas représentatives des concentrations plasmiques chez le poisson. Les niveaux d'ARNm des trois récepteurs activés par les proliférateurs de peroxysomes (PPAR) ont été établis dans les tissus du poisson rouge et de la truite arc-en-ciel en utilisant la PCR avec transcriptase inverse (RT-PCR). La détection des niveaux d'ARNm de PPARα s'est avérée ne pas être un bon indicateur d'exposition au GEM. Par contre, l'ARNm de PPARβ semble varier de façon comparable dans les deux espèces et PPARγ varie uniquement chez la truite arc-en-ciel. Les effets résultant d'une exposition aux fibrates pouvant s'avérer nocifs comprennent 1) une stimulation du système antioxydant du foie du poisson rouge résultant, entre autres, d'une exposition à une concentration environnementale qui a été déterminée par dosages spectrophotométriques et 2) une altération de la réponse cortisolique chez la truite arc-en-ciel

exposée à de hautes concentrations de CLO et FENO déterminées par des stimulations *in-vitro* des cellules antérieures du rein. Une étude du mécanisme de la corticostéroïdogénèse a permis d'identifier les étapes ciblées par l'exposition aux fibrates, et par le fait même, a contribué à la compréhension des mécanismes endocriniens. Cette thèse constitue une importante contribution au domaine émergeant des effets des produits pharmaceutiques retrouvés dans l'environnement aquatique en démontrant que les concentrations environnementales sous-estiment grandement la quantité de médicaments *in-vivo* et que l'exposition à ces produits pharmaceutiques peut altérer, voir compromettre, certaines fonctions physiologiques essentielles chez les poissons. Des études supplémentaires seront nécessaires pour explorer les effets d'expositions à plus long terme et à de plus faibles concentrations.

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LIST OF ABBREVIATIONS

ACO	acyl-coA oxidase
ACTH	adrenocorticotropic hormone
BCF	bioconcentration factor
BEZA	bezafibrate
cAMP	cyclic adenosine monophosphate
CAT	catalase
CLO	clofibrate
EC_{50}	effective concentration 50%
FENO	fenofibrate
GEM	gemfibrozil
GPx	glutathione peroxidase
GSH	reduced glutathione
GSSG	glutathione disulfide
GST	glutathione-s-transferase
HDL	high density lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPI	hypothalamic-pituitary-interrenal
IP	intraperitoneal injection
Kow	octanol-water partition coefficient
LC_{50}	lethal concentration 50%
LDH	lactate dehydrogenase
LOEC/L	lowest observed effect concentration/level
MDA	malonaldehyde
NOEC/L	no observed effect concentration/level
NSAID	non-steroidal anti-inflammatory drug
P450ssc	cholesterol side chain cleavage enzyme
PEC	predicted environmental concentration
PNEC	predicted no effect concentration
PP	peroxisome proliferator
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
ROS	reactive oxygen species
RXR	retinoid X receptor
StAR	steroidogenic acute regulatory protein
TBARS	thiobarbituric reactive substance
TC	total cholesterol
TG	triglyceride
WWTP	wastewater treatment plant

CHAPTER 1

General introduction

The presence of human pharmaceuticals in the environment is an emerging field in ecotoxicology and knowledge about the consequences of their exposure in fish was scarce at the onset of this thesis. The discovery of fibrate drugs in Canadian aquatic systems triggered this research. Studies were first designed to assess the potential uptake of a fibrate drug from the aquatic environment by a fish species. Although the classic approach for initial assessments of potential impacts of contaminants is through traditional endpoints such as the lethal concentration that kills 50% of a group of individuals (LC50), the continual addition of human pharmaceuticals to the aquatic environment and their occurrence at low concentrations justified the need for non-traditional endpoints (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999). Studies were designed to assess non lethal impacts of fibrate drug(s) exposure on nuclear receptors and on essential and well known systems including the cellular oxidative stress defence system and the endocrine stress response.

1.1. Human pharmaceuticals in the environment

The first evidence for the occurrence of human drugs in the environment was published in the early 1980s (Richardson and Bowron, 1985) but the field significantly expanded over the last decade. The sudden interest in these previously unsuspected environmental contaminants was triggered by the improved detection limits of analytical instruments and by two key review papers. Halling-Sørensen et al. (1998) reported on the occurrence, fate and effects of human and veterinarian pharmaceutical substances in the

environment. The inherent ability of medicinal compounds to elicit biological effects prompted them to review existing toxic effects on plankton, plants, algae and invertebrates. Acute endpoints revealed effect concentrations (mg/L to g/L) to be much higher than environmental occurrence (ng/L to μg/L) and life cycle and/or physiological endpoints were therefore determined to be more relevant than the traditional LC50 endpoint to study the effects of pharmaceuticals in the environment on aquatic organisms. Daughton and Ternes (1999) reiterated the need for non-traditional toxicological endpoints and highlighted the continual addition of human drugs into aquatic systems, a concept now referred to as pseudo-persistence (Daughton, 2002). Since these two review papers were published, many publications on source, occurrence, exposure, effects and risk assessment of human pharmaceuticals in the environment have contributed to the scientific literature.

1.1.1. Source and fate

Human pharmaceuticals can enter the aquatic environment by several routes and their different formulations make it difficult to generalize their environmental fate. Parent compounds and metabolites of human drugs make their way to sewage treatment plants (STPs) mainly through domestic and hospital sewage systems as drugs are excreted in urine and feces with absorption ranging from 5 to 100% following oral administration (Halling-Sørensen et al., 1998; Holford, 2001). In urban areas, the main point of entry into the aquatic environment is through STP effluent waters and subsequent dilutions into rivers, lakes and coastal zones (Trudeau et al., 2005). Several pharmaceuticals are released in post-treated effluent waters as the efficiency of removal of human drugs by STPs is highly variable, ranging from 7% (carbamezapine) to 99% (salicyclic acid) (Ternes, 1998). The variation in

the efficiency of STPs to remove drugs from input water depends on the type of treatment used by the STP and on the properties of the drug. Removal of human drugs in STPs occurs mainly by sorption to suspended particles and by biodegradation (Kummerer et al., 2005). Sorption being a reversible process, drugs can also reach the environment through landfill disposal or agricultural applications of sewage sludge from which drugs can leach out to groundwater (Trudeau et al., 2005). Other entry routes of pharmaceuticals in aquatic environments include the inappropriate disposal of unused and/or expired drugs in the domestic sewage system or in garbage (Halling-Sørensen et al., 1998).

As with other contaminants, the fate of human drugs once released into the aquatic environment depends on the physicochemical properties of the chemical and the properties of the receiving system (Rand et al., 1995). Water solubility, dissociation constant, partition coefficient and vapour pressure may all impact their environmental fate. Other factors including the rates of biotransformation, photolysis, hydrolysis, oxidation and reduction will also affect the fate of human drugs in the environment by affecting their rate of transformation (Kummerer et al., 2005). Even though it is difficult to predict the environmental fate of drugs as a group due to their variable chemical formulations, Daughton and Ternes (1999) predicted their environmental distribution to mainly occur through aqueous transport and food-chain dispersal due to their low volatility. All these parameters contribute to the degradation or the persistence of the parent compound and metabolites. In addition, some inactivated molecules can be reactivated to their biologically active form once in the environment (Trudeau et al., 2005).

1.1.2. Occurrence

The occurrence of human drugs in the environment depends on the total population and the amount of drugs sold and consumed in a given area (Trudeau et al., 2005). While clear indicators of drug use such as the number of drug prescriptions in Canada are not readily available, the total Canadian population has increased linearly since 1941, hence the entry of human pharmaceuticals into the aquatic environment is likely to increase relative to population numbers. Since the late 1990s, several scientific studies reported the presence of human drugs in STP effluents and in surface waters in concentrations ranging from ng/L to low μg/L. Detection of drugs was first reported in Europe but several studies from Canada and the US. confirmed their occurrence in North American waters (Table 1.1) (Kolpin et al., 2002; Metcalfe et al., 2003a; Metcalfe et al., 2003b; Pedersen et al., 2005; Lishman et al., 2006; Verenitch et al., 2006).

Table 1.1. Examples of human pharmaceutical classes reported in the aquatic environment and their medical applications. *Adapted from Daughton and Ternes (1999) and Trudeau et al. (2005).*

Class	Application(s) / treatment(s)	Examples
Analgesics/Anti-inflammatories	Pain, allergies, migraines, cold	Acetaminophen, acetylsalicyclic acid, diclofenac, ibuprofen
Antibiotics	Bacterial infections	Ampicilin, kanamycin, sulfonamides, tetracycline
Antidepressants	Depression	Fluoxetine
Antiepileptics	Epileptic seizures	Carbamezapine
Beta-blockers	Heart disorders	Proponolol
Blood lipid and cholesterol regulators	Lipid disorders	Bezafibrate, Clofibrate, fenofibrate, Gemfibrozil, Atorvastatin
Hormones	Birth control, hormone therapy	17α-ethinylestradiol
Tranquilizers	Anxiety	Diazepam

1.1.3. Exposure and effects

Significant exposure parameters include bioavailability, duration, frequency and concentration of contaminants (Rand et al., 1995). Effects of human drugs on non-target aquatic organisms are important despite their low detected environmental concentrations in the environment (ng/L to low μg/L) due to their pseudo-persistent characteristic. The continual addition of drugs into the aquatic environment results in chronic exposure of aquatic organisms being captives of their environment (Daughton and Ternes, 1999).

Assessing the route of exposure of toxic compounds is also an essential component to determine potential effects. In an aquatic environment, potential exposure routes are through water, sediments and food and in the case of human pharmaceuticals, water would probably be the most important exposure route given their aqueous occurrence (Daughton and Ternes, 1999). Despite several studies on human pharmaceuticals in the environment, information on exposure route and bioavailability of human drugs in aquatic life remain scarce.

Potential effects of human pharmaceuticals on non-target aquatic species are likely to occur as pharmaceuticals are specifically designed to be biologically active as opposed to other environmental contaminants. Drugs acting as hormone mimics or having high affinity for cellular receptors are likely to affect vertebrates other than humans as most of these target systems are well conserved across vertebrates (Trudeau et al., 2005). A global review of the effects of human pharmaceuticals on non-target species is beyond the scope of this introduction and can be found elsewhere (Crane et al., 2006; Fent et al., 2006).

Traditional acute ecotoxicological endpoints including LC50s, effective concentrations affecting 50% of individuals (EC50s) or lowest observed effect concentrations (LOECs) for mortality or growth are usually preferred to evaluate potential impacts of

environmental contaminants. Nevertheless, the ecological relevance of traditional endpoints in the case of human pharmaceuticals is uncertain considering that their reported effect concentrations are orders of magnitude higher than their concentrations in the environment (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999; Nunes et al., 2005).

1.1.4. Risk assessment and regulation

The ultimate goal of ecotoxicological research is to protect the environment from contaminant effects. Environmental risk assessments are conducted to evaluate the potential threat and subsequently determine if regulation is required. To date, the few environmental risk assessments for human pharmaceuticals used acute toxicity data to derive predicted environmental concentrations (PEC) over predicted no effect concentration (PNEC), the PEC/PNEC ratio (Stuer-Lauridsen et al., 2000; Jones et al., 2002). Nevertheless, the use of acute endpoints leads to inaccurate conclusions and underestimates the real environmental risk once more reflecting the need for more appropriate and environmentally-relevant ecotoxicity tests (Ferrari et al., 2004).

New pharmaceuticals in Canada are regulated by the Environmental Assessment Unit of the New Substances Assessment and Control Bureau of Health Canada which is responsible for conducting environmental and health risk assessments for all new substances regulated under the Food and Drug Safety Act, including pharmaceuticals, prior to import or manufacture in Canada. Nevertheless, this procedure initiated under the Canadian Environmental Protection Act only applies to substances on the market as of September 2001.

1.2. Fibrate drugs in the environment

This thesis deals principally with the fibrate drug gemfibrozil (GEM). Other fibrates (bezafibrate, BEZA; clofibrate, CLO; fenofibrate, FENO) were also included in Chapter 5. The structures (Fig. 1.1), pharmacological actions, mode of action, and environmental occurrence of this class of drugs will be addressed.

1.2.1. Fibrate drugs

Fibrate drugs are blood lipid regulators mainly prescribed to reduce triglycerides (TGs) and/or to increase high density lipoproteins (HDLs). Optimal human plasma lipid profiles are characterized by less than 200 mM total cholesterol (TC), 100 mM low density lipoprotein (LDLs), 150 mM TGs and more than 60 mM HDLs (Merck, 2007). Lipid regulating drugs such as fibrates are therefore prescribed when human diets and/or life styles are not adequate to achieve these optimal lipid profiles. GEM, being the principal drug used in this thesis, will be used to describe the pharmacological and mode of actions of the fibrate class of drugs.

1.2.2. Pharmacodynamics and pharmacokinetics of GEM

GEM is administered orally at a maximum dose of 600 mg twice a day. The drug is rapidly and completely absorbed through the intestine and a steady state is achieved within 2 weeks of treatment with an average peak plasma concentration of 15 to 25 mg/L (Todd and Ward, 1988; Spencer and Barradell, 1996). GEM, as other fibrate drugs, is absorbed from the gastrointestinal tract and the reported plasma half-life for GEM in humans varies between 1.5 to 7.6 h (Todd et al., 1988; Malloy and Kane, 2001). For all fibrate drugs, elimination

occurs through the hepatic biotransformation of the parent compounds into glucuronide-conjugated metabolites and are predominately eliminated in urine (Spencer and Barradell, 1996).

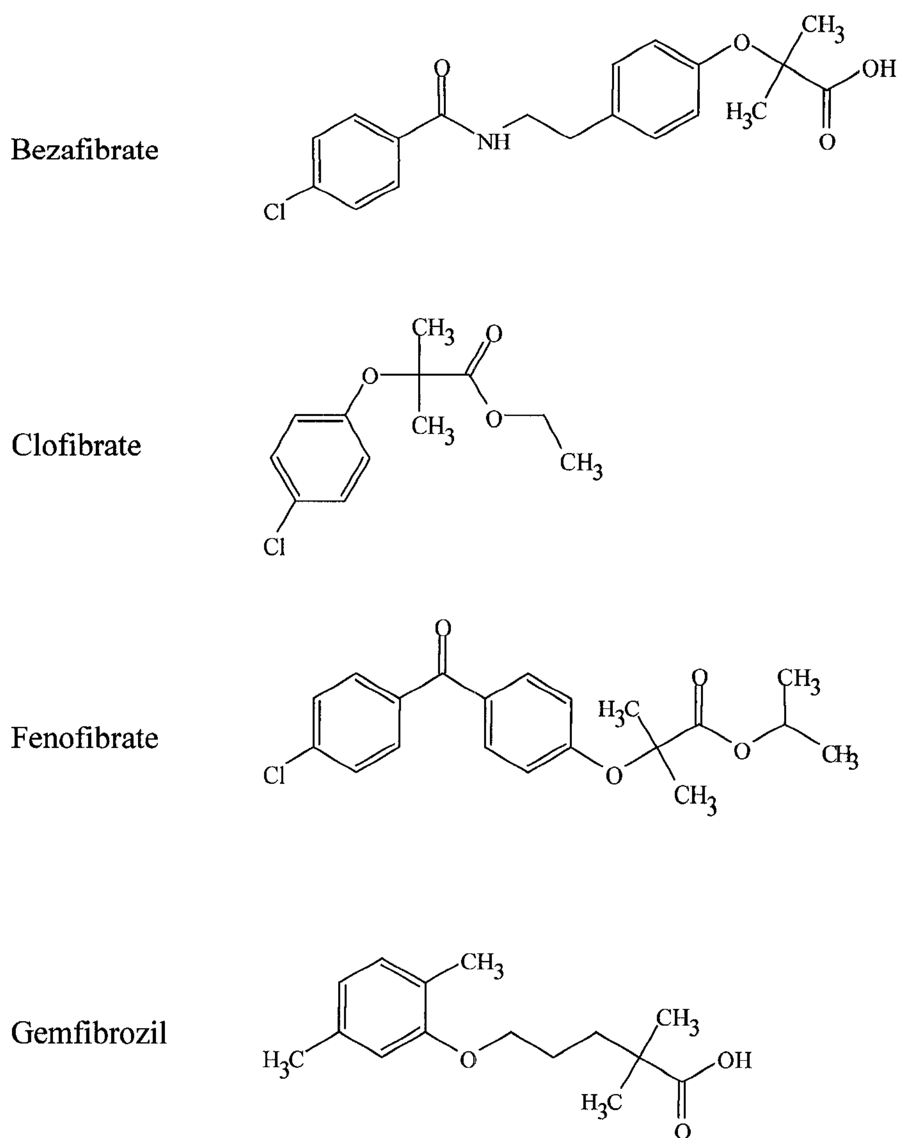

Figure 1.1. Chemical structures of the fibrate drugs used in this thesis.

The major effect of GEM treatment is a reduction in plasma TGs that occurs as a result of an increased activity of the lipoprotein lipase enzyme (Spencer and Barradell, 1996). Other significant effects include an increase in HDLs and in some cases, a decrease in total cholesterol (Todd and Ward, 1988; Spencer and Barradell, 1996). Spencer et al. (1996) also reported that GEM can affect levels of apolipoproteins and reduce *in-vitro* activity of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase leading to cholesterol decreases. The mode of action of GEM was elucidated and occurs through the peroxisome proliferator-activated receptor (PPAR).

1.2.3. Mode of action of GEM

Fibrate drug administration results in hepatomegaly and increases in the size and numbers of peroxisomes in rodents, hence the classification of fibrate drugs as peroxisome proliferators (PPs) (Lalwani et al., 1983). Peroxisomes are single membrane-bound organelles found in the cytoplasm of most eukaryotic cells but are particularly abundant in liver and renal cells. These organelles contain several enzymes and their principal metabolic functions are β-oxidation of fatty acids and the detoxification of reactive oxygen species (ROS) (Orbea et al., 1999). Peroxisomal β-oxidation specifically breaks down very long chain fatty acyl-CoAs by the shortening of 2 carbons per cycle (Fig. 1.2) (Moran et al., 1994; Gonzalez, 1997). Methods to identify peroxisome proliferation include the visual assessment of histological preparations or alternatively, the measurement of the activity of acyl-CoA-oxidase (ACO), the first and rate-limiting enzyme of peroxisomal β-oxidation, an accepted biomarker of peroxisome proliferation (Lalwani et al., 1983; Gonzalez, 1997; Orbea et al., 1999; Cajaraville et al., 2003).

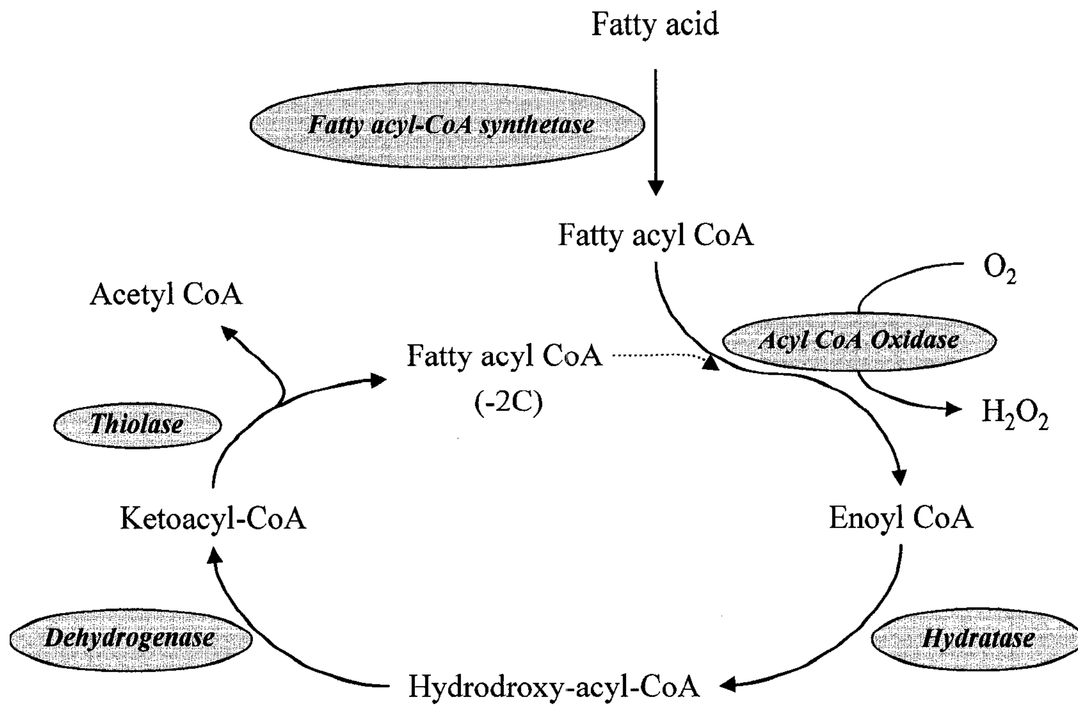

Figure 1.2. Peroxisomal β-oxidation. *Adapted from Moran et al. (1994).*

The biological effects of PPs are exerted through the activation of the nuclear receptors and transcription factors called PPARs (Issemann and Green, 1990; Desvergne and Wahli, 1999), for which three subtypes (PPARα, PPARβ and PPARγ) are identified with distinct tissue distributions and functions in mammals (Corton et al., 2000). PPARα is expressed mainly in liver, kidney, heart and skeletal muscle where it is involved in fatty acid catabolism (β-oxidation). PPARβ is expressed ubiquitously but its physiological function(s) is still not entirely clear although it also seems to be involved in metabolism (Corton et al., 2000). Finally, PPARγ is reported principally in adipose tissue where it influences lipid

storage and adipocyte differentiation (Desvergne and Wahli, 1999; Corton et al., 2000; Lee et al., 2003; Leaver et al., 2005).

Several endogenous and synthetic PPAR ligands have been identified. PPARs are primarly located in the nucleus (Patel et al. 2005). Natural ligands include fatty acids, eicosanoids and leukotrienes while synthetic ligands include hypolipidemic agents (including fibrate drugs) and non-steroidal anti-inflammatory drugs (NSAIDs) (Desvergne et al., 1999). Some ligands preferentially bind a specific PPAR subtype such as hypolipidemic fibrate drugs which preferentially bind to PPARα with weak binding to both the β and γ subtypes (Desvergne and Wahli, 1999; Leaver et al., 2005). Upon ligand binding, the PPAR heterodimerizes with the retinoid X receptor (RXR) within the nucleus and gene expression is regulated through subsequent binding of the complex to a peroxisome proliferator-response element (PPRE) in the promoter region of target genes (Fig. 1.3). The pharmacological effects of GEM are therefore the result of the presence of a PPRE upstream of critical genes including the lipoprotein lipase and the ACO genes (Mandard et al., 2004).

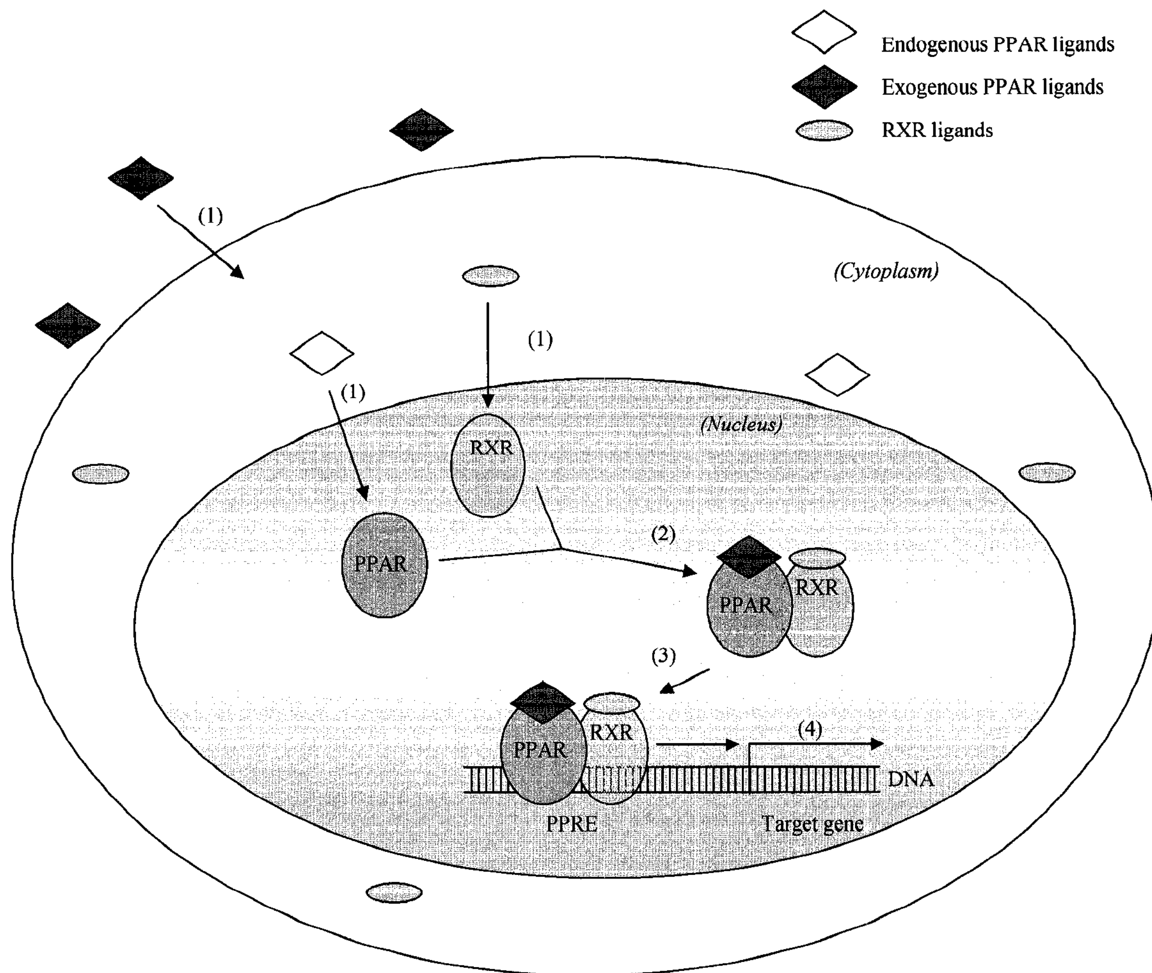

Figure 1.3. Mode of action of ligands on the peroxisome proliferator-activated receptor (PPAR). (1) Exogenous ligands such as fibrate drugs or other PPs enter the cell and nucleus to activate PPAR through binding of the receptor in the cytoplasm. The retinoic X receptor is also activated through binding of its own ligands. (2) Ligand-bound PPAR dimerizes with RXR. (3) The heterodimer binds to a PP response element (PPRE) in the promoter region of target genes. (4) Transcription of target genes is altered. *Adapted from Schoonjans et al. (1996), Corton et al. (2000), Gervois et al. (2000) and Patel et al. (2005).*

1.2.4. Fibrate occurrence and fate in the environment

Fibrate drugs are reported to persist in the aquatic environment and their concentrations in aquatic systems seem to be more a function of dilution than degradation (Daughton and Ternes, 1999). Evidence for fibrate drug persistence includes 1) the highly variable STP removal efficiencies (Fent et al., 2006), 2) the persistence of some fibrate drugs in both aerobic and anoxic conditions (Heberer, 2002), and, 3) the presence of GEM in water runoff from agricultural fields irrigated with treated wastewaters (Pedersen et al., 2005). In addition, fibrate drugs are generally less potent than statins, the other highly prescribed class of hypolipidemic/cholesterolemic drugs, hence doses are consequently higher for fibrates than for statins (Table 1.2). Both the persistent characteristic of fibrate drugs and the high dosage contribute to their occurrence in the aquatic environment. Fibrates are reported in domestic wastewater, surface water and drinking water in North America, South America and Europe at concentrations ranging from high ng/L to low μg/L (Table 1.3) (Ternes, 1998; Kolpin et al., 2002; Metcalfe et al., 2003a; Metcalfe et al., 2003b).

Table 1.2. Maximum prescribed daily doses of fibrate and statin drugs. *Modified from Staels et al. (1998) and Merck (2007).*

Class	Drug	Trade name	Maximum daily doses (mg)
Fibrates	Bezafibrate	Bezalip	600
	Clofibrate	Atromid-S	2000
	Fenofibrate	Tricor	200
	Gemfibrozil	Lopid	1200
Statins	Atorvastatin	Lipitor	80
	Simvastatin	Zocor	80
	Rosuvastatin	Crestor	40

Photodegradation is one process through which environmental contaminants can dissipate from aquatic systems. Clofibric acid, a metabolite of the fibrate drug clofibrate, was shown to photodegrade when exposed to sunlight (Doll and Frimmel, 2003); this process is known to be dependent on temperature and altitude (Andreozzi et al., 2003). Other factors that affect the rate of photodegradation include the presence of nitrate, humic acids and other pharmaceuticals (Andreozzi et al., 2003; Doll et al., 2003).

Table 1.3. Worldwide and Canadian occurrence of fibrate drugs in STP influent and effluent waters, surface waters and agricultural runoff waters. References are indicated by superscript letters. NR indicates that the drug was not reported in the scientific literature.

		Fibrate concentration in water (µg/L)			
Distribution	**Pharmaceuticals**	**STP influent**	**STP effluent**	**Surface**	**Runoff**
Worldwide	Bezafibrate	4.7[c]	4.6[a,e]	3.1[e]	NR
	Clofibrate	NR	0.8[a]	NR	NR
	Fenofibrate	NR	0.2[a,d]	1.4[d]	0.7[d]
	Gemfibrozil	2.1[c]	4.8[a,d,f]	0.8[b]	0.8[d]
Canada	Bezafibrate	4.7[c]	0.6[c]	0.2[c]	NR
	Clofibrate	NR	NR	NR	NR
	Fenofibrate	NR	NR	NR	NR
	Gemfibrozil	2.1[c]	4.8[f]	0.2[f]	NR

References: (a) Andreozzi et al. (2003); (b) Kolpin et al. (2002); (c) Metcalfe et al. (2003a); (d) Pedersen et al.(2005); (e) Ternes (1999); (f) Verenitch et al. (2006).

1.2.5. Effects of fibrate drugs in the environment

Published studies to date on the effects of fibrate drugs on non-target aquatic species include: 1) studies assessing impacts on PPARs, the mode of action of fibrate drugs (Ruyter et al., 1997; Pretti et al., 1999; Liu et al., 2005; Mimeault et al., 2006); 2) studies using the traditional approach to assess toxicity (Nunes et al., 2005; Emblidge and Delorenzo, 2006); and, 3) studies assessing non-traditional endpoints such as biochemistry and endocrine disruption (Yang et al., 1990; Donohue et al., 1993; Laville et al., 2004; Nunes et al., 2004; Mimeault et al., 2005; Emblidge and Delorenzo, 2006; Mimeault et al., 2006; Nunes et al., 2006; Thibault et al., 2006; Zurita et al., 2007). More details regarding the conclusions of these studies will be provided in appropriate chapters of this thesis.

1.3. Bioavailability of human pharmaceuticals in the aquatic environment

Bioavailability is the portion of a chemical that is available for biological action such as uptake by an aquatic organism (Rand et al., 1995). The bioavailability of a chemical is consequently an important component of exposure as it establishes the actual contact an organism has with a particular chemical (Rand et al., 1995). Assessing the bioavailability of new classes of environmental contaminants such as human pharmaceuticals is therefore a crucial step in defining the potential toxicity of chemicals.

Parameters affecting the bioavailability of chemicals include aspects of the environmental fate of the toxicants and the biological characteristics of the exposed organisms. The work presented in this thesis assesses the uptake of a fibrate drug by a fish species, by evaluating the transfer of the chemical from the aquatic medium to the organism. Factors that affect the uptake of chemicals include physicochemical parameters such as the

octanol-water partition coefficient (K_{ow}), and biological parameters such as membrane permeability.

Few papers to date have addressed the bioavailability of human pharmaceuticals to non-target species. Nevertheless, some authors report that bioaccumulation of human drugs in aquatic organisms is not likely to occur since these molecules are designed to be eventually metabolized (Trudeau et al., 2005). The work presented in Chapter 2 of this thesis provides evidence that the fibrate drug GEM can efficiently be taken up and bioconcentrate by goldfish (*Carassius auratus*) exposed to waterborne GEM.

1.4. Endpoints of interest

The choice of relevant endpoints is crucial in assessing the impacts of contaminants in non-target species. Ecotoxicological studies traditionally use endpoints such as lethal concentration (LC), effective concentration (EC), lowest-observed-effect concentration (LOEC) and no-observed-effect concentration (NOEL) for which the main observed effects are mortality, growth and reproduction (Rand et al., 1995). As reported by Daughton and Ternes (1999), investigations of human drug effects on non-target species should go beyond acute testing. Acute toxicity tests should not be the primary concern and subtle effects that could have profound implications over long exposure periods should be the priority.

The selected non-traditional endpoints to evaluate the potential effects of fibrate drugs in non-target fish species in this thesis are (1) the intended human pharmacological actions of GEM at therapeutic doses; (2) the assessment of the mRNA levels of PPARs; (3) the induction of oxidative stress responses; and, (4) the disruption of *in-vitro* cortisol response in rainbow trout. Each endpoint is briefly described below.

1.4.1. Human pharmacological effects of GEM in goldfish

Pharmaceuticals are manufactured specifically to induce biological response(s) in humans as opposed to other environmental contaminants. It is thus interesting to first determine if the same physiological response for which the drugs are designed could be observed in non-target aquatic species. Therefore, before addressing the potential effects of environmentally-relevant concentrations of GEM, the effects of a human equivalent dose of GEM, a fibrate drug, on goldfish triglyceride plasma levels were investigated (Appendix B).

1.4.2. Effects of GEM on PPAR mRNA levels

Transcription is the first step by which gene expression is regulated and it can be used to monitor contaminant exposure or to identify the mode of action of chemicals in non-target organisms (Levine and Oris, 1999; Wintz et al., 2006). In addition, measuring mRNA levels provides biologically relevant information regarding the molecular response to contaminant exposure and the high sensitivity of transcriptional biomarkers contribute to lowering the detection limits in assessing contaminant exposure (McClain et al., 2003).

PPARs belong to the steroid/thyroid/retinoid receptor superfamily and are ligand-induced transcription factors involved primarily in lipid metabolism (Desvergne and Wahli, 1999). As transcription factors, PPARs have physiological roles through PPRE binding and subsequent regulation of target genes (Mandard et al., 2004). PPARα is the most studied subtype. Genes identified with a PPRE in their promoter region implicate PPARα in several biological processes including fatty acid metabolism, lipogenesis, lipoprotein metabolism, glucose metabolism and biotransformation (Mandard et al., 2004). Interestingly, a PPRE has

also been identified upstream of PPARα itself, suggesting this receptor is subject to auto-regulation (Mandard et al., 2004).

At the onset of this study very little was known regarding the tissue distribution of the different PPAR subtypes in any fish species. Therefore, the PPAR expression profiles were characterized in fish and compared to those in mammals. In addition, the effects of GEM, a fibrate drug, on PPAR mRNA levels was investigated as a potential biomarker of fibrate drug exposure. Hypolipidemic fibrate drugs are known to preferentially bind to PPARα and to weakly bind to the other two subtypes (Desvergne and Wahli, 1999), so the effects of GEM were assessed on mRNA levels of all three PPAR subtypes. The results of the PPAR piscine tissue distribution and the effects of GEM exposure on PPAR mRNA levels are presented in Chapter 3 (in goldfish) and Chapter 4 (in rainbow trout).

1.4.3. Effect of GEM on oxidative stress

Oxidative stress is a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage (Sies, 1981) and occurs when there is an imbalance in the generation and removal of oxyradical species within a cell (Kelly et al., 1998). The toxic and adaptive responses to oxidative stress include modifications in cellular defence status, lipid peroxidation and DNA damage (Di Giulio et al., 1989; Kelly et al., 1998).

Mammalian models have traditionally been used to study oxidative stress but toxicological and adaptive responses to oxidative stress are ubiquitous and similar across species including mammals and fish (Fig. 1.4) (Di Giulio et al., 1989; Kelly et al., 1998; Livingstone, 2001). Recent evidence demonstrates that the study of oxidative stress is also of

ecotoxicological relevance as aquatic environmental contaminants stimulate reactive oxygen species (ROS) and cause oxidative damage in aquatic species (Livingstone, 2001).

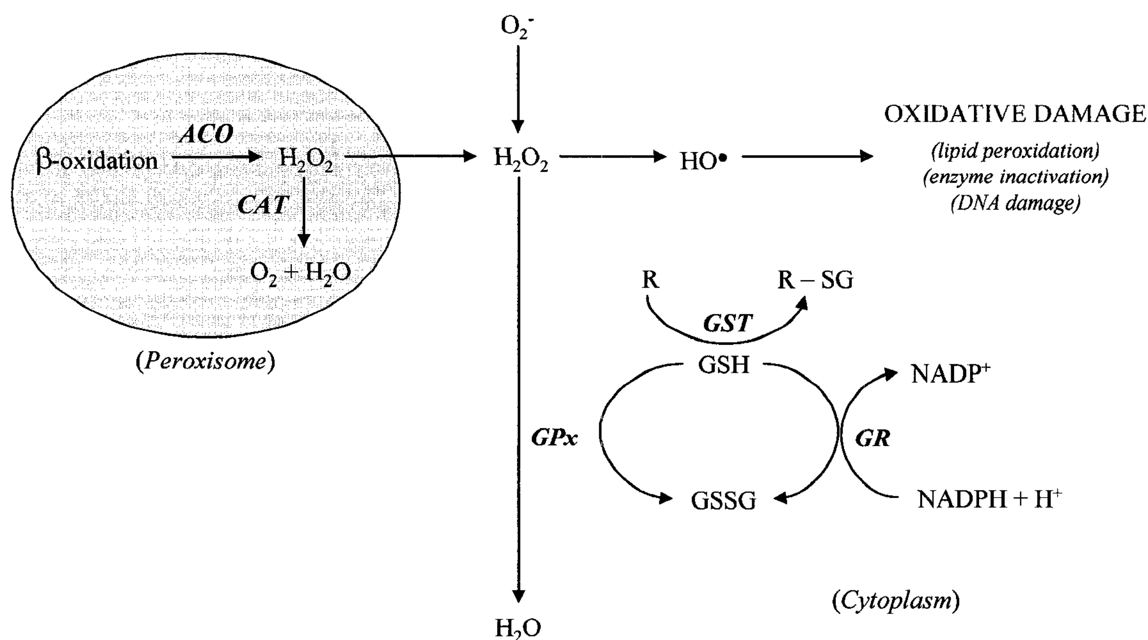

Figure 1.4. Peroxisomal production of H_2O_2 and cellular antioxidant defence system. *Abbreviations*: ACO: acyl-coA oxidase, CAT: catalase, GPx: glutathione peroxidase, GR: glutathione reductase, GSH: reduced glutathione, GSSG: glutathione disulfide and GST: glutathione-s-tranferase.

Several factors contribute to the rationale of assessing the effects of GEM on oxidative stress. Chronic exposure to hypolipidemic drugs, which are also PPs, causes liver tumor formation in mice and rats, hence raising the possible involvement of oxidative stress in hepatocarcinogenesis (Reddy et al., 1980; Peters et al., 2005). In addition, PPs can significantly increase ACO activity since this is a target gene of PPARα (Mandard et al.,

2004), and increased ACO activity could increase H_2O_2 production (Fig. 1.2) while only causing a slight increase in catalase (CAT) a gene for which no PPRE has yet been identified in its promoter region (Reddy et al., 1980). The effects of GEM on the oxidative stress system are presented in Chapter 3 (in goldfish) and Chapter 4 (in rainbow trout).

1.4.4. Effects of fibrate drugs on the stress axis

The endocrine system regulates several physiological functions including metabolism, osmoregulation, growth, stress and reproduction and is vital to living organisms. Organic and inorganic pollutants are known to disrupt hormonal regulation and this hormone-dysfunction is now used as a sub-lethal indicator of toxicant effects (Hontela et al., 1992; 1993). Endocrine endpoints provide useful and early assessment of exposure to environmental pollutants.

The stress axis is one specific endocrine system that is perturbed by contaminant exposure and is used as an indicator of toxic stress in fish (Hontela et al., 1993). The normal stress response following exposure to an acute stressor consists of an elevation of blood cortisol levels, the most common and reliable measure of stress (Hontela et al., 1992; Wendelaar Bonga, 1997; Mommsen et al., 1999). However, fish chronically exposed to contaminants fail to increase blood cortisol concentrations in response to acute stress thus demonstrating an impaired cortisol response and providing a novel biomarker of endocrine disruption (Hontela et al., 1992). An impaired cortisol response could significantly affect fish recovering from an acute stressor as cortisol is an important hormone involved in the regulation of energy metabolism (glucocorticoid functions) and hydromineral balance (mineralocorticoid functions) (Wendelaar Bonga, 1997).

Some environmental contaminants have been demonstrated to be adrenal disruptors and to impair the biosynthesis of cortisol (Hontela, 1997). The work presented in Chapter 5 of this thesis demonstrates for the first time the effects of fibrate drugs on *in-vitro* head kidney cortisol production and attempts to identify the step within cortiosteroidogenesis affected by fibrates (Fig. 1.5).

1.5. Thesis outline

Human pharmaceuticals are present in the aquatic environment as current STPs are not designed to effectively remove them from sewage waters. In addition it is unlikely that human drug consumption will diminish in the coming years. The main objective of this thesis was therefore to determine the potential effects of human pharmaceuticals occurring in the environment on non-target aquatic species. To do so, fibrate drugs and in particular GEM were selected due to its Canadian and worldwide occurrences and apparent environmental persistence. Two species of fish were used as test animals in this thesis. The goldfish, *Carassius auratus* was chosen being a laboratory model for other members of the cyprinid family of fishes (Blazquez et al., 1998) and the rainbow trout, *Oncorhynchus mykiss* was chosen because it is widely studied and represents a good fish metabolic model. Studies were designed to achieve and test the following objectives and hypotheses.

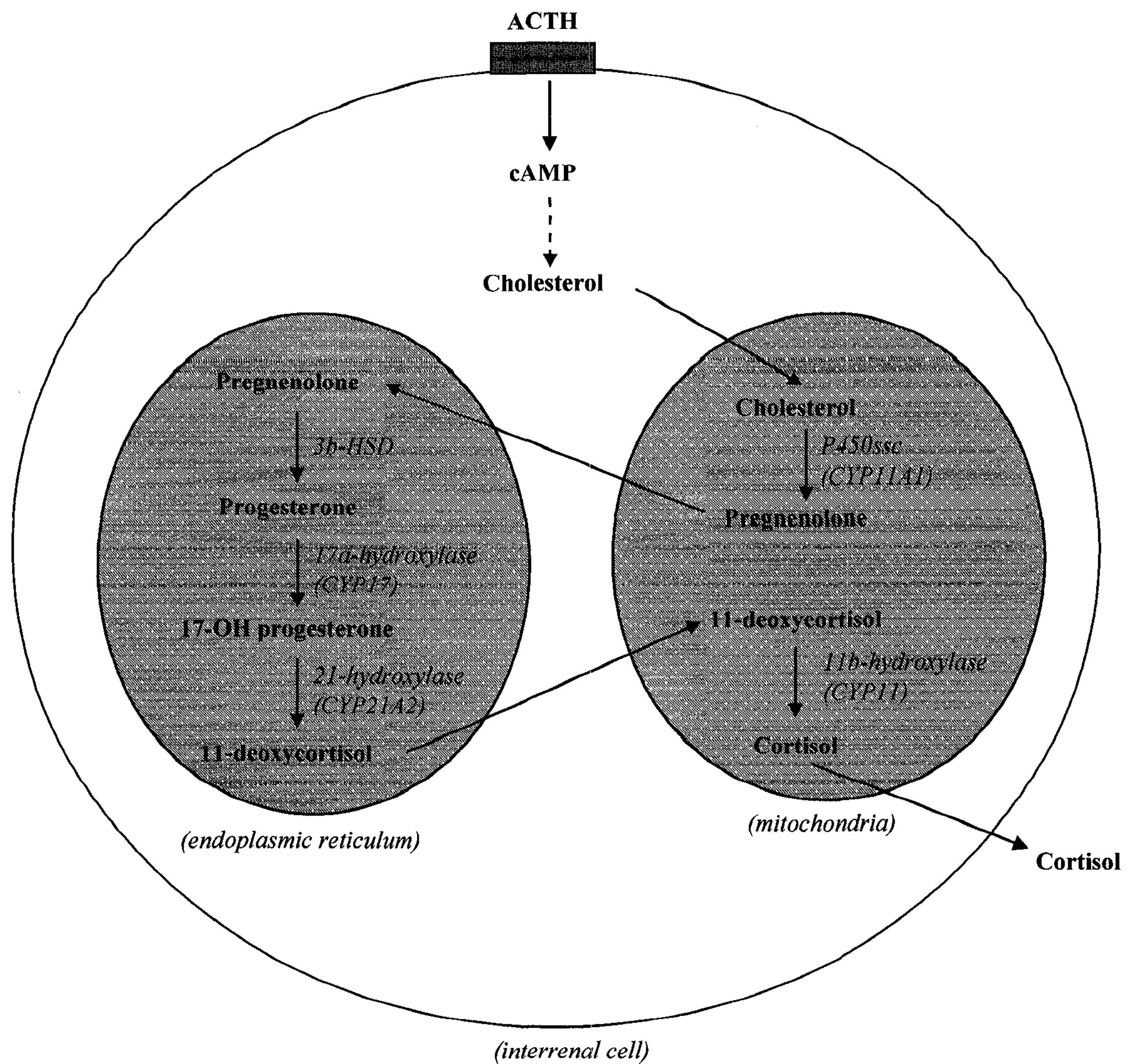

Figure 1.5. Cortisol biosynthesis in the fish interrenal cell. A dashed line was used to illustrate that cAMP does not have a direct action on cholesterol. *Abbreviations*: P450ssc, cholesterol side chain cleavage enzyme; 3β-HSD, 3β-hydroxy steroid dehydrogenase. *Adapted from Hontela et al. (1997) and Mommsen et al. (1999).*

Objective 1: Demonstrate that human pharmaceuticals are bioavailable to non-target aquatic species

Hypothesis 1: Goldfish will take up human drugs from the surrounding environment

Prediction 1: GEM will be detectable in the blood of goldfish exposed to waterborne GEM

Objective 2: Demonstrate that human pharmaceuticals can impact non-target aquatic species

Hypothesis 2a: GEM will increase the transcription of nuclear receptors

Prediction 2a: PPAR mRNA levels will increase with exposure to GEM

Hypothesis 2b: GEM exposure will result in oxidative stress in goldfish

Prediction 2b: The goldfish antioxidant defence system will increase following GEM exposure

Hypothesis 2c: Fibrate drugs will impair cortisol secretion

Prediction 2c: ACTH-induced cortisol production will be lower in rainbow trout head kidney cells in the presence of fibrate drugs

CHAPTER 2

Gemfibrozil (GEM) bioconcentrates in goldfish (*Carassius auratus*)

Chapter adapted from: Mimeault C, Woodhouse AJ, Miao XS, Metcalfe CD, Moon TW and VL Trudeau. 2005. The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, *Carassius auratus*. **Aquatic Toxicology** 73: 44-54.

2.1. Introduction

Pharmaceuticals, including anti-inflammatories, antibiotics, oral contraceptives and blood lipid regulators are detected in the aquatic environment at concentrations ranging from μg/L in the effluents of wastewater treatment plants to ng/L in surface waters (Daughton and Ternes, 1999; Kolpin et al., 2002; Metcalfe et al., 2003a; 2003b). Detection of environmental contaminants often prompts research to determine potential effects on non-target species without assessing bioavailability, which is an essential component of exposure as it determines the actual contact an organism has with a chemical (Rand et al., 1995).

The bioavailability depends on the physicochemical properties of the chemicals and is also related to the biological characteristics of the organism (Spacie et al., 1995). Aquatic organisms, being trapped into their environment, are exposed to contaminants through diet uptake (gastro-intestinal absorption), or through aqueous uptake (gill filtration) (Spacie et al., 1995). Following uptake into organisms, chemicals can be eliminated, can bioconcentrate or bioaccumulate. While no information was available on the elimination and bioconcentration of human pharmaceuticals in aquatic species at the onset of this thesis, bioaccumulation was reported unlikely to occur since human drugs are specifically designed to be metabolized (Trudeau et al., 2005).

The principal physicochemical parameter used to evaluate the potential of a contaminant to bioconcentrate is the octanol-water partition coefficient (K_{ow}), a concept captured in the hydrophobicity model supported by the fact that bioconcentration factor increases with increased log K_{ow} (Barron, 1990). Nevertheless, this model does not capture physiological parameters such as gill uptake or rate of metabolism of the chemicals nor does it apply to all chemicals. Human pharmaceuticals are a new category of aquatic contaminants so some case-by-case assessments are necessary before predictive models can be developed.

Drugs from the fibrate class of lipid regulators have been detected in domestic wastewater, surface water and drinking water in both North America and Europe, and gemfibrozil (GEM) is one of the most widely distributed compounds of this class (Ternes, 1998; Metcalfe et al., 2003a; 2003b). Reports on the amount of unmodified GEM excreted in human urine range widely from 5 to 70% (Zimetbaum et al., 1991; Malloy and Kane, 2001) and GEM is reported at concentrations as high as 2.1 μg/L in treated wastewaters and 0.5 μg/L in surface waters (Ternes, 1998; Daughton and Ternes, 1999; Metcalfe et al., 2003a; 2003b).

The fact that GEM is present and persistent in the aquatic environment and that no previous studies report human pharmaceutical uptake by any non-target aquatic species, prompted this study. The goldfish, *Carassius auratus* was chosen as it is a laboratory model for other members of the cyprinid family of fishes which are widely distributed across North America (Blazquez et al., 1998). The objective of this study was to determine if the goldfish, and presumably other fish species, take up GEM from the water. To test this, goldfish were

exposed to GEM either by intraperitoneal injections or through the water and GEM concentration in water and plasma were measured.

2.2. Materials and Methods

2.2.1. Experimental design

2.2.1.1. Fish

Common goldfish, *Carassius auratus*, were purchased from a tropical fish supplier (Aleong's International, Mississauga, ON). Fish were acclimated at 18 ± 1 °C for more than 2 weeks in 70-L fibreglass tanks that received treated, oxygenated City of Ottawa water. Only males were chosen, with an average weight of 25.6 ± 0.8 g (n = 46) and 26.4 ± 0.7 g (n = 62) for the acute and the sub-chronic experiments (described in section 2.2.1.2), respectively. In both experiments, fish were kept at a density of 10 to 12 fish per 70-L tank. All experiments were conducted under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhere to guidelines established by the Canadian Council on Animal Care (CCAC) for the use of animals in research.

2.2.1.2. GEM stability in water

GEM was dissolved in dimethylsulfoxide (DMSO, Sigma; final concentration 0.003%) and added to 30-L glass aquaria. GEM concentrations for the acute experiment were 1,500 and 10,000 µg/L. Water samples were collected in absence of fish after 0, 24, 48, 72 and 96 h without GEM renewal. Samples were extracted on the day of collection.

2.2.1.3. Acute experiment

Male goldfish were exposed to GEM (Sigma Aldrich, St. Louis, MO, USA) in February by either intraperitoneal injections or through the water. At time zero, goldfish exposed by injection received a single injection of GEM dissolved in DMSO at a volume of 0.5 μl/g fish. Fish received either 10,000 μg GEM per L of DMSO for a final dose of 5 ng GEM/g of fish or 100% DMSO (control). For waterborne exposures, GEM was dissolved in DMSO and added at time zero to 70-L fiberglass aquaria at nominal concentrations of 0, 1,500 or 10,000 μg GEM per L. The final DMSO concentration in the aquaria was 0.003 %.

After a 96 h exposure, all fish were anesthetized with 200 mg/L MS-222 (Sigma) prior to blood sampling from the caudal vessels using a heparinized 1ml syringe and a 26-gauge needle. Fish were sacrificed by trans-spinal sectioning after blood sampling. Plasma was separated from red cells by a 10 min centrifugation at 12,000 g and aliquots stored at –80 °C until analyzed for GEM.

2.2.1.4. Sub-chronic experiment

Male goldfish were exposed in November for 14 days to nominal waterborne GEM concentrations of 0, 1.5 or 1,500 μg/L. GEM was dissolved in DMSO and added to the aquaria as noted above. Water and GEM concentrations were renewed every 72 h as a precaution since water concentration remained stable for at least 96 h. Following 14 and 28 days of exposure, two 1-L water samples were collected from each tank in amber glass bottles. In addition, fish were anesthetized with MS-222 prior to blood and tissue sampling, as described above.

2.2.2. Extraction and analysis of GEM in plasma and water

GEM was extracted from plasma using Waters HLB Oasis™ (Vac RC 30 mg) solid phase extraction cartridges (Waters Corporation, Milford, MA, USA) according to the manufacturer's protocol. Briefly, after cartridge conditioning, 100 μl aliquots of plasma were acidified to pH 2.0 with 3.5 M H_2SO_4 and then applied to the cartridge. The cartridge was then rinsed three times with HPLC-grade water and once with 5% methanol in water prior to elution with 1 ml ethanol. Average percent recovery of GEM from goldfish plasma was 89 ± 6% (n = 5). GEM was extracted from water samples using the same HLB Oasis™ extraction cartridges according to the manufacturer's protocol. Briefly, after cartridge conditioning, 10 ml aliquots of water were acidified to pH 2.0 with 3.5 M H_2SO_4 and then applied to the cartridge. The cartridge was rinsed three times with HPLC-grade water prior to elution with 1 ml of ethanol.

Methods for analysis of GEM were adapted from those described by Miao et al. (2002) using liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of acidic drugs in water. Briefly, separations were conducted with an Alliance 2695 liquid chromatograph (Waters, Milford, MA, USA) with a Genesis C_{18} (50 × 2.1 mm i.d., 3 μm) column (Jones Chromatography, Hengoed, Mid Glamorgan, UK). The mobile phases were acetonitrile (A) and 20 mM ammonium acetate (B), operated with gradient elution at a flow rate of 0.3 ml/min. The sample injection volume was 20 μl. Mass spectrometry was performed using a Quattro LC tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Z-Spray electrospray ionization source and operated in negative ion mode. MassLynx v 3.4 software was used for data acquisition and processing. For mass spectrometric analysis of GEM, the deprotonated molecular ion ($[M-H]^-$, m/z 249)

was selected for collision-induced dissociation using argon collision gas at 45 eV. The deprotonated molecular ion at m/z 249 and product ion at m/z 121 were monitored with unit resolution on both the first and second quadrupole analyzers.

2.2.3. Statistical analysis of data

Experimental results are presented as means ± standard error of the mean (SEM). All statistical analyses were conducted using SigmaStat™ 2.0 software (SPSS Corp., Chicago, IL). As appropriate, statistical significance was tested with t-test or one or two-way ANOVAs followed by Student-Newman-Keuls or Tukey tests for multicomparisons. A value of at least $P < 0.05$ was considered significant.

2.3. Results

2.3.1. GEM stability in water

The concentration of GEM in water samples collected from glass aquariain absence of fish remained stable over 96 h at both concentrations (Fig. 2.1).

2.3.2. Acute experiment: GEM uptake

The concentration of GEM in goldfish plasma for the two different exposure routes was determined. GEM was not detected in the plasma of any fish from the control treatments. Fish exposed to 10,000 µg GEM/L by injection achieved plasma concentrations of 10,300 ± 100 µg/L after 24 h which decreased rapidly to less than 5% of the initial concentration at 96 h (Fig. 2.2). The highest plasma GEM concentrations occurred in fish

exposed to GEM through water (either 1,500 or 10,000 μg/L), with concentrations >75,000 μg/L in fish from both treatments (Fig. 2.2). Plasma GEM concentrations remained relatively constant with time and were slightly, but significantly different ($P = 0.02$) between fish exposed to the two waterborne doses.

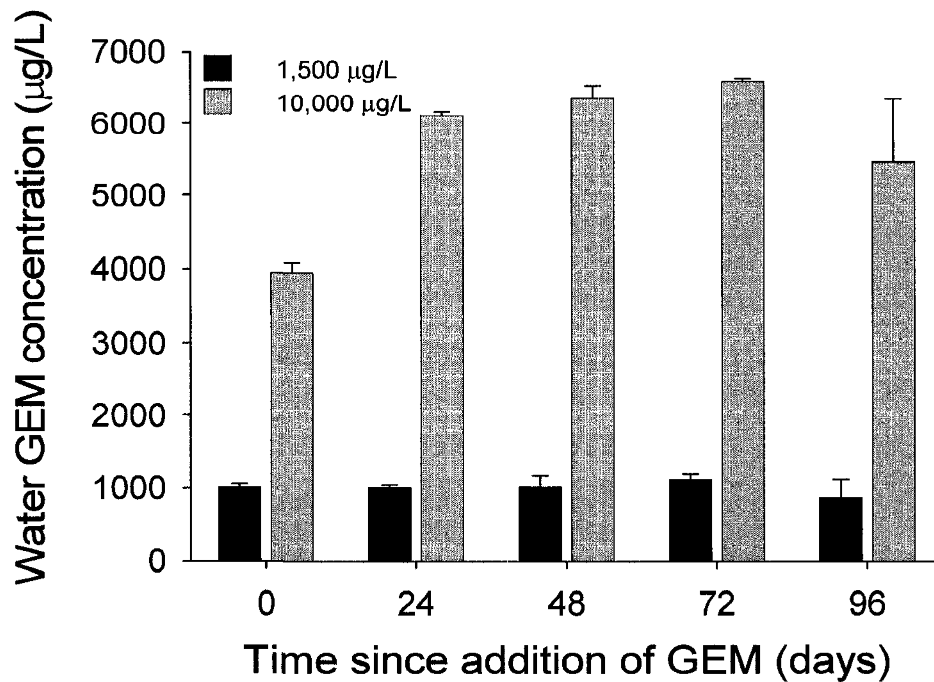

Figure 2.1. GEM stability (in absence of fish) over time in water. GEM (1,500 and 10,000 μg/L) was dissolved in DMSO (final concentration of 0.003%) and added to the tanks at time zero. Bars represent means + SEM (n = 2 to 4).

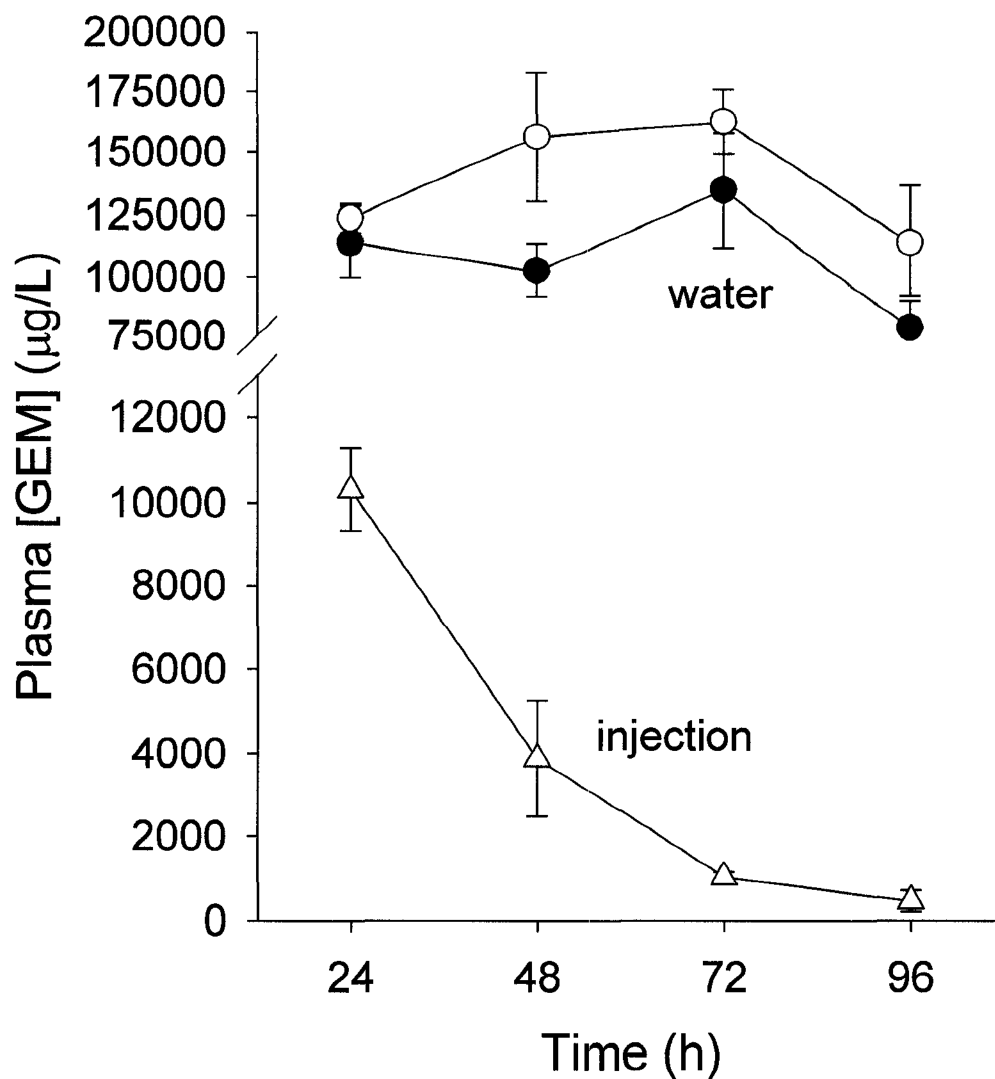

Figure 2.2. GEM concentrations in plasma of goldfish exposed by either intraperitoneal injection or through the water. Triangles represent detected GEM concentrations following a single GEM injection of 10,000 µg GEM/L; circles represent measured GEM plasma concentration following water exposure (closed circles, 1,500 and opened circles, 10,000 µg GEM/L). No GEM was detected in any control sample. Fish were exposed for a total of 96 h and plasma was sampled every 24 h. Data represent means ± SEM (n = 4-5). For waterborne exposure, the dose had a significant effect on plasma concentration ($P < 0.02$) but not time ($P = 0.07$).

2.3.3. Sub-chronic experiment: GEM uptake from water

GEM concentrations were determined in both water and plasma samples at the end of a 14 and 28 day exposure period. GEM concentration in the water of the control treatment for both 14 and 28 days were below detection limits (Fig. 2.3A). At the nominal concentration of 1.5 μg/L, the measured concentration of GEM in water at the end of the 14 and 28 day period were 0.34 and 0.78 μg/L, respectively. Similarly, at the nominal concentration of 1,500 μg/L, the measured concentration at the end of the 14 and 28 day exposure period were 851.9 and 520.3 μg/L, respectively (Fig. 2.3A).

Concurrent estimates of plasma GEM found control goldfish had an average concentration of 16 ± 5 and 4 ± 1 μg/L after 14 and 28 days of exposure, respectively (Fig. 2.3B). After 14 days of GEM exposure, plasma concentrations in goldfish were 170 ± 20 and 78,000 ± 5000 μg/L when exposed to nominal concentrations of 1.5 and 1500 μg/L, respectively (Fig. 2.3B). After 28 days, plasma concentrations in goldfish were 280 ± 50 and 59,000 ± 3,000 when exposed to nominal concentrations of 1.5 and 1,500 μg/L, respectively (Fig. 2.3B). Statistical analysis revealed a significant effect of exposure concentration ($P < 0.001$) on GEM plasma concentration.

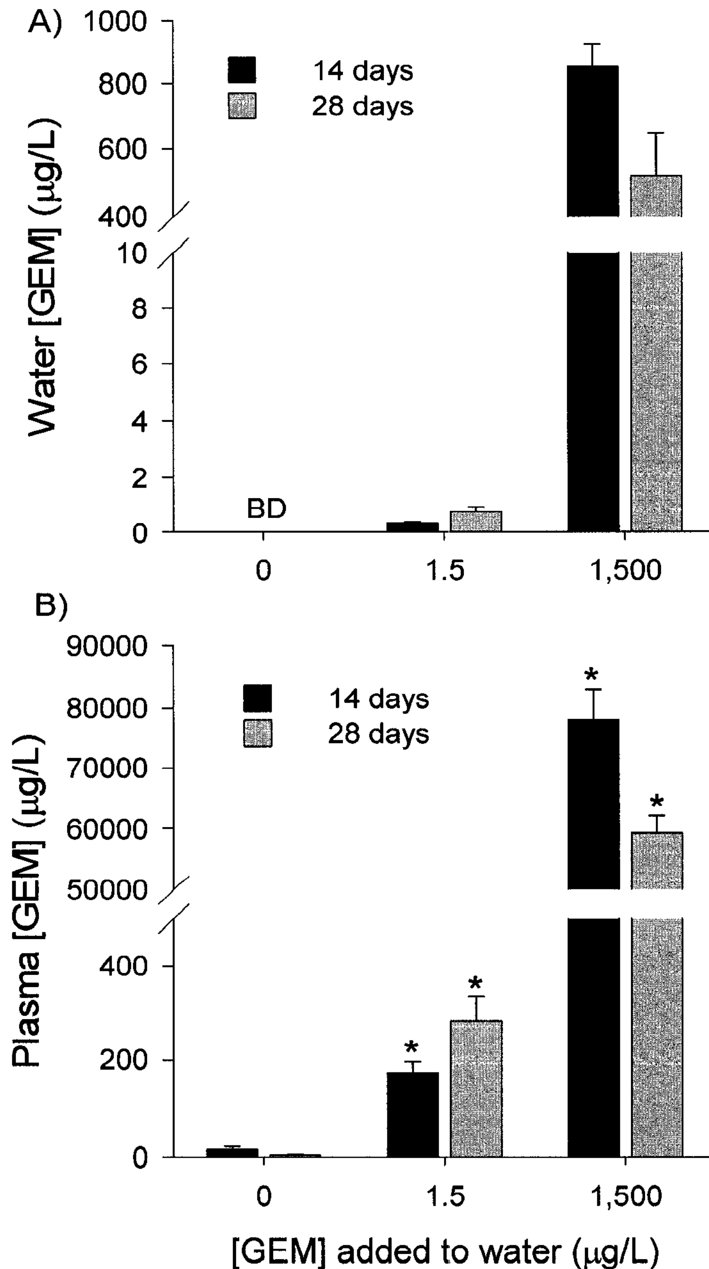

Figure 2.3. GEM concentrations detected in water and in plasma of goldfish exposed to waterborne GEM for 14 and 28 days. **A)** Measured GEM concentrations in water compared with nominal concentrations to which goldfish were exposed. Data represent means ± SEM (n = 2). **B)** Measured GEM concentrations in plasma of goldfish exposed to waterborne GEM. Data represent means + SEM (n = 8 to 12). BD indicates below detection limit (0.1 μg GEM/L). One-way ANOVA revealed a significant difference for both dose groups compared with the control as indicated by * ($P < 0.001$).

2.4. Discussion

This study demonstrates that the lipid-regulating drug, gemfibrozil has the potential to be taken up from water and concentrate in fish blood. In the acute experiment with aqueous GEM exposure, bioconcentration factors (BCF) in plasma relative to the nominal concentrations in water were 89 and 16 following exposure to 1,500 and 10,000 μg GEM/L, respectively. These results imply that uptake through the gills is an important route for bioconcentration of this pharmaceutical in fish blood. Qiao et al. (1995) assessed the relative contribution of aqueous versus dietary uptake of hydrophobic chemicals with log K_{ow} values ranging from 3.98 to 7.55 and concluded that aqueous uptake by the gills predominates for tested chemicals with log K_{ow} values up to 5.05. GEM has a K_{ow} of 4.77 (Sanderson et al., 2003), so according to Qiao et al. (2000), GEM should be primarily taken up by the gills. At this point, it remains unknown if GEM passes across the gill membrane by passive diffusion or active uptake (Gobas and Morrison, 2000). Further experiments are required to evaluate the relative efficiencies of uptake of GEM from dietary and aqueous sources. Preliminary results suggest that GEM may also be taken up across the digestive tract (data not shown).

Knowing that GEM can bioconcentrate in goldfish plasma, we investigated the potential for uptake of GEM at an environmentally relevant concentration. Even though GEM was not detected in the water used to hold the goldfish (de-chloraminated City of Ottawa water), GEM was detected at low concentrations in the plasma of control fish after 14 (16 ± 6 μg/L) and 28 (5 ± 1 μg/L) days. The ability of the goldfish to bioconcentrate GEM from the water explains the low, but detectable GEM concentration in the control, non-exposed group. In the same experiment, fish exposed through water to nominal GEM concentrations of 1.5 and 1,500 μg/L, had plasma GEM concentrations of 170 ± 20 and

78,000 ± 5000 μg/L, respectively after 14 days and 280 ± 50 and 59,000 ± 3000 μg/L after 28 days. Relative to the nominal concentrations, plasma BCFs were 113 and 52 for the 1.5 and 1,500 μg/L GEM treatments, respectively after 14 days and 188 and 40 after 28 days (Table 2.1). Based upon the actual assayed GEM concentration in the water of 0.34 and 851.9 μg/L for the nominal concentrations of 1.5 and 1,500 μg/L, plasma BCFs are 500 and 92 for each GEM treatment, respectively after 14 days and 382 and 114 after 28 days (Table 2.1).

Table 2.1. Measured GEM bioconcentration factors (BCFs) in goldfish plasma following acute and sub-chronic waterborne exposures. BCFs calculated with both the nominal and measured water concentrations are reported. *NA*: not available.

Water [GEM] (μg/L)	Acute experiment		Sub-chronic experiment			
	4 days (96 h)		**14 days**		**28 days**	
	Nominal	Measured	Nominal	Measured	Nominal	Measured
10,000	16	NA	—	—	—	—
1,500	89	NA	52	92	40	114
1.5	—	—	113	500	188	382

The finding that GEM can be taken up from surrounding water is further supported by a comparable BCF value of 63 reported in rainbow trout exposed to clean water aquaria containing 510 μg GEM/L (Brown et al., 2007). Caged rainbow trout in final treated effluent from a STP in Sweden containing 1.05 μg GEM/L also lead to a blood concentration of 210 μg/L and a BCF value of 199, which once more is comparable to results presented in this study and demonstrates the uptake of GEM in the field (Brown et al., 2007). BCF values for another pharmaceutical, the anti-inflammatory drug diclofenac, ranged between 12 and 2,700

in different tissues of the rainbow trout, including liver, kidney, gills and muscles, but no plasma values were reported (Schwaiger et al., 2004). While evidence demonstrate that GEM has the potential to bioconcentrate in a fish laboratory model, further work is required to assess the bioavailability of this drug in the aquatic environment.

No information exists regarding the pharmacokinetics of GEM in goldfish or any other fish species. GEM exposure through a single intraperitoneal injection allowed determination of the rate of GEM elimination from plasma. The half-life of GEM in plasma was approximately 19 h in the goldfish, as compared with 1.5 h in human plasma (Malloy and Kane, 2001). It is unknown if GEM binds to plasma proteins or whether elimination occurs mainly through the kidney, as it does in humans (Malloy and Kane, 2001). In order for a drug to exert its intended pharmacodynamic properties the presence of an appropriate receptor is required. The peroxisome proliferator-activated receptor alpha (PPARα), the nuclear receptor through which GEM exerts its effects, has been reported in goldfish (GenBank Accession no. AY198322), suggesting that this human drug has the potential to induce effects in this non-target aquatic species. In fact, PPARs are reported in a variety of fish species (Andersen et al., 2000; Ibabe et al., 2002; 2004) and are also thought to be involved in the enhanced peroxisomal β-oxidation reported to occur in salmon hepatocytes following fibrate drug exposure (Ruyter et al., 1997).

2.5. Conclusions

For the first time, this study provides evidence that a human drug can persist in water and be taken up by a non-target species. GEM, a fibrate drug detected in the aquatic environment, was demonstrated to persist in water for at least 96 h and to be taken up from

the water by goldfish. Environmental risk assessments of pharmaceuticals should therefore consider that environmental drug concentrations are not indicative of concentrations in fish as GEM bioconcentrate in blood plasma. Exposure is likely to be higher than reported aquatic concentrations and are thereby more likely to elicit biological response(s).

While this chapter was not an attempt to model or determine the bioavailability of GEM, it demonstrates that GEM, and potentially other fibrate drugs and human drugs can be taken up across the gills by aquatic species. Finally, the bioconcentration factor of GEM seems to be in accordance with the hydrophobicity model in which a chemical with a log K_{ow} of 4.77 should bioconcentrate by factors between 100 and 10,000 (Barron, 1990). Further research will be required to determine if other pharmaceuticals with the similar properties, such as other fibrates, are likely to follow the same model.

CHAPTER 3

Effects of waterborne GEM on the hepatic antioxidant defense system and peroxisome proliferator-activated receptors in goldfish (*Carassius auratus*)

Chapter published as: Mimeault C, Trudeau VL and TW Moon. 2006. Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-activated receptor beta (PPARβ) mRNA levels in male goldfish (*Carassius auratus*). **Toxicology** 228:140-50.

3.1. Introduction

The presence of pharmaceuticals in the environment is now well established (Daughton and Ternes, 1999; Kolpin et al., 2002; Metcalfe et al., 2003a; 2003b). Recent studies report evidence of *in vivo* effects in non-target species of drugs including the lipid regulator gemfibrozil (GEM) in goldfish (*Carassius auratus*) (Mimeault et al., 2005) and the anti-inflammatory diclofenac in rainbow trout (*Oncorhynchus mykiss*) (Schwaiger et al., 2004; Triebskorn et al., 2004).

GEM, prescribed to improve human lipid profiles, is consistently found in the aquatic environment at concentrations as high as 2.1 μg/L in effluent waters of wastewater treatment plants (WWTPs) (Daughton and Ternes, 1999; Kolpin et al., 2002; Metcalfe et al., 2003a; 2003b). The presence of GEM in WWTP effluents and surface waters is partially attributed to its continual addition and to its persistence in the environment (Daughton and Ternes, 1999; Metcalfe et al., 2003). The half-life of GEM in river water has been reported to be 15 h (Lin and Reinhard, 2005), demonstrating that GEM resists degradation longer than some other pharmaceuticals (ketoprofen, naproxen and propanolol) and estrogens (17β-estradiol, estriol, estrone and 17α-ethinylestradiol) with reported half-lives of less than 4 h.

Furthermore, GEM is detected in water runoff from agricultural fields irrigated with treated wastewaters and sludge (Pedersen et al., 2005). Finally, GEM was shown to bioconcentrate (Chapter 2) and to reduce plasma testosterone levels by 50% in male goldfish at an environmentally appropriate exposure (Mimeault et al., 2005).

As a fibrate drug, GEM is also classified as a peroxisome proliferator (PP) as it increases the size and numbers of liver peroxisomes in rodents (Yang et al., 1990). PPs bind to nuclear transcription factors, the peroxisome proliferator-activated receptors (PPARs) for which three subtypes, PPARα, PPARβ and PPARγ are identified with distinct tissue distributions and functions (Desvergne and Wahli, 1999; Leaver et al., 2005). Upon ligand binding, the PPAR heterodimerizes with the retinoid X receptor and regulates gene expression through its binding to a peroxisome proliferator-response element (PPRE) in the promoter region of target genes (Desvergne and Wahli, 1999). Hypolipidemic fibrate drugs, which include GEM, bind preferentially to PPARα with weak binding to PPARβ and PPARγ (Desvergne and Wahli, 1999). The PPARα-target genes include amongst others, PPARα itself and lipoprotein lipase through which GEM exerts its pharmaceutical actions, and acyl-CoA oxidase (ACO), the first enzyme of the peroxisomal β-oxidation pathway (Mandard et al., 2004).

Acyl-CoA oxidase oxidizes very long chain-fatty acids releasing H_2O_2 that can imbalance cellular oxidative homeostasis of the exposed organism through the eventual formation of hydroxyl radicals (•OH) (Gonzalez, 1997). As a defense system, cells possess both enzyme and non-enzyme antioxidant components (Kelly et al., 1998). Briefly, antioxidant enzymes include the peroxisomal enzyme catalase (CAT) and the cytosolic enzymes glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-s-

transferase (GST) (Kelly et al., 1998). The cytosolic non-enzyme antioxidants include, amongst others, glutathione (GSH) that is oxidized to glutathione disulfide (GSSG). The antioxidant defense system ensures that exposure to oxidants, including some xenobiotics, does not result in oxidative damage, which includes lipid peroxidation, DNA damage and protein degradation.

The effects of PPs in mammals are well documented. Gonzalez et al. (1998) reported that chronic exposure to chemicals that significantly induce peroxisome proliferation (measured through increased ACO activity) may cause liver cancer in mice and rats. Furthermore, a compromised antioxidant defence following PP treatment has been reported in rats and hamsters (O'Brien et al., 2001a; 2001b), as well as a a positive correlation between liver expression of PPARα and ACO mRNA levels in rats fed a polyunsaturated fatty acid diet and exposed to the PP bezafibrate (Inoue et al., 1997; 1998). Studies on the effects of PPs in fish are scarce, but fish generally respond to hypolipidemic drugs by an increase in ACO activity (Trudeau et al., 2005). Ruyter et al. (1997) showed an increase in ACO activity and PPAR mRNA level in salmon (*Salmo salar*) hepatocytes exposed to the two hypolipidemic drugs clofibric acid and bezafibrate. Finally, induction of reactive oxygen species (ROS) production was previously reported in immature rainbow trout hepatocyte cultures exposed to two hypolipidemic drugs, fenofibrate and clofibrate (Laville et al., 2004). Authors of the study acknowledged that the *in vitro* concentrations used for these experiments were higher than that found in the environment and that *in vivo* studies were required before reaching any conclusions regarding physiological significance.

Knowing that the mammalian ACO promoter contains a PPRE and that waterborne GEM bioconcentrates in fish led us to investigate the potential effects of GEM on the

antioxidant system in goldfish. In addition, since the PPARα promoter has also been reported to contain a PPRE, we investigated whether GEM would affect PPAR mRNA levels. Male goldfish were exposed to either an environmentally relevant or a high waterborne GEM concentration for 14 and 28 days. The results demonstrate that an environmental level of GEM significantly challenges the hepatic antioxidant defence system without leading to oxidative damage in the form of lipid peroxidation.

3.2. Materials and methods

3.2.1. Fish and experimental design

3.2.1.1. Fish

Goldfish were purchased from a fish supplier (Aleong's International, Mississauga, ON). Fish were acclimated for more than 2 weeks in 70-L fibreglass tanks that received oxygenated and dechloraminated City of Ottawa water at approximately 18 °C. Both sexes were used for the tissue distribution study (n = 6); however, only males were used for the dosing experiment as female mammals are reported to be more tolerant of PP effects (Amacher et al., 1997). All fish used had an average mass of 26.4 ± 0.7 g (n = 62). In both experiments, fish were kept at a density of 10 to 12 fish per 70-L tank. All experiments were conducted under a protocol approved by the University of Ottawa Animal Care Protocol Committee and adhere to guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research.

3.2.1.2. Waterborne exposure experiment

Male goldfish were exposed in November 2003 for 14 and 28 days to nominal waterborne GEM (Sigma-Aldrich, St. Louis, MO, USA) concentrations of 0, 1.5 or 1,500 μg/L. Water and GEM concentrations were renewed every 72 h throughout the entire experiment as previously described (Chapter 2; Mimeault et al., 2005). GEM was initially dissolved in dimethyl sulfoxide (DMSO; Sigma) before being added to the 70-L fiberglass aquaria. The final DMSO concentration in the aquaria was 0.0003%. Fish were feed daily a commercial rainbow trout food (Classic Floating Trout Grower; Martin Mills, Tavistock, ON, Canada) at 1% body weight. After 14 or 28 days of exposure, fish were anesthetized with MS-222 prior to euthanizing by trans-spinal sectioning, and the livers were removed, weighed, snap frozen in liquid nitrogen and kept at -80°C until analysed. Condition factor (K = (body weight / length^3) x 100) and hepatosomatic index (HIS = (liver mass / body mass) x 100) were calculated for each fish.

3.2.2. Semi-quantitative PCR of PPARs, CYP1A1 and β-actin and their tissue distributions

Total RNA was extracted from goldfish tissues using TRIzol reagent as per the manufacturer's protocol (Invitrogen, Life Technologies, Burlington, ON, Canada). RNA was treated with DNase (DNAse I Amplification Grade; Invitrogen) and reverse transcribed into cDNA using oligo dT as per the manufacturer's protocol (M-MLV-RT kit, Invitrogen, Life Technologies, Burlington, ON, Canada). All PCR reactions used an Eppendorf Mastercycler EP.

PCR primers (Invitrogen) for PPARα, -β and -γ were developed based on sequences that we previously cloned for goldfish and submitted to GenBank (accession numbers

AY198322, AY894894, AY894893, respectively). PCR primers for cytochrome P450 1A1 for goldfish were developed based on the carp sequence (accession number AB048939). The primer sequences were: PPARα, 5'- GCGTCGTGCATGAATAAAGA -3' (F) and 5'-TGCACATGAAGCACGTTTAC -3' (R); PPARβ, 5'- TGGCTTTGTGGATCTCTTCC -3' (F) and 5'- GATCTCGCTGAAAGGTTTGC -3' (R); PPARγ, 5'-TTCCACAGCTGTCAGTCTCG -3' (F) and 5'- CATGAAGATCTGTCCGTAGG -3' (R); CYP1A1, 5'-CTCATCAACCACTGCGAAGA-3' (F) and 5.-GAGGAATCGGTCCAGATTGA-3' (R). The PCR conditions were 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1 min. Cycle gradient curves were used to determine the optimal cycle number (based on the linear phase of amplification for which the R^2 varied between 0.96 and 1.00) for each PPAR subtype: PPARα, -β and -γ optimal cycle numbers were 28, 30 and 27 for the tissue distribution and 28, 28 and 27 for the sub-chronic experiment, respectively. The optimal cycle number for CYP1A1 was 27. Product sizes were 269, 178, 201 and 447 bps for PPARα, -β, -γ, and CYP1A1, respectively. PCR products were confirmed through sequencing and subsequent gene identification with a BLASTX search.

β-Actin was chosen as the internal control as no quantitative changes in its expression in the liver following GEM exposure were detected. Primers for β-actin were developed using available sequences for goldfish (GenBank accession number AB039726). The primer sequences were 5'- CTGGGATGATATGGAGAAGA -3' (F) and 5'-CCAGTAGTACGACCTGAAGC-3' (R), and PCR conditions were 94 °C for 45 s, 60.0 °C for 45 s and 72 °C for 1 min. β-Actin primers were optimized as above at 24 and 27 cycles for the tissue distribution and the exposure experiment, respectively. The size of the product was 216 bps and was confirmed by sequencing.

Three male and three female goldfish were randomly chosen from a tank and anesthetized with 200 mg/L MS-222 (Sigma) prior to euthanizing as above. Tissues (brain, gonads, heart, intestine, kidney, liver, spleen, red and white muscles) were rapidly removed, separately snap frozen in liquid nitrogen and kept at -80 °C until analysed. β-Actin expression varied between tissues so the data for the tissue distribution are presented as percent averages of the total expression detected for each PPAR subtype in each individual (Doyon et al., 2003).

3.2.3. Enzyme assays

3.2.3.1. Homogenate for antioxidant enzyme assays

Tissue homogenates for enzyme activities were prepared according to Lushchak et al., 2001) with minor changes. Briefly, frozen tissues (1:10 wt/vol) were homogenized in ice-cold 50 mM potassium-phosphate buffer containing 0.5 mM EDTA and adjusted to pH 7.0. A protease inhibitor cocktail (Sigma, P2714) (diluted 100 times) was added just prior to homogenization. Homogenization was done in 1.5 ml plastic conical centrifuge tubes with a Kontes Motor Cordless tissue homogenizer and centrifuged for 15 min at 15,000 g (Beckman Coulter Microfuge R) and 4 °C. The supernatant was collected and used for enzyme assays at the appropriate dilution as defined as the dilution that gave at least 10 min of linear kinetic activity. Activities were estimated using a microplate reader (Molecular Device SpectraMax Plus 384; Sunnyvale, CA) at room temperature (22 °C).

3.2.3.2. Antioxidant enzyme assays

Assays for the antioxidant enzymes glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-s-tranferase (GST) were adapted from Lushchak et al. (2001) to a 96-well microplate format. Briefly, GPx activity was determined by a coupled assay following the oxidation of 0.25 mM NADPH in 50 mM potassium-phosphate buffer, 4 mM sodium azide (ICN, Orangeburg, NY, USA), 1 U/ml GR (Sigma), 15 mM GSH (Sigma) and 10 μl of the diluted supernatant; 0.2 mM H_2O_2 (Sigma) initiated the reaction. GR activity followed the oxidation of 0.25 mM NADPH in 50 mM potassium-phosphate buffer and 10 μl of the tissue supernatant; the reaction was initiated with 1 mM oxidized glutathione (GSSG; Sigma). GST activity was measured by monitoring the conjugation of GSH and 1-chloro-2,4-dinitrobenzene (CDNB; Sigma). The reaction mixture contained 50 mM potassium-phosphate buffer, 1 mM CDNB and 10 μl of the diluted tissue supernatant; the reaction was initiated by the addition of 5 mM GSH. All assays were optimized for substrate concentration. The assay for catalase (CAT) was performed in a 3-ml quartz cuvette and monitored the initial rate of H_2O_2 decomposition by CAT at 240 nm in 50 mM potassium-phosphate buffer, 50 mM H_2O_2 and 20 μl of tissue supernatant. All enzyme activities are reported as mU or U/mg protein, where U is activity per min.

3.2.3.3. Acyl-CoA oxidase assay

The spectrophotometric assay for peroxisomal acyl-CoA oxidase (ACO) enzyme activity (Small et al., 1985) was adapted to a microplate format. Briefly, peroxisome-enriched homogenates were prepared by ultra-centrifugation from fresh tissues minced with a razor blade and homogenized (1:10 wt/vol) in ice-cold SI buffer (350 mM sucrose,1.5 mM

imidazole, pH 7.4) with three strokes by hand in a Radnoti Glass homogenizer (Monrovia, CA, USA). A protease inhibitor cocktail (diluted 100 times) was added just prior to homogenization. Homogenates were centrifuged for 10 min at 6,000 g and 4 °C. The supernatant was collected and kept on ice. Pellets were resuspended by gentle homogenization in SI medium and re-centrifuged as above. The two supernatants were combined and centifuged for 30 min at 30,000 g (Beckman Coulter OptimaTM XL-100K Ultracentrifuge) and 4 °C. The resulting pellet was resuspended in 1/10 wt/vol of the initial SI medium to which was added 9 volumes of 250 mM sodium azide (ICN, Orangeburg, NY, USA) to inhibit catalase. The peroxisome-enriched fractions were used to determine ACO activity by H_2O_2 production coupled with the oxidation of the dye leuco-dichlorofluorescein (leuco-DCF; Cayman Chemicals, Cedarlane Laboratories, Hornby, ON, Canada) to DCF catalyzed by peroxidase. The assay mixture contained 0.05 mM leuco-DCF (prepared as a stock at 11 mM in *NN*-dimethylformamide, diluted to 1.1 mM with 0.01 M NaOH, stored in a light-tight container under N_2 gas until 1 ml was added to the assay mixture just before loading samples on to microplates), 0.125 mg/ml horseradish peroxidase, 26 mM sodium azide, and 13 mM potassium phosphate buffer, pH 7.4. Twenty μl of the peroxisome-enriched fraction was added to each well containing 200 μl of the assay mixture followed by 5 min incubation in the dark. The reaction was initiated by the addition of 83 μM palmitoyl-CoA (Sigma) and continuously monitored at 502 nm for 15 min. Activities are calculated as DCF produced per min per mg protein and reported as a percent relative to controls after 14 and 28 days of exposure.

3.2.4. Glutathione measurements

Total glutathione (TGSH) and glutathione disulfide (GSSG) assays were adapted to a microplate format from Hermes-Lima and Storey (1996) and Lushchak et al. (2001). Frozen tissues were homogenized in 10 volumes of ice-cold 5% sulfosalicyclic acid (Sigma) and centrifuged at 5000 g at 5 °C for 5 min (Beckman Coulter Microfuge R). Supernatants were used to measure TGSH and GSSG; GSH content was derived from the TGSH and GSSG measurements (TGSH = GSH + 2 GSSG).

To estimate GSSG, supernatant was added to 24 mM 2-vinylpyridine (Lancaster, Boston, MA) and 250 mM potassium-phosphate buffer (pH 7.0), the pH adjusted to 7.0 with NaOH and incubated at room temperature for 1 h before monitoring changes at 412 nm. GSSG concentrations were determined by comparison with a GSSG standard curve. TGSH was assessed within minutes of homogenization by monitoring at 412 nm the rate of reduction of 0.6 mM 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB; Sigma) by GSH in 100 mM potassium-phosphate buffer (pH 7.0) containing 0.25 mM NADPH, 1 mM EDTA, 1 U/ml yeast GR (Sigma) and 10 µl tissue supernatant. GSH concentrations were determined by comparison with a GSH standard curve treated in an equivalent manner. Glutathione concentrations are reported as nmol/g wet tissue weight.

3.2.5. Lipid peroxidation

Lipid peroxidation was quantified as thiobarbituric reactive substances (TBARS) and adapted to a 96-well microplate format from Hermes-Lima and Storey (1996). Frozen liver samples and malonaldehyde (MDA; Sigma) standards were homogenized with a tissue homogenizer in 9 volumes of ice-cold 1.1% phosphoric acid. Two hundred µl of this tissue

homogenate was mixed with 0.2 ml of a solution containing 1% thiobarbituric acid (Sigma), 50 mM NaOH and 0.1 mM butylated hydroxytoluene (BHT; Sigma). All solutions were kept on ice. One hundred μl of 7% phosphoric acid was then added prior to adjusting the pH to 1.5 to 2.0. Samples were boiled for 15 min and cooled on ice prior to the addition of 0.75 ml butanol. Tubes were vortexed and centrifuged at 3000 rpm for 20 min (GLC-2 Sorvall centrifuge). The organic layer was collected and 200 μl added to a microplate and the absorbance measured at 529 nm. TBARS were quantified with a MDA standard curve and reported as nmol/g wet tissue weight.

3.2.6. Protein assays

Protein concentration was determined for each tissue homogenate with the bicinchoninic acid protein assay kit (Sigma-Aldrich, TPRO-562) using bovine serum albumin as standard.

3.2.7. Statistical analysis

All parametric statistical analyses were conducted using the SigmaStat™ 3.1 software (SPSS Corp., Chicago, IL). Non-parametric analyses were performed with the S-PLUS™ 7.0 software (Insightful Corp., Seattle, WA, 2005). Data are presented as mean ± standard error of the mean (SEM) of *n* experiments. Two-way ANOVAs were used to determine the effects of the waterborne GEM concentration (referred to as concentration) and of the duration of exposure (referred to as duration). ANOVAs were followed by Bonferonni tests for multiple comparisons. A value of $P < 0.05$ was considered significant.

3.3. Results

Goldfish body weight (26.4 ± 0.7 g, n = 62), K factor (2.20 ± 0.05, n = 62) and HSI (2.83 ± 0.12, n = 62) were not affected by waterborne GEM exposure (data shown for all groups combined).

3.3.1. Tissue distribution of PPARα, -β and -γ

β-actin mRNA was detected in all samples and, as expected levels varied between tissues (Fig. 3.1A), and thus could not be used to compare relative PPAR transcripts between tissues. Therefore, data on tissue distribution of the three PPAR subtypes (Fig. 3.1B, C, D) as determined using reverse-transcriptase PCR (RT-PCR) is presented as average percentages of the total band intensity for the PPAR subtype detected for each tissue. Transcripts for each of the three PPAR subtypes were detected in all nine tissues examined (brain, gonads, heart, intestine, kidney, liver, spleen, red and white muscles). There were no significant differences between males and females in the expression levels of any subtype in any tissue (data not shown) and results for both sexes were therefore pooled.

The highest mRNA levels of PPARα were found in the intestine, kidney, liver and spleen; brain, heart, red and white muscles had intermediate and gonads the lowest levels (Fig. 3.1B). Highest levels of PPARβ mRNA were found in the brain, gonads, intestine and spleen; heart, kidney, red and white muscles revealed intermediate while liver expressed the lowest mRNA levels (Fig. 3.1C). Highest levels of PPARγ mRNA were found in the intestine, kidney, liver and spleen; intermediate to weak expression was found in the brain, gonads, heart, red and white muscles (Fig 3.1D).

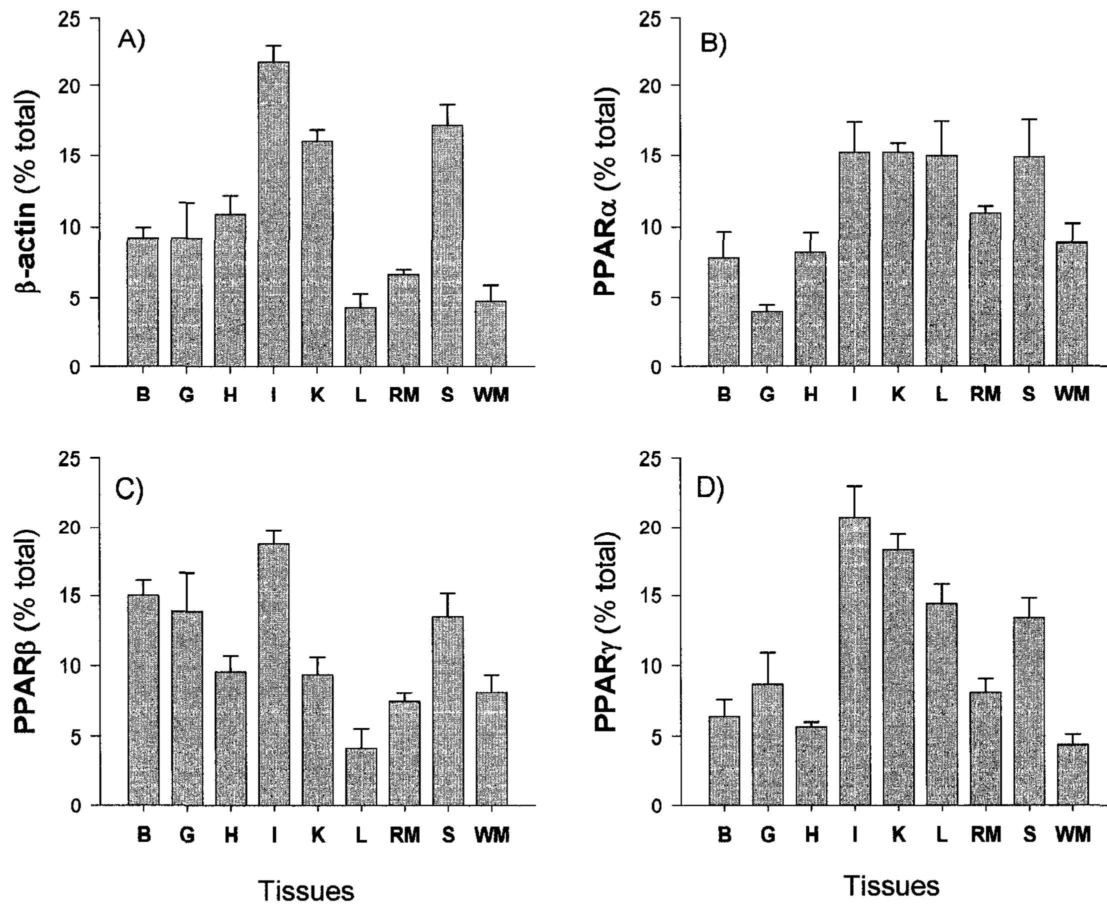

Figure 3.1. Tissue distribution of (A) β-actin, (B) PPARα, (C) PPARβ and (D) PPARγ mRNA in various tissues of the goldfish using RT-PCR (see Section 3.2). The tissue abbreviations are: (B) brain, (G) gonads, (H) heart, (I) intestine, (K) kidney, (L) liver, (RM) red muscle, (S) spleen, and (WM) white muscle. Tissue samples from males (n = 3) and females (n = 3) were first analyzed separately but then pooled (n = 6) because no significant difference was found between the sexes ($P > 0.05$; t-test). Data are average percentages (+ SEM) of the total band intensity for the PPAR subtype detected in each tissue.

3.3.2. Effect of waterborne GEM on hepatic PPARs and CYP1A1 mRNA levels

Relative transcript levels of the three hepatic PPAR subtypes were assessed by semi-quantitative reverse-transcriptase PCR (RT-PCR) after 14 and 28 days of exposure to 1.5 and 1500 μg/L GEM (Fig. 3.2). β-Actin levels were unaffected by GEM treatment, hence was an appropriate internal control (two-way ANOVA: P = 0.342, 0.070 and 0.609 for concentration, duration and interaction, respectively). Two-way ANOVA revealed no effect on PPARα mRNA levels following GEM exposure (Fig. 3.2A) (P = 0.052, 0.125 and 0.754 for concentration, duration and interaction, respectively). PPARβ transcript levels were significantly lower in the liver of fish exposed to high concentration (1,500 μg/L) of GEM (P for concentration < 0.001) (Fig. 3.2B) and overall levels were higher after 28 days compared to 14 days (P for duration = 0.008). PPARβ mRNA levels in fish exposed to high concentration of GEM were 50% lower that in unexposed fish after 14 days and 25% lower after 28 days. No interaction between the concentration and the duration of exposure was detected (P = 0.452). Relative PPARγ mRNA levels did not vary with concentration (P = 0.105) or with duration of exposure (P = 0.125) and no interaction was observed (P = 0.879) (Fig. 3.2C). Relative CYP1A1 expression levels were unaffected by treatment (P for concentration = 0.119, P for duration = 0.460 and P for interaction = 0.302; Fig. 3.2D).

3.3.3. Effect of waterborne GEM on acyl CoA oxidase (ACO) activities

Both the concentration and the duration of exposure had no effect on ACO activity (P = 0.160 and 0.076, respectively) but there was a significant interaction between the two factors (P = 0.007) (Fig. 3.3). ACO activity was therefore not different from the control

values after 14 days of exposure but a significant 54% reduction was observed after 28 days of exposure to the environmentally relevant concentration (1.5 μg/L).

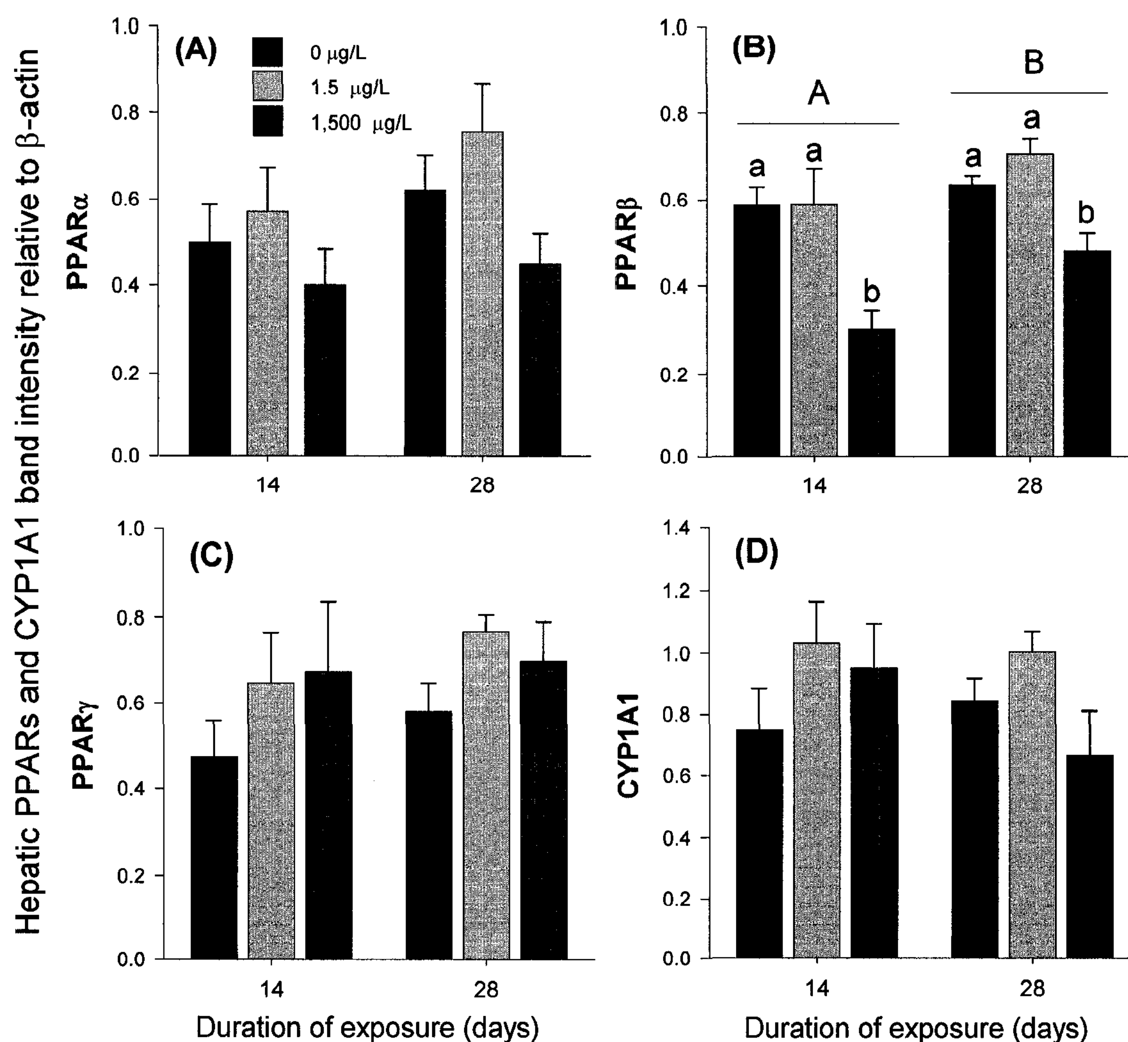

Figure 3.2. Relative transcript levels of (A) PPARα, (B) PPARβ (C) PPARγ and (D) CYP1A1 in goldfish liver exposed to 0, 1.5 and 1,500 μg/L GEM for 14 and 28 days. Data represent means ± SEM (n = 6). Two-way ANOVA revealed no effect on PPARα (P for concentration = 0.052, P for duration = 0.125, and P for interaction = 0.754). Both, the concentration and the duration had a significant effect on PPARβ ($P < 0.001$ and $P = 0.008$) but not the interaction ($P = 0.452$). Two-way ANOVA on log-transformed data revealed no effect on PPARγ (P for concentration = 0.105, P for duration = 0.125, and P for interaction = 0.879). Relative CYP1A1 expression was unchanged (P for concentration = 0.119, P for duration = 0.460 and P for interaction = 0.302). Letters denote statistical difference detected with a Bonferroni test. Results were obtained by RT-PCR.

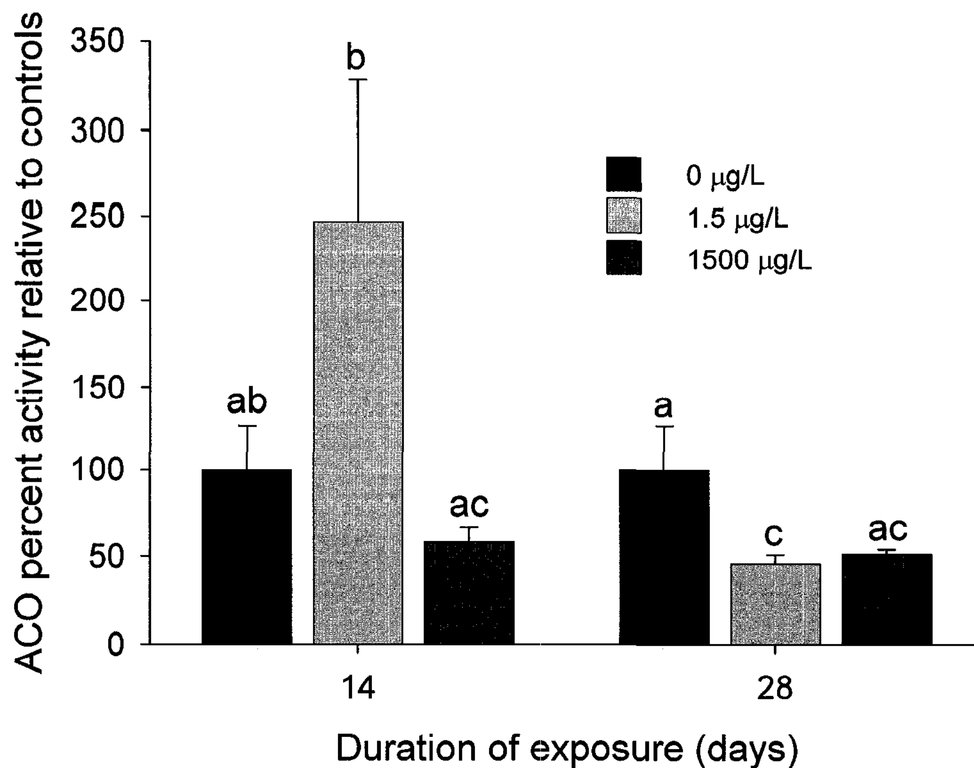

Figure 3.3. Acyl-CoA oxidase (ACO) activity (expressed as percent of control) in goldfish livers exposed to waterborne GEM for 14 and 28 days. Data represent means ± SEM (n = 8 to 11). Non-parametric two-way ANOVA revealed no effect of concentration (P = 0.076), no effect of duration (P = 0.160) but a significant interaction between the concentration and the duration of exposure (P = 0.007). Different letters denote statistical differences (P < 0.05). Hepatic ACO specific activities in control goldfish were 13 ± 3 and 30 ± 10 mU/mg protein after 14 and 28 days, respectively.

3.3.4. Effect of waterborne GEM on oxidative defence mechanisms

The activities of enzymes involved in the hepatic antioxidant defense system were measured as indicators of a possible oxidative stress response to GEM exposure. The specific activities for GPx, GST, GR and CAT in control animals (Fig. 3.4) were all comparable with a previous study in goldfish (Lushchak et al., 2001). The concentration of GEM had a significant effect on GPx activity ($P < 0.001$) that depended on the duration of exposure ($P = 0.039$) (Fig. 3.4A). A significant increase was observed in the control groups between 14 and 28 days of exposure. The 1.5 μg/L concentration of GEM also resulted in a significant increased of GST enzyme activity ($p = 0.006$) (Fig. 3.4B). Neither concentration nor duration of exposure had an effect on GR ($p = 0.152$) (Fig. 3.4C). GEM significantly increased CAT activities at both water concentrations compared with the control values ($P < 0.001$) (Fig. 3.4D).

The status of the non-enzymatic antioxidant molecules was also assessed (Table 3.1). Control values for TGSH were comparable with those reported in goldfish (Lushchak et al., 2001). Results demonstrate a significant effect of GEM concentration on GSH content ($P < 0.001$) that depended on the duration of exposure ($P < 0.001$). The high concentration of GEM resulted in a 34% decrease in GSH compared with control after 14 days but a 44% increase after 28 days. The concentration and the duration of GEM exposure significantly affected GSSG ($P = 0.004$ for both). GSSG levels were significantly lower than controls in fish exposed to the environmentally relevant GEM concentration after both 14 and 28 days. The concentration and the duration of GEM exposure also significantly affected the GSSG/TGSH ratio ($P = 0.007$ and $P = 0.004$, respectively).

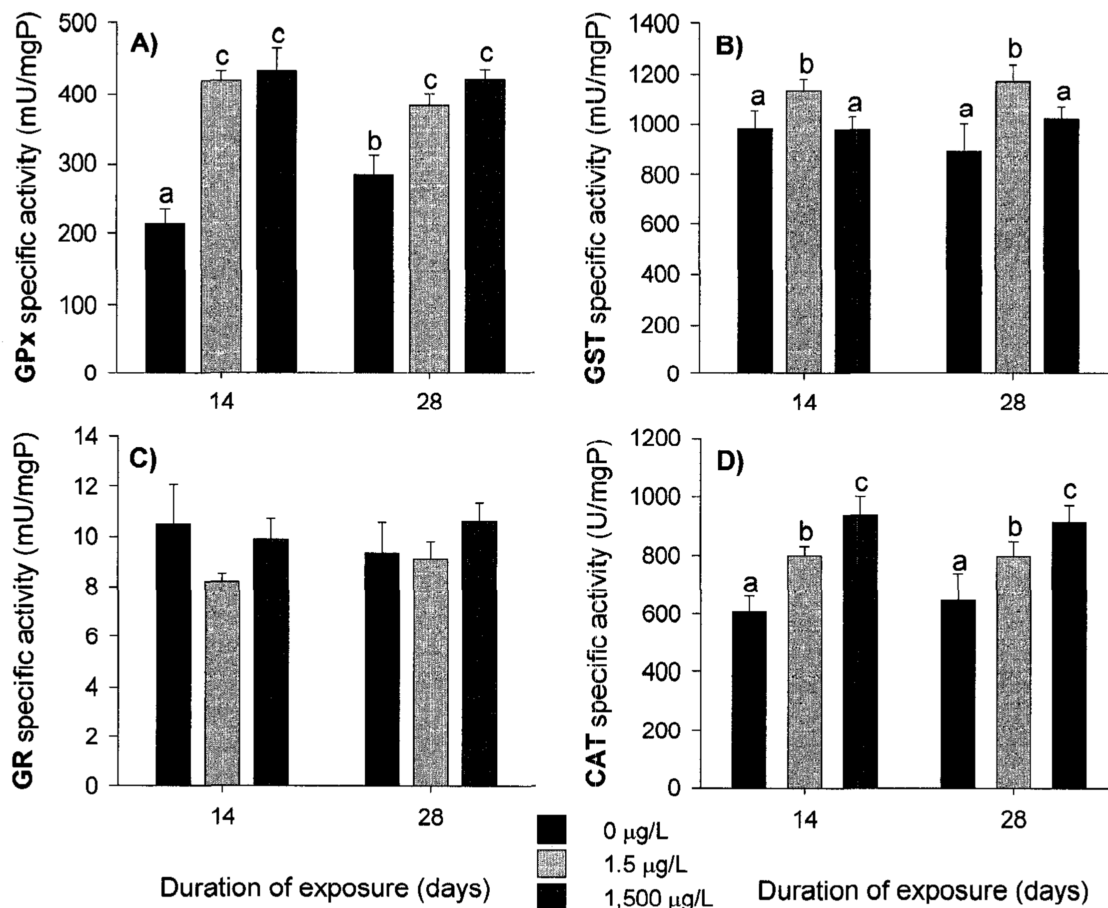

Figure 3.4. Hepatic antioxidant enzyme specific activities in goldfish following waterborne GEM exposures for 14 and 28 days: (A) glutathione peroxidase (GPx), (B) glutathione-s-tranferase (GST), (C) glutathione reductase (GR) and (D) catalase (CAT). Data represent means + SEM (n = 8 to 11). Two-way ANOVA revealed (A) that exposure duration had no effect on GPx (P = 0.624) as opposed to a significant increase due to the concentration (P < 0.001) and a significant interaction (P = 0.039); (B) a significant effect of concentration (P = 0.006) on GST but not of duration (P = 0.903) or the interaction (P = 0.537); (C) no significant effect on GR (P for concentration = 0.152, P for duration = 0.693, P for interaction = 0.539); (D) a significant effect of concentration on CAT (P < 0.001) but no effect of duration or interaction (P = 0.894 and 0.875, respectively). Bonferroni's multiple comparison tests were used to detect differences between means which are indicated by different letters (P < 0.05).

Table 3.1. Effect of a waterborne exposure to GEM for 14 and 28 days on goldfish hepatic oxidative stress status. Data represent means ± SEM (n = 8 to 11). Two-way ANOVA revealed a significant effect of duration and interaction (P < 0.001) on total glutathione (TGSH) as opposed to the concentration (P = 0.982). Concentration (P = 0.004) and duration (P = 0.004) affected glutathione disulfide (GSSG) and not the interaction (P = 0.077); only the concentration (P = 0.004) had a significant effect on the GSSG/TGSH ratio as opposed to duration (P = 0.915) and interaction (P = 0.149). Multiple comparison tests were Bonferroni and significant differences are indicated by different letters.

		GEM waterborne concentration		
	Days	**0 (µg/L)**	**1.5 (µg/L)**	**1,500 (µg/L)**
GSH (nmol/g wet wt)	**14**	1800 ± 100 (a)	1700 ± 100 (ab)	1200 ± 200 (b)
	28	1560 ± 70 (a)	2000 ± 200 (a)	2200 ± 200 (c)
GSSG (nmol/g wet wt)	**14**	200 ± 20 (a)	160 ± 10 (b)	170 ± 20 (a)
	28	310 ± 60 (c)	160 ± 20 (b)	320 ± 50 (c)
GSSG/TGSH (%)	**14**	10 ± 1 (a)	8 ± 1 (b)	13 ± 2 (a)
	28	14 ± 2 (a)	8 ± 1 (b)	12 ± 2 (a)

3.3.5. Oxidative damage

Oxidative damage was assessed as thiobarbituric acid reactive substances (TBARS), one index of lipid peroxidation (Table 3.2). GEM concentration did not significantly affect TBARS levels (P = 0.953) but the duration of exposure did (P = 0.020). TBARS levels were significantly higher after 28 days of exposure compared with 14 days.

Table 3.2. Thiobarbituric acid reactive substances (TBARS) expressed as malonaldehyde levels (nmol MDA/g wet tissue weight) in goldfish livers exposed to waterborne GEM for 14 and 28 days. Data represent means ± SEM (n = 8 to 11). Two-way ANOVA on transformed data revealed a significant effect of duration (P = 0.020) on MDA levels but not of concentration or interaction (P = 0.953 and 0.921, respectively). Different letters indicate statistical differences detected by Bonferroni.

	GEM waterborne concentration		
Days	**0 (µg/L)**	**1.5 (µg/L)**	**1500 (µg/L)**
14	22 ± 2 (a)	21 ± 1 (a)	22 ± 2 (a)
28	28 ± 4 (b)	26 ± 3 (b)	27 ± 3 (b)

3.4. Discussion

Previously results reported that environmental concentrations of waterborne GEM bioconcentrate in goldfish plasma (Chapter 2) and reduce plasma testosterone levels (Mimeault et al., 2005). Results in this chapter demonstrate that a waterborne exposure to GEM significantly challenges the hepatic antioxidant defense system of goldfish and modifies PPAR mRNA subtype expression.

The tissue distribution demonstrates the presence of transcripts for all three PPAR subtypes in all nine goldfish tissues tested; there were no difference in expression between the sexes. This latter finding is consistent with reports of no significant sex differences in PPAR mRNA tissue distribution in adult rats (Lemberger et al., 1996) and the identical expression patterns of PPARγ in male and female Atlantic salmon (*Salmo salar*) (Andersen et al., 2000). On the other hand, Jalouli et al. (2003) reported that sub-adult female rats expressed lower hepatic levels of PPARα mRNA and protein than males, suggesting that sex

differences in PPAR subtypes expression may be age-related as PPAR expression is reported to vary through development (Braissant et al., 1996).

The main objective of this study was to determine the effect of a waterborne GEM exposure on the goldfish liver. GEM, which is a peroxisome proliferator and a PPARα ligand, was predicted to affect PPARα mRNA levels and cause peroxisome proliferation in the liver. Results demonstrate that a waterborne exposure to GEM had no effect on PPARα mRNA levels. This lack of change in PPARα transcript levels suggests that GEM does not regulate the expression of PPARα in goldfish when exposed for 14 or 28 days to 1.5 or 1500 μg of GEM/L. Further research is needed to exclude any effect of GEM or other fibrate drugs on this PPAR subtype.

Even though PPARα is considered to be the principle mediator of peroxisome proliferation, hypolipidemic drugs may also act as weak PPARβ and γ ligands (Schoonjans et al., 1996; Desvergne and Wahli, 1999). Waterborne exposure to a high concentration of GEM (1,500 μg/L) significantly reduced PPARβ mRNA levels by 50% and 25% after 14 and 28 days, respectively. PPARβ function is the least understood of the three PPAR subtypes but has been previously reported to be expressed earlier in development than other PPARs (Braissant and Wahli, 1998). Nevertheless, such a reduction in PPARβ transcripts is not likely to result in developmental or fat regulation problems *in vivo* as it occurred only at a concentration 1000-fold higher than environmentally relevant concentrations. The lack of effect of GEM on PPARγ mRNA reported here differs from the increase observed in cultures of Atlantic salmon hepatocytes following exposure to the PPs, clofibric acid and bezafibrate (Ruyter et al., 1997). Longer studies at environmentally relevant concentrations should be

performed before making conclusions on the effects of GEM on PPAR mRNA levels as the observed differences may relate to age, species, route, concentration or duration of exposure.

Given that ACO is a PPARα target gene (Mandard et al., 2004) its hepatic specific activity following GEM exposure was assessed. A 2.5-fold increase relative to ACO control activity was observed at the environmentally relevant concentration after 14 days. Despite the fact that this result was not statistically significant ($P = 0.076$), it may suggest that ACO activity increased earlier during PP exposure. Cajaraville et al. (2003) previously reported that the effects of PPs on ACO disappeared with time of exposure. That ACO activity was significantly reduced in goldfish exposed to the environmental GEM concentration at 28 days suggests an adaptive response to GEM exposure. ACO is an accepted marker of peroxisome proliferation (Cajaraville et al., 2003), thus exposure to an environmentally relevant concentration of GEM may initially trigger peroxisome proliferation in the first two weeks of exposure but not over a longer period of time.

We also tested the hypothesis that exposure to GEM would generate ROS and thereby cause oxidative stress in goldfish. While ACO did not significantly increase with GEM exposure, CAT activity increased when exposed to both GEM concentrations as did GPx and GST. Both CAT and GPx are antioxidant enzymes that serve as a primary defense against oxidative stress by neutralizing free radicals. CAT, which is located primarily to peroxisomes but is also found in the cytosol, detoxifies H_2O_2 to H_2O while GPx, which is located in the cytosol, detoxifies H_2O_2 and organic peroxides (Kelly et al., 1998). O'Brien et al. (2005) reported that PP treatments in rats and mice increased ACO activities resulting in weak or no increase in CAT activity and in lower GPx activity. The hepatic oxidative response observed in goldfish exposed to waterborne GEM for 14 and 28 days is different

than what is generally observed with PPs in rats and mice. This may result from the time points chosen in this study as peroxisome proliferation may have occurred earlier or it may suggest that the oxidative challenge imposed on the liver is not related to peroxisome proliferation and could be ACO-independent in the goldfish. If the observed induction of antioxidant enzymes (GPx, GST, CAT) in goldfish exposed to GEM results in ROS generation by an ACO-independent mechanism, a cytochrome P-450-dependent mechanism may be responsible as reported by Otto and Moon (1995) in rainbow trout and black bullhead. Hepatic mRNA levels of CYP1A1 did not vary following a waterborne exposure to GEM in the present study suggesting that CYP1A1 is not involved in the observed oxidative response. O'Brien et al. (2005) reported that peroxisome proliferators increased the activity of the cytochrome P-450 4A family which can generate ROS in mammals. The induction of these enzymes is furthermore supported by the reported presence of a PPRE in the promoter regions of mammalian cytochrome P450 4A1 and 4A6-Z (Mandard et al., 2004). Unfortunately, CYP4A has yet to be cloned in goldfish and further research will be required to evaluate its role in the hepatic oxidative response in goldfish exposed to waterborne GEM.

GSH is essential to maintain the antioxidant defense system by providing reducing equivalents to GPx and GST which, in the process, transform GSH into GSSG (Dickinson and Forman, 2002). Exposure of mice and rats to PPs generally increases GSH (O'Brien et al., 2005) which differs from the effects observed in goldfish in this study. A waterborne exposure to the highest GEM concentration (1,500 μg/L) resulted in a 33% reduction and in a 44% increase in GSH relative to control after 14 and 28 days, respectively. These results suggest an adaptive response over time of the glutathione system to the oxidative challenge,

but it must be emphasized that this high GEM concentration (1,500 μg/L) would not be encountered by a fish in its native environment. On the other hand, the environmentally relevant concentration of GEM (1.5 μg/L) significantly reduced GSSG in goldfish by 21% and 48% compared with the control after 14 and 28 days of exposure, respectively. This consistent reduction of GSSG suggests that an environmentally relevant exposure to GEM may weaken the hepatic antioxidant defenses without recovery during the 28 days exposure period. The observed reduction in GSSG levels probably accounts for the lack of response of GR; GSH levels were therefore most likely replenished through *de novo* synthesis, the main replacement mode of GSH (Dickinson and Forman, 2002). The reduced GSSG levels at the environmentally relevant GEM concentration with a concomitant increase in GPx could then be attributive to GSSG efflux from the liver cells. This explanation is supported by the study of Bartoli and Sies (1978) showing that rat liver cells release GSSG when exposed to oxidative stress. Further research would be required to clarify the mechanism of action of GEM on GSH metabolism in goldfish.

This study provides evidence that a waterborne GEM exposure in goldfish challenges the oxidative status of the fish but does not lead to oxidative damage, at least in the form of lipid peroxidation as measured as TBARS. This finding is in contrast with the significant negative correlation between TBARS levels and liver PPARα mRNA levels reported in rats (Inoue et al., 1997). This further supports the hypothesis that fish and rodents respond very differently to PP exposure. Alternative oxidative damage endpoints should be assessed, such as DNA damage or protein degradation, before reaching any conclusion about the absence of adverse oxidative effects.

3.5. Conclusions

Results from this study confirm the presence of the three PPAR subtypes in all tested tissues in goldfish. Investigation of the effects of GEM on PPAR transcripts suggests that the monitoring of hepatic PPAR mRNA levels is not a good indicator of GEM exposure in goldfish for ecotoxicological studies. This report demonstrates that the oxidative defense system in goldfish is activated by an exposure to GEM thus triggering an adaptive response that exerts a protective role against oxidative damage over a longer period of time. Nevertheless, further research is needed to determine if CYP4A would play a role in the antioxidant defense mechanisms in the goldfish liver following GEM waterborne exposure. This study further demonstrates that GEM, a human pharmaceutical prescribed to regulate blood lipid levels, may lead to unexpected effects in non-target species.

CHAPTER 4

Effects of waterborne GEM on the hepatic antioxidant defense system and peroxisome proliferator-activated receptors in rainbow trout (*Oncorhynchus mykiss*)

4.1. Introduction

Gemfibrozil (GEM) is a human pharmaceutical prescribed to regulate plasma lipid levels and, as other fibrate drugs, is classified as a peroxisome proliferator (PP). Environmental concentrations for human drugs are relatively low (high ng/L to low µg/L for GEM) compared to the concentrations at which they are designed to elicit pharmacological effects (15 to 25 mg/L for GEM) (Todd and Ward, 1988; Spencer and Barradell, 1996). Investigations of human drug effects on non-target species should therefore go beyond acute testing (Daughton and Ternes, 1999; Fent et al., 2006). With the detection of GEM and other pharmaceuticals in the aquatic environment, interest in their effects on normal physiological processes including the oxidative stress defense system have increased (Trudeau et al., 2005; Mimeault et al., 2005).

The antioxidant defense system is essential to prevent xenobiotic exposure damage including lipid peroxidation, protein inactivation and DNA damage that can lead to cancer (Kehrer, 1993). The reported effects of PPs in rodents include increases in the size and number of peroxisomes, compromised antioxidant capabilities and hepatocarcinomas (Yang et al., 1990; Gonzalez, 1997; O'Brien et al., 2001). The two fibrate drugs, fenofibrate and clofibrate induced reactive oxygen species in rainbow trout (*Oncorhynchus mykiss*) hepatocyte cultures (Laville et al., 2004) and GEM significantly challenged the hepatic antioxidant defense system of goldfish potentially through an acyl-CoA oxidase (ACO)-

independent mechanism (Mimeault et al., 2006). This result arises from the fact that even though the mode of action of PPs is through the peroxisome proliferator-activated receptor (PPARs) subtypes and principally PPARα for fibrate drugs, the effects of GEM on the goldfish oxidative defense system did not appear to be related to mRNA levels of PPARα (Mimeault et al., 2006).

While aquatic species possess qualitatively similar oxidative stress defense systems as in mammals (Kelly et al. 1998), quantitative differences exist across fish species. Basal hepatic antioxidant enzyme activities in the black bullhead (*Ameiurus melas*) as compared to the rainbow trout are 19 to 28 times higher for glutathione peroxidase (GPx), 4 to 5 times higher for glutathione-*S*-transferase (GST) and 50% lower for catalase (CAT) (Otto and Moon, 1996). In addition, antioxidant enzyme activity responses following exposure to contaminants are transient and variable for different species, enzymes and contaminants (Rand et al., 1995; Livingstone, 2001).

The main purpose of this study was to investigate the effects of a waterborne GEM exposure on antioxidant enzymes, oxidative stress and PPAR mRNA levels of rainbow trout and to compare these to the previously evaluated goldfish response (*Carassius auratus*) presented in Chapter 3. Rainbow trout were chosen as they are a widely used model in aquatic toxicology research (Rand et al., 1995) and their life history traits are very distinct from those of the goldfish. Fish were exposed to 1,500 μg GEM/L for 1, 7 and 14 days and the results demonstrate that the antioxidant defense system of rainbow trout responds differently from that of the goldfish as GEM did not lead to oxidative damage or significantly impact any hepatic antioxidant enzyme. Nevertheless, the lack of an effect of GEM on

PPARα, as reported in goldfish, confirms that mRNA levels of the principal PPAR involved in the mode of action of fibrate drugs is not a good indicator of GEM exposure in fish.

4.2. Material and methods

4.2.1. Fish and experimental design

4.2.1.1. Fish

Female rainbow trout (*Oncorhynchus mykiss*) were purchased from Linwood Acres Trout Farm (Campellcroft, ON, Canada). Fish were acclimated for more than 2 weeks in 1275-L fibreglass tanks that received oxygenated and dechloraminated City of Ottawa water at 13 °C with a constant 12-h light/12-h dark photoperiod. Fish used in these studies had an average mass of 87.5 ± 3.65 g (n = 35). Seven days prior to an experiment, fish were randomly transferred to 100-L tanks at a density of 12 fish per tank. Fish were fed 0.65% body weight daily with commercial trout pellets (Classic Floating Trout Grower, Martin Mills, Tavistock, ON, Canada). All experiments were conducted under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhered to guidelines established by the Canadian Council on Animal Care (CCAC) for the use of animals in research.

4.2.1.2. Waterborne exposure experiment

Rainbow trout were exposed in February 2006 for 1, 7 and 14 days to nominal waterborne GEM (Sigma-Aldrich, St. Louis, MO, USA) concentrations of 0 or 1,500 μg/L.

Water and GEM concentrations were renewed every 24 h throughout the entire experiment as previously described (Mimeault et al., 2005). GEM was initially dissolved in dimethyl sulfoxide (DMSO; Sigma) before being added to the 100-L fiberglass aquaria. The final DMSO concentration in the aquaria was 0.0003%. Fish were feed daily during the experiment as above. After 1, 7 or 14 days of exposure, fish were anesthetized with benzocaine prior to euthanizing by trans-spinal sectioning; livers were then removed, weighed, snap frozen in liquid nitrogen and kept at -80 °C until analysed. Condition factor (K = (body weight / length^3) x 100) and hepatosomatic index (HSI = (liver mass / body mass) x 100) were calculated for each fish.

4.2.2. Semi-quantitative PCR of PPARs and β-actin and their tissue distributions

The tissue distribution of PPARs were done using three rainbow trout randomly chosen from the holding tank and anesthetised with 30 to 35 mg/L benzocaine (Sigma) prior to euthanasia by trans-spinal sectioning. Tissues (brain (anterior, bottom, posterior, top), fat, gills, head kidneys, heart, intestine, kidney, liver, red and white muscles) were rapidly removed, separately snap frozen in liquid nitrogen and kept at -80 °C until analysed. β-Actin mRNA expression was measured but not used as an internal control to normalize data since expression varied between tissues. The data for tissue distribution are presented as percent averages of the total expression detected for each gene in each individual (Doyon et al., 2003).

Total RNA was extracted from tissues using TRIzol reagent as per the manufacturer's instructions (Invitrogen, Life Technologies, Burlington, ON, Canada). RNA was treated with DNase (DNAse I Amplification Grade; Invitrogen) and reverse transcribed into cDNA using

oligo dT as per the manufacturer's instructions (M-MLV-RT kit, Invitrogen, Life Technologies, Burlington, ON, Canada). All PCR reactions used an Eppendorf Mastercycler EP.

PPAR fish sequences from GenBank (Table 4.1) were used to design degenerate PCR primers (Table 4.2) (purchased from Invitrogen) for PPARα, -β and -γ. Amplified products were run on agarose gels, product bands were cut, cleaned and sequenced. Non-degenerate primers (Table 4.2) were designed based on sequencing results. Cycle gradient curves were used to determine the optimal cycle number for each PPAR subtype: optimal cycle numbers were 33, 33 and 31 for PPARα, -β and -γ in the tissue distribution (Fig. 4.1) and 33, 34 and 34 for PPARα, -β and -γ in the GEM waterborne exposure experiment, respectively. PCR products were confirmed through sequencing and subsequent gene identification with a BLASTX search.

Table 4.1. Sequences with NCBI-GenBank accessions numbers used to design goldfish PPAR-α, -β and –γ degenerated primers.

PPAR alpha	PPAR beta	PPAR gamma
Goldfish (AY198322)	Goldfish (AY894894)	Goldfish (AY894893)
Sea Bass (AY590300)	Salmon (AF342945)	Plaice (AJ243956)
Sparus (AY590299)	Salmon (AJ416953)	Salmon (AJ292962)
	Sea Bass (AY590302)	Salmon (AJ416951)
	Sparus (AY590301)	Salmon (AJ416952)
	Trout (AY356399)	Sea Bass (AY590303)
	Zebrafish (AF342937)	Sparus (AY590304)
	Zebrafish (AF342938)	

Table 4.2. Primer sequences and PCR conditions used to amplify PPARs in rainbow trout. Conditions were 94 °C for 45 s, various temperatures (T) for 45 s and 72 °C for 1 min. The hybridization temperatures were 55, 50 and 60 °C for PPARα, β and γ, respectively.

	Gene	Strand	Sequence	Size (bp)	$MgCl_2$ (mM)	T (°C)
Degenerate	PPARα	F	WKS AGA RGG AGG CTG ARG YC	331	2	55
		R	TCA CTR TCR TCY AGC TCS AG			
	PPARβ	F	CAT GGA GCC CAA GTT TGA GT	311	2	50
		R	GGT GKA GYG AYG TCT CTG AC			
	PPARγ	F	GGR TTC ACH GAK CTG GAY MT	461	2	60
		R	TTC TTS AGC AGS TGG ATG WG			
Not degenerate	PPARα	F	CGG TCA CAG AGC TGA CTG AG	248	1	60
		R	ACT TCA TGG CGA ACT GGA AC			
	PPARβ	F	CAT GGA GCC CAA GTT TGA GT	311	1	60
		R	GGT GAA GCG AGG TCT CTG AC			
	PPARγ	F	ATG AAC AAG GAC GGC ACA CT	310	1	60
		R	GCA GTA GCT TGG CGA ACA G			

β-Actin was chosen as the internal control gene for the GEM waterborne exposure experiment as no quantitative changes in its expression in the liver following GEM exposure were detected. Primers for β-actin were developed using available sequences for rainbow trout (GenBank accession number AJ438158). The primer sequences were 5'-CGCCGCACTGGTTGTTGACA -3' (F) and 5'- GCGGTGCCCATCTCCTGCT -3' (R), and PCR conditions were 94 °C for 45 s, 60.0 °C for 45 s and 72 °C for 1 min with 1mM $MgCl_2$. β-Actin primers were optimized as above at 23 and 29 cycles for the tissue distribution and

the exposure experiment, respectively. The size of the product was 674 bps and was confirmed by sequencing.

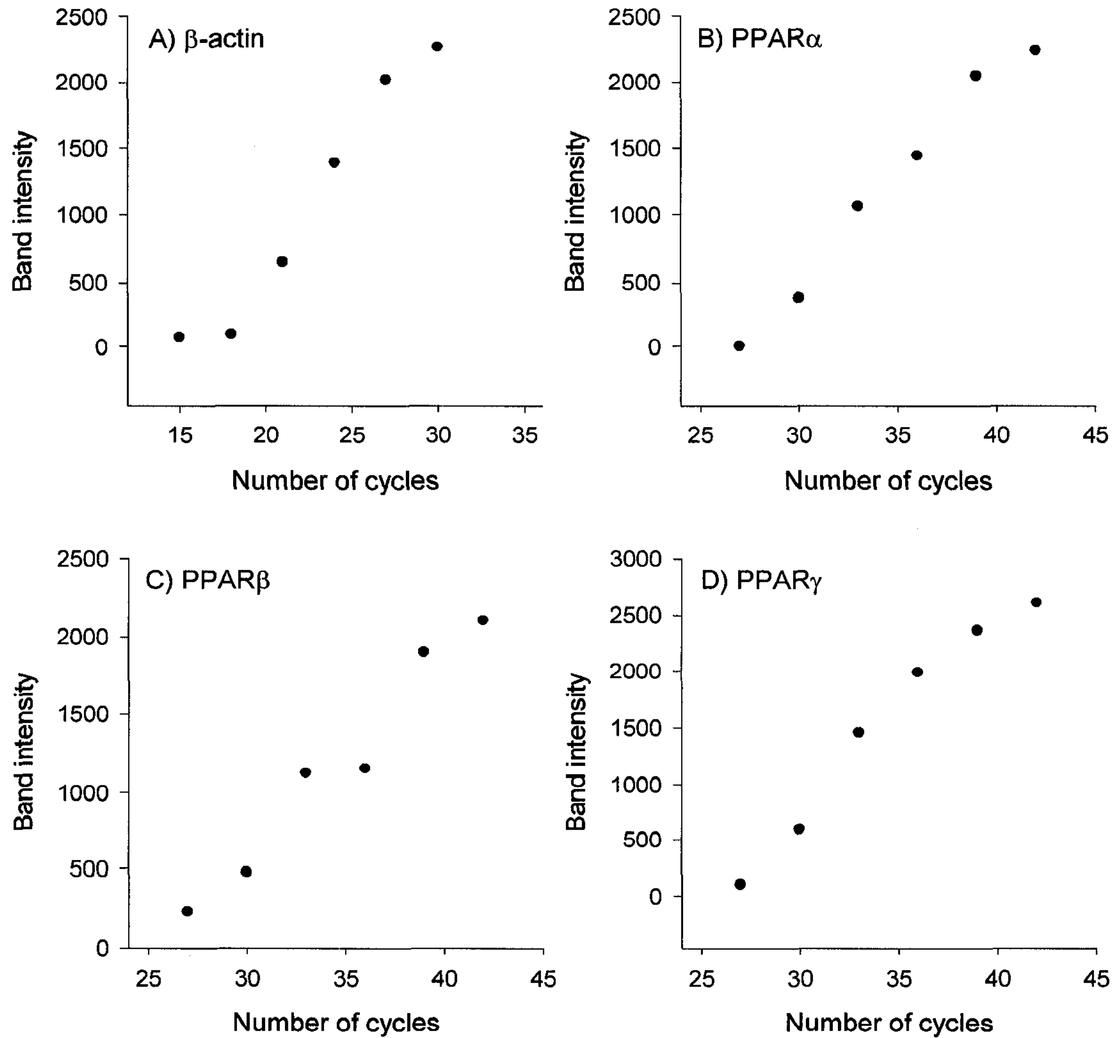

Figure 4.1. Cycle number optimization for (A) β-actin, (B) PPARα, (C) PPARβ and (D) PPARγ cDNA amplification for the tissue distribution quantification in rainbow trout. Optimal cycle numbers were determined on the linear phase of the curve (R^2 for β-actin, PPARα, PPARβ and PPARγ were 0.997, 0.993, 0.963 and 0.983, respectively).

4.2.3. Antioxidant enzyme assays

Methods to prepare homogenate for antioxidant enzyme assays and the antioxidant assays were the same as the ones presented in Chapter 3.

4.2.4. Lipid peroxidation

Lipid peroxidation was quantified as thiobarbituric reactive substances (TBARS) and adapted to a 96-well microplate format from Hermes-Lima and Storey (1996) as reported in Chapter 3.

4.2.5. Protein assay

Protein concentration was determined for each tissue homogenate with the bicinchoninic acid protein assay kit (Sigma-Aldrich, TPRO-562) using bovine serum albumin as standard and a microplate reader.

4.2.6. Statistical analysis

All statistical analyses were conducted using SigmaStat™ 3.1 software (SPSS Corp., Chicago, IL). Data are presented as means ± standard error of the mean (SEM) of n independent experiments. Two-way ANOVAs were used to determine the effects of the waterborne GEM (referred to as drug) and of the duration of exposure (referred to as duration). ANOVAs were followed by Bonferroni tests for multiple comparisons. A value of $P < 0.05$ was considered significant.

4.3. Results

No mass difference between groups was detected in the initial random distribution of trouts in tanks (one-way ANOVA, $p = 0.729$). Rainbow trout body mass (87.5 ± 3.65 g, $n = 35$), K factor (1.10 ± 0.03, $n = 35$) and HSI (1.5 ± 0.1, $n = 35$) after exposure were not affected ($P > 0.05$) by waterborne GEM exposure (data for all groups combined).

4.3.1. Tissue distribution of PPARα, -β, and –γ

β-actin mRNA could not be used to compare relative PPAR transcript levels between tissues as expression levels varied between tissues (Fig. 4.2A) hence the tissue distribution data are presented as the average percentage of the total band intensity for the PPAR subtype detected in each tissue, as previously done for other tissue distribution studies (Doyon et al., 2003; Mimeault et al., 2006). Transcripts for each of the three PPAR subtypes were detected in all thirteen tissues examined.

PPARα was detected in all tissues at comparable levels with the exception of red muscle which had relatively higher levels and head kidney which had lower levels than the average value (Fig. 4.2B). PPARβ mRNA levels were ubiquitously expressed in all tested tissues at comparable levels (Fig. 4.2C). PPARγ mRNA tissue distribution was the most distinct between tissues with the highest levels detected in kidney and liver, intermediate levels detected in brain and intestine and lowest levels in fat, gills, head kidney, heart and muscles (Fig. 4.2D).

4.3.2. Effect of waterborne GEM on rainbow trout hepatic PPARs mRNA levels

Relative transcript levels of the three hepatic PPAR subtypes were assessed by semi-quantitative reverse-transcriptase PCR (RT-PCR) after 1, 7 and 14 days of exposure to 0 or 1,500 μg/L GEM (Fig. 4.3). β–Actin was considered an appropriate internal control gene as expression levels were unaffected by GEM treatment (data not shown, $P = 0.565$) (Mimeault et al., 2006). Two-way ANOVA revealed no effect on PPARα mRNA levels following GEM exposure (Fig. 4.3A) ($P = 0.861$, 0.659 and 0.585 for duration, drug and interaction, respectively). PPARβ transcript levels were also not affected in the liver of fish exposed to GEM ($P = 0.098$, 0.094 and 0.343 for duration, drug and interaction, respectively) (Fig. 4.3B). A significant interaction between exposure to the drug and the duration explained that the relative PPARγ mRNA levels were reduced by 63% at day 7 of waterborne exposure, but did not change after 1 or 14 days ($P = 0.665$, 0.246 and 0.031 for duration, drug and the interaction, respectively) (Fig. 4.3C).

4.3.3. Effect of waterborne GEM on oxidative defence mechanisms

The activities of enzymes involved in the hepatic antioxidant defense system were measured as indicators of a possible oxidative stress response to GEM exposure. The specific activities for GPx, GST, GR and CAT in control animals (Fig. 4.4) were in comparable ranges of activities reported previously for fish (Otto and Moon, 1996; Mimeault et al., 2006). Exposure to GEM did not affect GPx activity ($P = 0.586$) but activity significantly increased over time ($p = 0.001$) (Fig. 4.4A). Neither drug nor duration of exposure had an effect on GST ($P = 0.164$ and 0.275, respectively), GR ($P = 0.204$ and 0.194, respectively) or CAT ($P = 0.299$ and 0.703, respectively) activities (Fig. 4B,C and D).

4.3.4. GEM and oxidative damage

Oxidative damage was assessed as thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation (Fig. 4.5). Exposure to GEM did not significantly affect TBARS levels (P = 0.982) but TBARS levels were significantly lower after 14 days compared to day 1 of exposure (P = 0.025).

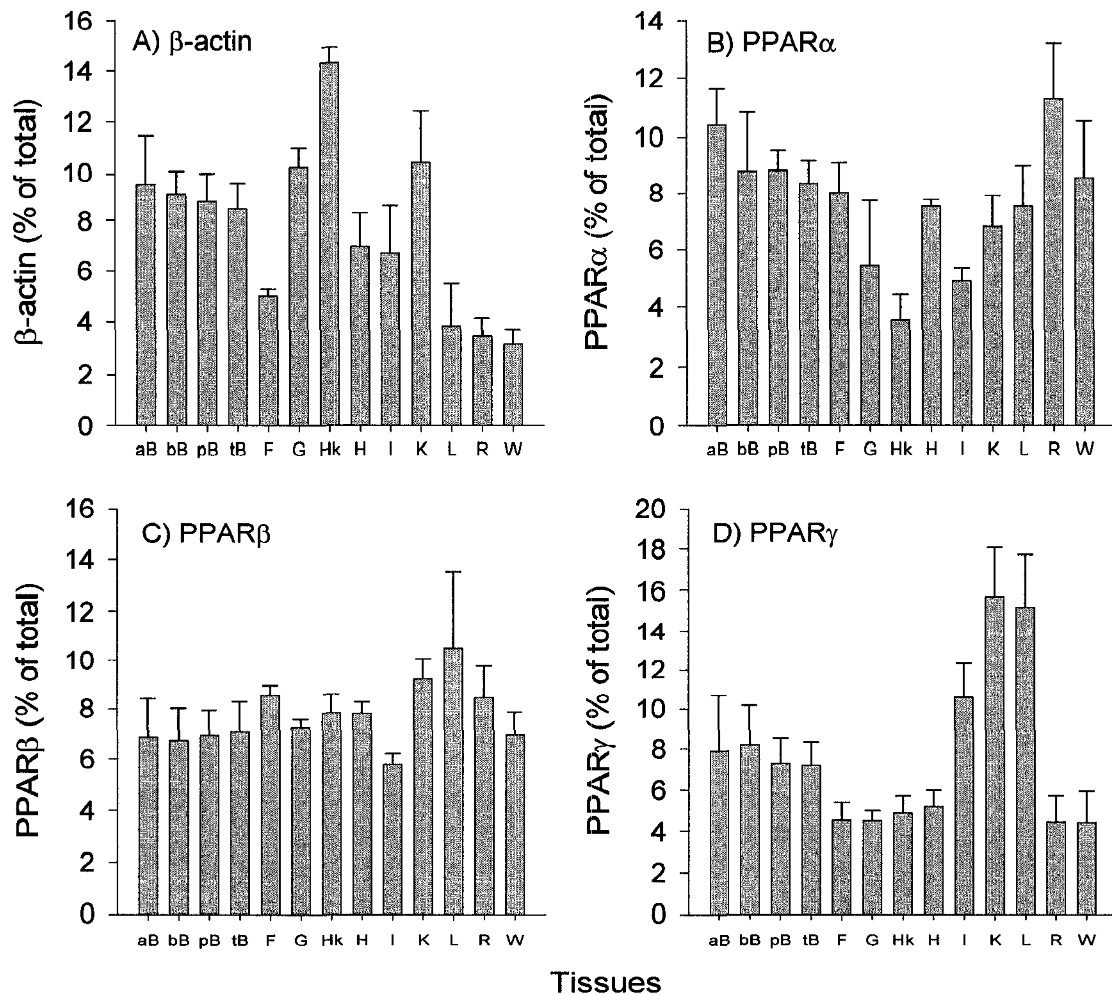

Figure 4.2. Tissue distribution of (A) β-actin, (B) PPARα, (C) PPARβ and (D) PPARγ mRNA in various tissues of the female rainbow trout using RT-PCR. The tissue abbreviations are: (aB) anterior brain, (bB) bottom brain, (pB) posterior brain, (tB) top brain, (F) fat, (G) gills, (Hk) head kidney, (H) heart, (I) intestine, (K) kidney, (L) liver, (R) red muscle and (W) white muscle. Bars represent average percentages (+ SEM) of the total band intensity for the particular transcript detected in each tissue (n = 3).

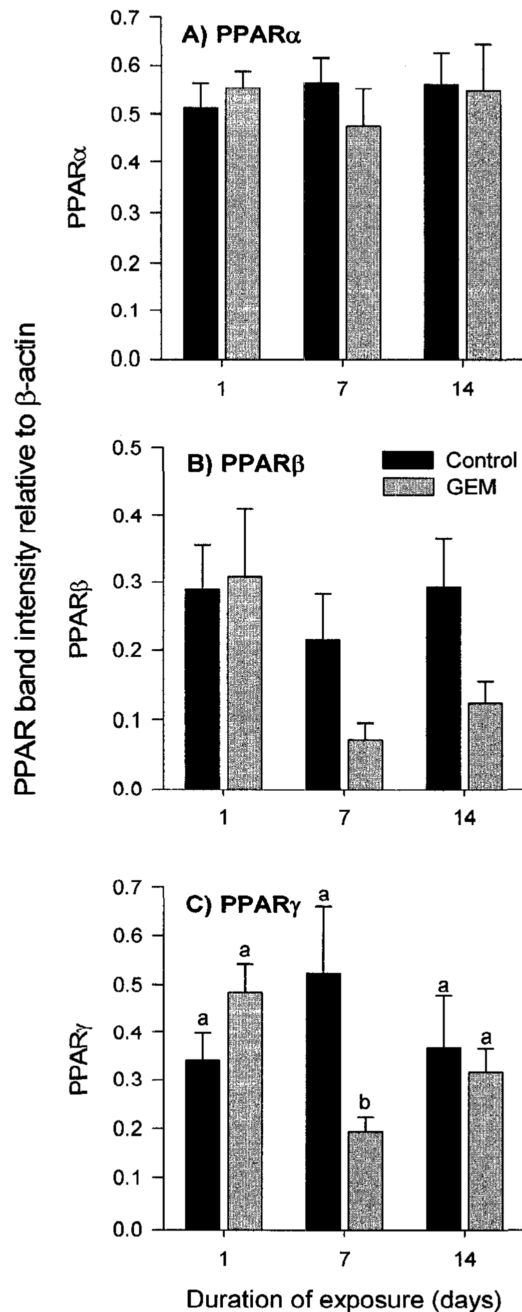

Figure 4.3. Relative transcript levels of (A) PPARα, (B) PPARβ and (C) PPARγ in rainbow trout liver exposed to 0 or 1,500 μg/L GEM for 1, 7 and 14 days. Data represent means + S.E.M. (n = 4). Two-way ANOVA revealed (A) no effect on PPARα ($P = 0.861$, 0.659 and 0.585 for duration, drug and the interaction), (B) no effect on PPARβ ($P = 0.098$, 0.094 and 0.343 for duration, drug and the interaction), and (C) a significant interaction of duration and drug in PPARγ ($P = 0.665$, 0.246 and 0.031 for duration, drug and the interaction). Bonferroni's multiple comparison tests were used to detect differences between means and these are indicated by different letters ($P < 0.05$).

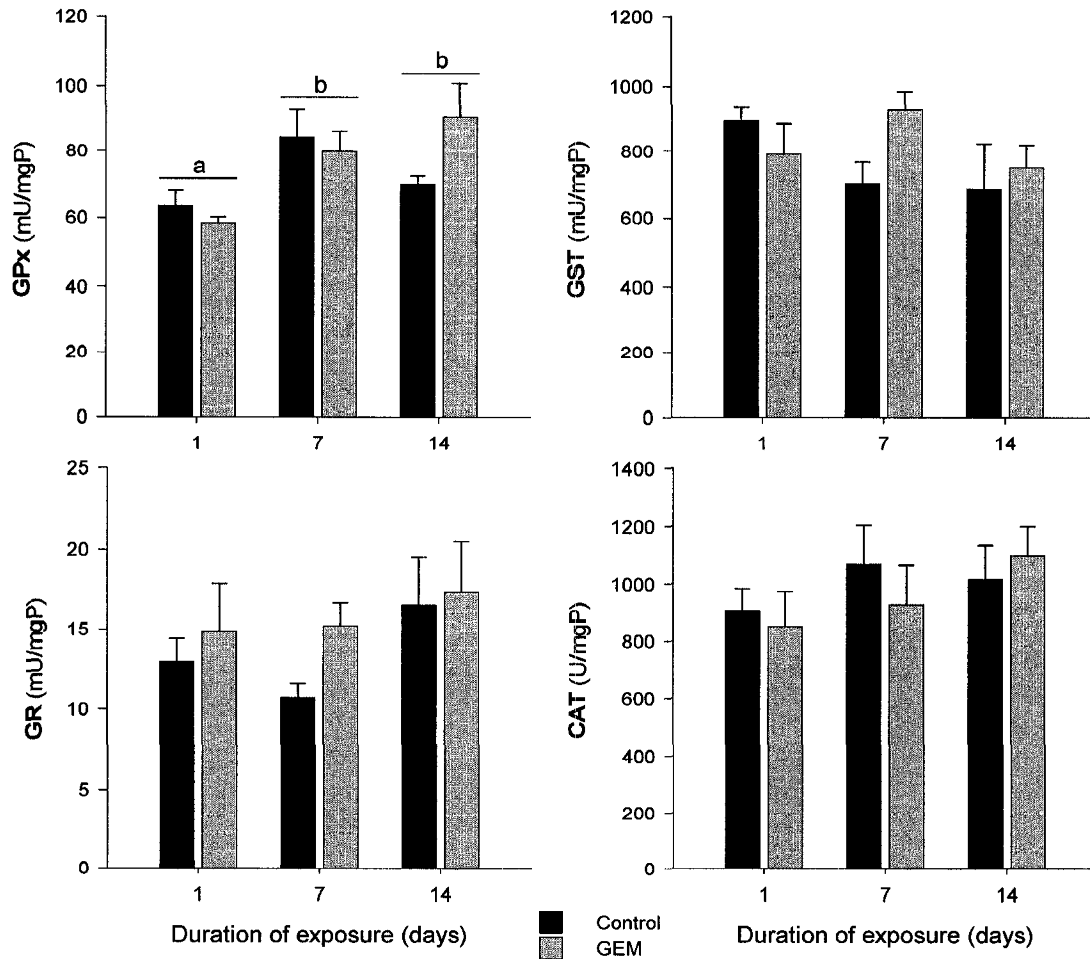

Figure 4.4. Hepatic antioxidant enzyme specific activities in rainbow trout following waterborne GEM exposures for 1, 7 and 14 days: (A) Glutathione peroxidase (GPx), (B) glutathione-*S*-tranferase (GST), (C) glutathione reductase (GR), and (D) catalase (CAT). Data represent means + S.E.M. (n = 6). Two-way ANOVA revealed (A) that duration had an effect on GPx ($P = 0.001$) as opposed to the drug which had no effect ($P = 0.586$), (B) no significant effect on GST (P for duration = 0.164 and P for drug = 0.275), (C) no significant effect on GR (P for duration = 0.204, P for drug = 0.194), and (D) no significant effect on CAT (P for duration = 0.299, P for drug = 0.703). Interactions between factors were not significant for any of the enzymes. Bonferroni's multiple comparison tests were used to detect differences between means where appropriate and these are indicated by different letters ($P < 0.05$).

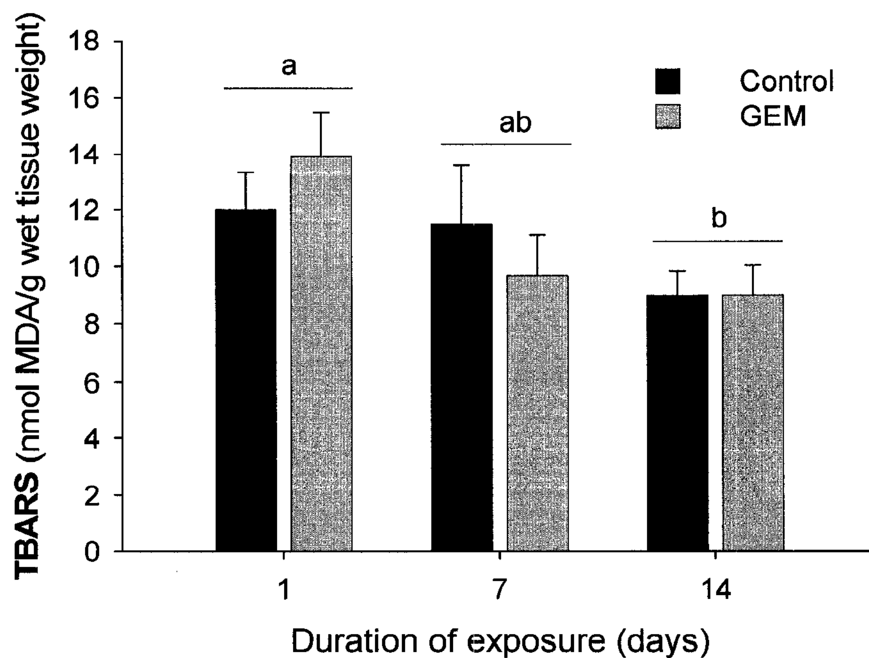

Figure 4.5. Thiobarbituric acid reactive substances (TBARS) expressed as malonaldehyde levels (nmol MDA/g wet tissue weight) in rainbow trout liver following exposure to waterborne GEM for 1, 7 or 14 days. Two-way ANOVA revealed that duration had an effect (P = 0.025) as opposed to the drug which had no effect (P = 0.982) nor were there any interactions detected (P = 0.422). Data represent means + S.E.M. (n = 6). Bonferroni's multiple comparison tests were used to detect differences between means and these are indicated by different letters ($P < 0.05$).

4.4. Discussion

GEM has the ability to challenge the antioxidant defence system in goldfish without leading to oxidative damage in the form of lipid peroxidation (Chapter 3; Mimeault et al., 2006). This study indicates that rainbow trout respond differently to the same waterborne GEM concentration as used in the goldfish study.

Assessing the tissue distribution of the different PPAR subtypes allows understanding their significance (Desvergne and Wahli, 1999). Results from this study demonstrate the presence of transcripts for all three PPAR subtypes in all rainbow trout tissues tested. PPARα is not expressed at higher levels in liver relative to other tissues in the rainbow trout. This observation is in accordance with another freshwater fish, the goldfish (Mimeault et al., 2006) but differs from the two marine fish: the sea bream (*Sparus aurata*) and the plaice (*Pleuronectes platessa*) (Leaver et al., 2005) as well as in mammals (Braissant et al., 1996; Desvergne and Wahli, 1999). This lack of higher mRNA expression of PPARα in tissues with high β-oxidation capacity such as the liver, may partially explain the lack of effect of GEM, primarily a PPARα ligand, on PPARα mRNA levels. The ubiquitous expression of PPARβ in all tissues at comparable levels is consistent to that previously reported for this subtype in other vertebrate species (Desvergne and Wahli, 1999). Finally, although PPARγ has the most distinct distribution of all three PPARs in rainbow trout, its expression is not as restricted as in mammals. PPARγ mRNA is expressed in all tissues in rainbow trout with relatively higher levels in the intestine, liver and kidney as in the goldfish (Mimeault et al., 2006) as opposed to a more restricted pattern to adipose tissues in mammals (Braissant et al., 1996; Lemberger et al., 1996; Desvergne and Wahli, 1999; Leaver et al., 2005).

The main objective of this study was to determine the effect of a waterborne GEM exposure on the rainbow trout liver PPAR mRNA levels and antioxidant defense system. GEM, as a PP, was expected to increase PPARα mRNA levels, but failed to do so as in goldfish (Mimeault et al., 2006). This result further demonstrates that GEM is a poor inducer of PPARα mRNA levels in trout and goldfish. As species with different basal PPARα expression levels, such as human compared with rat and mouse, were shown to react differently to PP exposure (Holden and Tugwood, 1999), further research is needed to exclude any effect of GEM on PPARα on other fish species such as the marine sea bream and plaice which seem to have higher basal hepatic PPARα mRNA levels (Leaver et al., 2005).

Despite a lack of statistical difference ($P = 0.094$), most likely due to a small sample size), the 58% reduction of PPARβ mRNA levels in livers of rainbow trout exposed to 1,500 μg GEM/L for 14 days corroborates the previously reported 50% reduction of PPARβ mRNA levels in goldfish liver exposed to the same conditions (Mimeault et al., 2006). This result suggests an effect of GEM on PPARβ mRNA levels rather than on PPARα as initially expected due to the presence of a peroxisome proliferator-response element in the promoter region of the PPARα gene. Further studies are required to elucidate the potential mode of action of GEM on PPARβ mRNA levels and the potential physiological implications.

In rodents, PPARγ plays a key role in lipogenesis stimulating the uptake of glucose and fatty acids (Desvergne and Wahli, 1999; Kersten et al., 2000; Anghel et al., 2007). High levels of PPARγ are present in mammals in the nutritional fed state (Kersten et al., 2000) which has also been observed in a fish species, the sea bream (Leaver et al., 2005) suggesting the same function of the subtype in the different species. As waterborne GEM reduced PPARγ mRNA levels by 63% after 7 days of exposure, the conversion of glucose and fatty

acids into triglycerides could be affected. Nevertheless, this reduction seems to be transient as levels of PPARγ mRNA levels recovered after 14 days of exposure. Impaired lipogenesis would therefore not be expected in the aquatic environment as exposure is likely to be over longer periods and most importantly, reported environmental concentrations are 1000-fold lower than the one used in this study.

Results from this experiment also demonstrate that GEM does not challenge the hepatic antioxidant defense mechanisms of rainbow trout when exposed to 1,500 μg GEM/L, a concentration higher than those reported in the environment. The complete lack of effect of waterborne GEM on both the primary and secondary defense antioxidant enzymes combined with the absence of oxidative damage in the form of lipid peroxidation further suggest that GEM did not generate reactive oxygen species in rainbow trout as opposed to goldfish (Mimeault et al., 2006). These results further confirm species differences in antioxidant responses to environmental contaminants (Ferreira et al., 2005).

As GEM bioconcentrates in goldfish (Mimeault et al., 2005) and rainbow trout (Brown et al., 2007) with comparable bioconcentration factors, the lack of response observed in this experiment is very unlikely due to a lack of uptake of GEM from the surrounding water. In addition to species differences, gender is a potential explanation for the lack of oxidative stress response as only female trout were available for this experiment and PPs have been previously demonstrated to be more potent in males than in female rodents (Amacher et al., 1997). Other oxidative stress damage endpoints such as DNA damage or protein degradation should also be assessed before concluding that GEM does not lead to oxidative damage in rainbow trout.

4.5. Conclusions

Results from this study confirm the presence of the three PPAR subtypes in all tested tissues of the rainbow trout and that monitoring of the hepatic PPARα mRNA levels is not a good indicator of GEM exposure in fish for ecotoxicological studies. Further research should examine the possibility of using PPARβ or PPARγ mRNA levels as indicators. Finally, waterborne GEM did not challenge the antioxidant defense system or cause lipid peroxidation. Further research would be required to discriminate if the lack of effect of GEM on the rainbow trout antioxidant defense system is due to species or gender differences.

CHAPTER 5

In-vitro corticosteroidogenesis is impaired by fibrate drugs in rainbow trout (*Oncorhynchus mykiss*) head kidney cells

Chapter to be submitted as: Mimeault C, Chen X, Trudeau VL and TW Moon. 2007. In-vitro corticosteroidogenesis is impaired by fibrate drugs in rainbow trout (*Oncorhynchus mykiss*) head kidney cells. Manuscript to be submitted to: Aquatic Toxicology.

5.1. Introduction

Cortisol, the main corticosteroid in teleost fish, is involved in the regulation of intermediary metabolism, osmoregulation, growth and reproduction (Wendelaar Bonga, 1997; Mommsen et al., 1999). In fish, cortisol secretion is under the control of the hypothalamic-pituitary-interrenal (HPI) axis and cortisol biosynthesis occurs in the interrenal cells which are located amongst the anterior head kidney cells (Wendelaar Bonga, 1997). Through the activation of the second messenger cyclic adenosine monophosphate (cAMP) and a subsequent signalling cascade, cholesterol enters the mitochondrion where it is oxidized to pregnenolone by the P450 side chain cleavage enzyme (CYP11A1), the rate-limiting step of all steroid hormone biosynthesis (Payne and Hales, 2004). Pregnenolone is then transported to the endoplasmic reticulum where it is modified to progesterone, 17-OH progesterone and 11-deoxycortisol by 3-β-hydroxysteroid dehydrogenase (3-β-HSD), 17α-hydroxylase (CYP17) and 21-hydroxylase (CYP21A2), respectively. 11-Deoxycortisol is then transported back to the mitochondrion where 11β-hydroxylase (CYP11) converts it to cortisol (Fig. 1.5.) (Hontela, 2005).

Cortisol, as the final effector of the HPI axis, is crucial to overcome threats and to readjust the homeostatic equilibrium of organisms exposed to stressors (Wendelaar Bonga, 1997). However, the essential adaptive elevation of plasma cortisol concentrations after exposure to a stressor is compromised in the presence of contaminants in the aquatic environment. Fish have an impaired stress response when chronically exposed to contaminants as they fail to increase their cortisol levels as high as in clean lakes when exposed to an acute stress (Hontela, 1998). This interrenal endocrine dysfunction was also demonstrated using *in-vitro* head kidney cells exposed to several environmental contaminants including metals, pesticides and non-steroidal anti-inflamatories (Leblond and Hontela, 1999; Leblond et al., 2001; Lacroix and Hontela, 2003; Gravel and Vijayan, 2006). The reported mode of action of some contaminants to impair steroidogenesis is through inhibition of the steroidogenic acute regulatory protein (StAR) and P450scc, two proteins involved at the beginning of cholesterol conversion into steroid hormones (Aluru et al., 2005; Aluru and Vijayan, 2006; Gravel et al., 2006).

The recent detection of the human pharmaceuticals in the aquatic environment (Ternes, 1998; Metcalfe et al., 2003a; 2003b; Tixier et al., 2003) has prompted research on their potential effects in non-target species. Evidence for endocrine-disrupting properties of human drugs in fish is reported (Mimeault et al., 2005; Trudeau et al., 2005; Gravel and Vijayan, 2006; Lyssimachou and Arukwe, 2007). Salicylate, acetaminophen and ibuprofen were shown to disrupt corticosteroidogenesis in rainbow trout (*Oncorhynchus mykiss*) interrenal cells (Gravel and Vijayan, 2006). Gemfibrozil (GEM), a fibrate drug prescribed as a hypolipidemic agent, decreased plasma testosterone and gonadal StAR mRNA levels in

goldfish but the reduced testosterone levels could not be solely attributed to an impaired delivery of cholesterol to the inner mitochondrial membrane (Mimeault et al., 2005).

One objective of this study was to investigate the *in-vitro* effects of different fibrate drugs on the ACTH-induced cortisol response in head kidney cells of rainbow trout. A second objective was to identify the site of action of potent fibrate drugs within the interrenal steroidogenic pathway. This study reports that some fibrates act as endocrine disruptors by specifically targeting interrenal steroidogenesis and specific enzymes are identified as the potential sensitive steps within the pathway.

5.2. Material and methods

5.2.1. Chemicals

Bezafibrate (BEZA), clofibrate (CLO), fenofibrate (FENO), gemfibrozil (GEM), minimum essential media (MEM), adrenocorticotropic hormone (ACTH), dibutyryl cyclic AMP (dbcAMP), pregnenolone, progesterone, 17α-hydroxyprogesterone and 11-deoxycortisol were purchased from Sigma-Aldrich (St-Louis, MO, USA). Collagenase-dispase was purchased from Roche Canada (Mississauga, ON) and imidazole from BDH (VWR, Mississauga, ON). Cortisol kits and NADH were from ICN (Monrovia, CA, USA) while pyruvate was purchased from Boehringer (Ingelheim, Germany). Molecular biology chemicals (DNase treatment, M-MLV-RT kit, PCR and solutions) were obtained through Invitrogen Life Technologies (Burlington, ON).

5.2.2. Fish and experimental design

Female rainbow trout (*Oncorhynchus mykiss*) were purchased from Linwood Acres Trout Farm (Campellcroft, ON, Canada). Fish were acclimated for more than 2 weeks in 1275-L fibreglass tanks that received oxygenated and dechloraminated City of Ottawa water at 13 °C with a constant 12-h light/12-h dark photoperiod. Holding tanks initially contained 300 fish with an average weight of 121 ± 5 g. Fish were feed daily with commercial trout pellets (Classic Floating Trout Grower, Martin Mills, Tavistock, ON, Canada). All experiments were conducted under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhere to guidelines established by the Canadian Council on Animal Care (CCAC) for the use of animals in research.

5.2.3. Head kidney cell preparation

Head kidney cells were isolated by an adaptation of the method of Leblond et al. (2001). Briefly, fish were anaesthetized with benzocaine (30 to 35 mg/L), a blood sample for subsequent cortisol determination was quickly taken from the caudal vasculature using a heparinized 1 ml syringe and a 26-gauge needle. Fish were sacrificed by a quick blow to the head, the spine severed and the entire vasculature perfused with 2 X 20 ml Cortland saline (124 mM NaCl, 2.0 mM KCl, 4.1 mM $MgSO_4$, 5.0 mM $NaHCO_3$, 0.8 mM glucose, 1.2 mM KH_2PO_4 and 2.6 mM $CaCl_2$) through the caudal vasculature. Enriched MEM (eMEM) was prepared by supplementing MEM with 75 μM bovine serum albumin (MP Biomedicals, Canada, cat. no 105033) and 26 mM $NaHCO_3$ and pH-adjusted to 7.4. The entire head kidney was removed and immediately added to a 15-ml plastic culture tube containing 2 ml eMEM with 2 mg/ml collagenase/dispase. The tissue was disrupted by gently pressing it on

the interior side of the tube with a small spatula followed by a 60 min incubation at 13°C; after 30 min, cells were resuspended gently with a glass pipette every 10 min. After enzyme digestion, the cell suspension was passed successively through 200 and 70 µm nylon filters and centrifuged at 260 *g* (Hermle Z360K, Mandel Scientific Company Ltd.) for 5 min at 13°C. The supernatant was discarded and the cells were resuspended in 0.8 to 1.5 ml fresh eMEM depending on the size of the pellet. Cells were counted using a hemacytometer and cell density was adjusted to 50 million cells/ml. One hundred fifty µl of resuspended cells were added to wells of a 96-well plate and pre-incubated for 2 h at 13 °C with gentle shaking. The optimal cell density and incubation time were determined in preliminary experiments (Fig. 5.1A and 5.1B).

5.2.4. Exposure to fibrate pharmaceuticals

Following pre-incubation, the 96-well plate was centrifuged at 140 *g* (Hermle Z360K) for 3 min at 13 °C and the cells were resuspended in eMEM containing different concentrations of fibrate drugs. For ACTH-induction experiments, cells were exposed to 0, 0.004, 0.04, 0.4, 4, 40 or 400 µM BEZA, CLO, FENO or GEM dissolved in DMSO (final DMSO concentration 0.3% in wells); an additional well received only DMSO as a control treatment. For experiments in which cortisol was stimulated with different steroidogenic inducers, cells were exposed to 400 µM BEZA, CLO, FENO or GEM dissolved in DMSO. Cells were then incubated at 13 °C with gentle shaking for 1 h (Leblond and Hontela, 1999; Leblond et al., 2001; Lacroix and Hontela, 2003).

5.2.5. Stimulation of head kidney cells

After 1 h of incubation in the presence of the drugs, cortisol secretion was stimulated by the addition of different steroidogenic inducers. The optimal concentration of each inducer was determined in preliminary experiments (Fig. 5.1B, Fig. 5.3). Concentrations were adjusted so that adding 5 μl of the inducer to each well would result in the optimal concentration of the different steroidogenic inducers. Cells were incubated at 13 °C with gentle shaking for one additional hour after which cells were resuspended and transferred to 1.5 ml tubes. Forty μl of cell suspension were kept for the cell viability assay (lactate dehydrogenase assay) and the remaining suspension was centrifuged at maximum speed (Beckman Coulter Microfuge R) for 2 min. The supernatant was collected and snapped frozen in liquid nitrogen for subsequent cortisol analysis.

5.2.6. Cortisol assay

Cortisol was determined using a ^{125}I RIA kit as per the manufacturer's protocol (MP Biomedicals, Irvine, CA, USA). Reported cortisol kit-specific cross-reactivity with steroids was none, 0.25%, 1% and 12.3% for pregnenolone, progesterone, 17α-hydroxyprogesterone and 11-deoxycortisol, respectively.

5.2.7. Head kidney cell viability assay

Lactate dehydrogenase (LDH) was used as a cell viability indicator (Leblond et al., 2001). Briefly, the 40 μl aliquot of cell suspension was gently centrifuged at 200 *g* for 2 min at 4 °C and the supernatant collected to assess LDH activity. Deionized water was added to the pellet and the lysed cells were vortexed prior to centrifugation at maximum speed for 2

min to assess total LDH activity. LDH activity was determined in 96-well plates by following the oxidation of 0.20 mM NADH in 34 mM imidazole and 4.7 mM pyruvate with 10 μl of supernatant. Viability is reported as the percent of LDH detected in the presence of live cells (supernatant) compared with the total LDH activity (cell pellet and supernatant).

5.2.8. Head kidney cell RNA isolation

Total RNA was isolated from frozen rainbow trout head kidney cells using TRIzol reagent (GibcoBRL, Burlington, ON) according to the manufacturer's protocol with the following exception. Four μl linear acrylamide (2 mg/μl) (Ambion, Austin, TX) was added after the addition of isopropanol to increase RNA yield (Gaillard and Strauss, 1990). RNA concentration and quality were verified by spectrophotometry (OD at 260 nm) and gel electrophoresis, respectively.

5.2.9. Amplification of rainbow trout CYP21A2

An initial set of rainbow trout CYP21A2 clones was amplified using routine RT-PCR strategies. cDNA was synthesized using random primers with a First Strand cDNA Synthesis Kit (Roche Molecular Biologicals, Laval, QC). Primers were all purchased from Invitrogen. The first round of PCR amplification was performed using degenerate primers CYP21F and CYP21R1 and the second round of PCR using CYP21F with CYP21R2 (Table 5.1.). Degenerate primers were designed using the CODEHOP program (http://blocks.fhcrc.org/codehop.html) based on sequences for Japanese eel, fugu fish and zebrafish CYP21A2 (GenBank accession numbers – BAC76051.1; CAD45005.1 and XP_001345422.1, respectively). The PCR products were cloned using the TOPO TA

cloning kit (Invitrogen). Trout clones were sequenced at the Ottawa Genome Centre. To confirm the cDNA sequence, the rainbow trout CYP21A2 gene was cloned three times using different PCR reactions and each clone was sequenced on both strands. The sequence was submitted to GenBank (accession number EU246942). All PCR amplifications described above used the following regimen of denaturing, annealing and extension: 1x 2 min at 94 °C, 35x (30 sec at 94 °C, 30 sec at 55 °C, 1 min at 72 °C), and 1x 10 min at 72 °C; annealing temperature was 55 °C (Table 5.1).

Table 5.1. Real-time PCR primer sequences used to amplify CYP genes and β-actin in rainbow trout head kidney cells.

Name	5' primers	3' primers	Tm (°C)
CYP21F	TGGACCTGTCCGAGGACTTYACNGTNGC		55
CYP21R1		GGGCACGGCCAGAGGNGCNACNGG	55
CYP21R2		CGGTGCAGCAGGAAGGCNACNGTCCA	55
CYP11QPFR	CTGAAGGGACTCGTCAAGGA	ACGACTTCTCCCCAGAGGAT	58
CYP17QPFR	ACATATTTGGAGCCGGAGTG	TAATGGTGGCCTCCAGGTAG	58
CYP21QPFR	TGCACGGCTGTCTTAATGAG	GGCTTCCTCTGATGTTTTGC	58
β-actinFR	CGTCCCAGGCATCAGGGAGT	TCTCCATGTCGTCCCAGTTG	58

5.2.10. Real-time PCR analysis of CYP11, 17 and 21 mRNAs

CYP genes (11, 17 and 21) and β-actin (internal control) were assessed using SYBR green (Molecular Probes, Eugene, OR) based real-time PCR analysis (Stratagene Mx4000) according to the manufacturer's protocol. The real-time PCR primers (Table 5.1) were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www_slow.cgi) and synthesized to yield amplicons of 150, 200 and 200 bp for CYP11, CYP17 and CYP21, respectively. Trout CYP11 and CYP17 cDNA sequences were obtained from GenBank (accession numbers AAF76752 and P30437,

respectively). Total RNA was isolated from head kidney cells exposed to DMSO (control) or 400 μM BEZA, CLO, FENO or GEM using the same method as described above. cDNA was synthesized as above. The real-time PCR products were cloned and sequenced to ensure that the amplification was identical to the original sequence. Forty cycles of a two-step PCR protocol were used: 1x 15 min at 95 °C, 40x (30 sec at 95 °C, 30 sec at 58 °C, 30 sec at 72 °C). A no template control for each master mix and a no reverse transcriptase control were included in each analysis. The slopes of the standard curves for CYP11, CYP17, CYP21 and β-actin were -3.362, -3.300, -3.168 and -3.015, respectively, yielding amplification efficiencies of 98.4%, 100.9%, 106.9% and 114.6%, respectively. Real-time PCR data are reported using the comparative ΔCt method (Livak and Schmittgen, 2001) and given by the formula 2(-ΔCt) where ΔCt = CtCYP - Ctβ-actin and Ct is defined as the cycle at which the level of fluorescence exceeds that of the baseline.

5.2.11. Statistical analysis

Experimental results are presented as means ± standard error of the mean (SEM). Most statistical analyses were conducted using SigmaStat™ 3.1 software (SPSS Corp., Chicago, IL, USA) with the exception of non-parametric analyses which were performed using S-PLUS™ 8.0 (Insightful Corp., USA). As appropriate, statistical significance was tested with t-test, one or two-way ANOVAs followed by Bonferroni multiple comparison tests. A value of at least $P < 0.05$ was considered significant.

5.3. Results

A linear relationship ($R^2 = 0.982$, $P < 0.001$) exists between increasing head kidney cell density and cortisol production (Fig. 5.1A). Subsequent drug exposure experiments were performed with 50 million cells per ml. A one-way ANOVA revealed a significant increase in cortisol secretion ($P < 0.001$) after a minimum of 30 min of incubation (Fig. 5.1B) but the analysis of percent increase compared to basal levels revealed time to reach maximum cortisol secretion to be 60 min (data not shown). A dose-response curve of cortisol secretion against increasing ACTH concentrations resulted in a significant increase compared to control ($P < 0.001$) at 0.005 IU/ml with a plateau after 0.01 IU/ml (Fig. 5.1C). For comparison with previously published studies, the concentration of 1 IU ACTH/ml was chosen for the drug exposure experiments (Leblond and Hontela, 2001).

The effects of increasing concentrations of fibrate drugs on ACTH-induced cortisol secretion and cell viability of head kidney cells are illustrated in Figure 5.2. All drugs were dissolved in DMSO and the final DMSO concentration was adjusted to 0.3%, a concentration at which it has no effect on cell viability and cortisol (one-way ANOVAs, $P = 0.63$ and 0.31, respectively; data not shown). Cortisol secretion and cell viability are reported as percent of average ACTH-induced secretion in the absence of drug. A concentration of 1 IU ACTH/ml significantly increased basal cortisol secretion in head kidney cells as demonstrated by optimization results ($P < 0.05$) (data not shown). Exposure to increasing concentrations of BEZA and GEM did not affect ACTH-induced cortisol secretion ($P = 0.09$ and 0.96, respectively) or cell survival ($P = 0.98$ and 0.94, respectively) (Fig. 5.2A and D). ACTH-induced cortisol secretion was significantly reduced by 79% when exposed to 400 μM CLO ($P < 0.001$) without modifying cell viability ($P = 0.112$) (Fig. 5.2B). FENO also

significantly reduced ACTH-induced cortisol secretion by 47% and 81% at 40 and 400 μM ($p<0.001$), respectively without affecting cell viability (P = 0.09) (Fig. 5.2C). The experiments were also repeated using a lower dose of ACTH and similar effects were observed (data not shown).

Different inducers and precursors were used to identify step(s) potentially impaired by the fibrate drugs within the steroidogenic pathway leading to cortisol. Head kidney cells were incubated in the presence of increasing concentrations of ACTH (Fig. 5.1C), dbcAMP (Fig. 5.3A), pregnenolone (Fig. 5.3B), progesterone (Fig. 5.3C), 17α-hydroxyprogesterone (Fig. 5.3D) and 11-deoxycortisol (Fig. 5.3E) to establish the individual dose-response relationship for each compound. The selected concentrations of inducers and precursors for cortisol secretion in presence of fibrate drugs were 1 IU/ml for ACTH, 4 mM for dbcAMP, 5 μM for pregnenolone, 10 μM for progesterone, 5 μM for 17α-hydroxyprogesterone and 1 μM 11-deoxycortisol.

Results of induced cortisol secretion following exposure to the different inducers and precursors in the presence of 400 μM of each fibrate drug are presented in figure 5.4. Both cortisol secretion (vertical bars) and cell viability (symbols and lines) are expressed as a percent of the control (DMSO). Cell survival (P = 0.46) and cortisol secretion (P = 0.89) induced by any of the compounds were not affected by BEZA exposure (Fig. 5.4A). On the other hand, 400 μM CLO reduced by 6% cell viability ($P < 0.01$) and significantly affected cortisol secretion (P = 0.03). Induced-cortisol secretion with ACTH, dbcAMP, pregnenolone and progesterone in the presence of CLO was significantly depressed by 78, 80, 67 and 57%, respectively, compared with the induction in the absence of the fibrate drug (Fig. 5.4B). Four hundred μM FENO also affected both cell viability ($P < 0.01$) and induced cortisol

secretion ($P < 0.01$). Cell viability was reduced by 10, 13 and 7% in cells exposed to dbcAMP, pregnenolone and 11-deoxycortisol, respectively. Induced cortisol secretion was impaired by 85, 85, 90, 83 and 49% in cells stimulated with ACTH, dbcAMP, pregnenolone, progesterone and 11-deoxycortisol, respectively (Fig. 5.4C). Finally, GEM exposure affected neither cell viability ($P = 0.65$) nor cortisol secretion ($P = 0.88$) for any of the compounds tested (Fig. 5.4D).

The effect of fibrate drug exposure on CYP mRNA expression in head kidney cells is presented on figure 5.5. There were no effects of BEZA, CLO, FENO or GEM on CYP17 ($P = 0.96$), CYP21 ($P = 0.20$) or CYP11 ($P = 0.10$) mRNA levels.

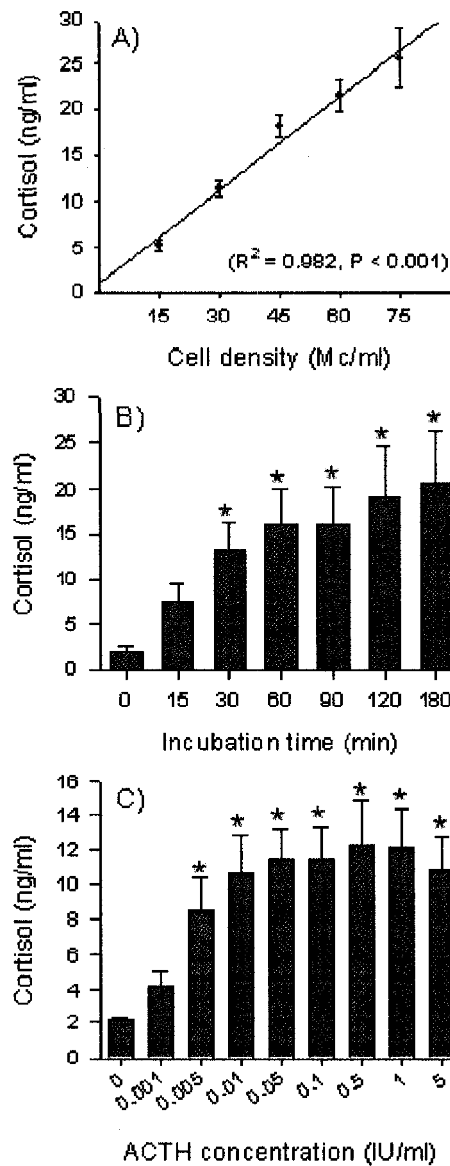

Figure 5.1. Optimization for (A) cell density, (B) incubation time and (C) ACTH concentration in the head kidney cell cortisol stimulation experiments. (A) Relationship between increasing concentrations of cells stimulated with 1 IU/ml ACTH for 60 min. Experiments with fibrate exposure were undertaken with 150 µl of 50 million cells/ml based on these results. (B) Cortisol secretion at increasing incubation periods. Cells were incubated at 75 million cells/ml and stimulated with 1 IU/ml ACTH. Subsequent experiments were incubated for 60 min. (C) Cortisol secretion with increasing ACTH concentrations. One hundred fifty µl of cells were plated at 75 million cells/ml and incubated for 60 min. Subsequent experiments were stimulated with 1 IU ACTH/ml. Data represent means ± SEM (n = 7). Asterisks (*) indicate statistical difference compared to the control detected with a one-way ANOVA followed by a Bonferroni test ($P < 0.001$).

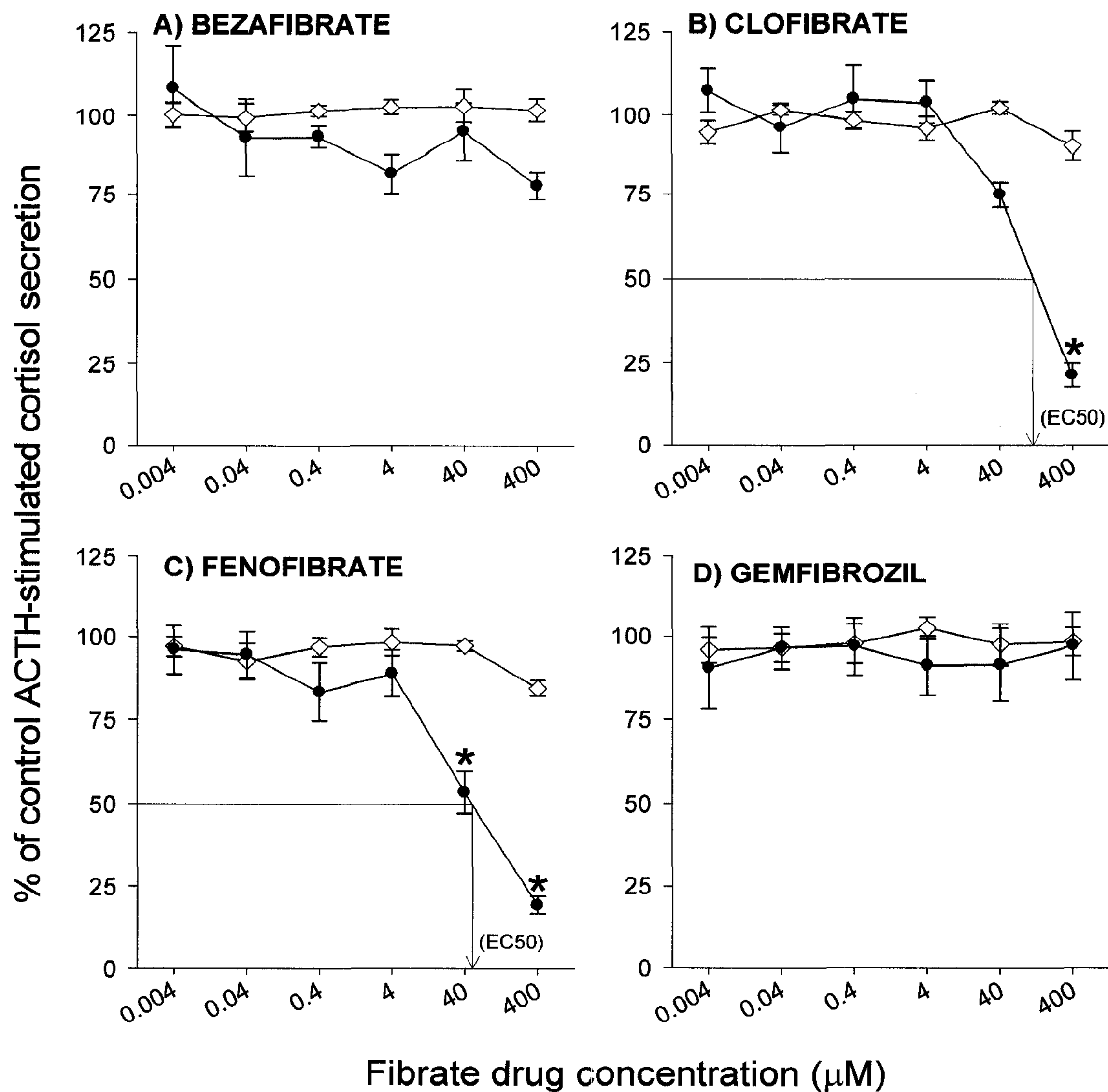

Figure 5.2. Cortisol secretion (closed circles) and cell viability (opened rhombus) in dispersed trout head kidney cells exposed to increasing concentrations of A) BEZA, B) CLO, C) FENO and D) GEM. Cells (50 million cells/ml) were stimulated with 1 IU ACTH/ml for 60 min. Data (means ± SEM, n = 5 to 6) are expressed as percent secretion compared to cells in the absence of drugs. Cell viability was determined through LDH enzyme activity. The noted EC50 value represents an interpolation of the data presented in the figures. Statistical differences, relative to control (no drug), were detected using one-way ANOVAs followed by Bonferroni tests and are indicated by an asterisk (*).

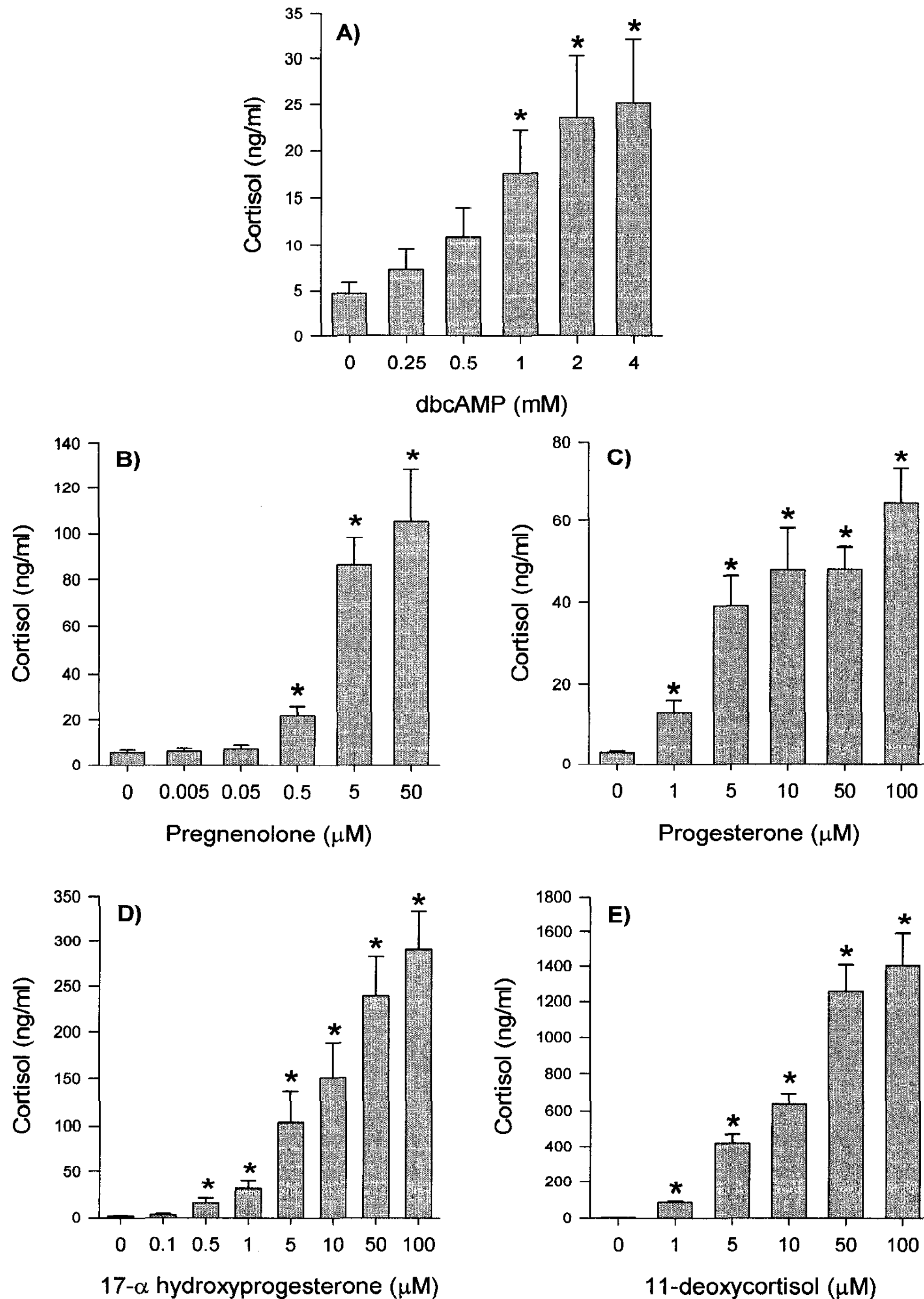

Figure 5.3. Cortisol secretion by dispersed trout head kidney cells (50 million cells/ml) stimulated with increasing concentrations of the inducer A) dbcAMP and the steroids used as precursors B) pregnenolone, C) progesterone, D) 17α- hydroxyprogesterone and E) 11-deoxycortisol. Cells were incubated for 60 min at 13 °C. Data represent means + SEM (*n* = 5 to 7). Asterisks (*) indicate significant differences from control detected using one-way ANOVA followed by a Bonferroni.

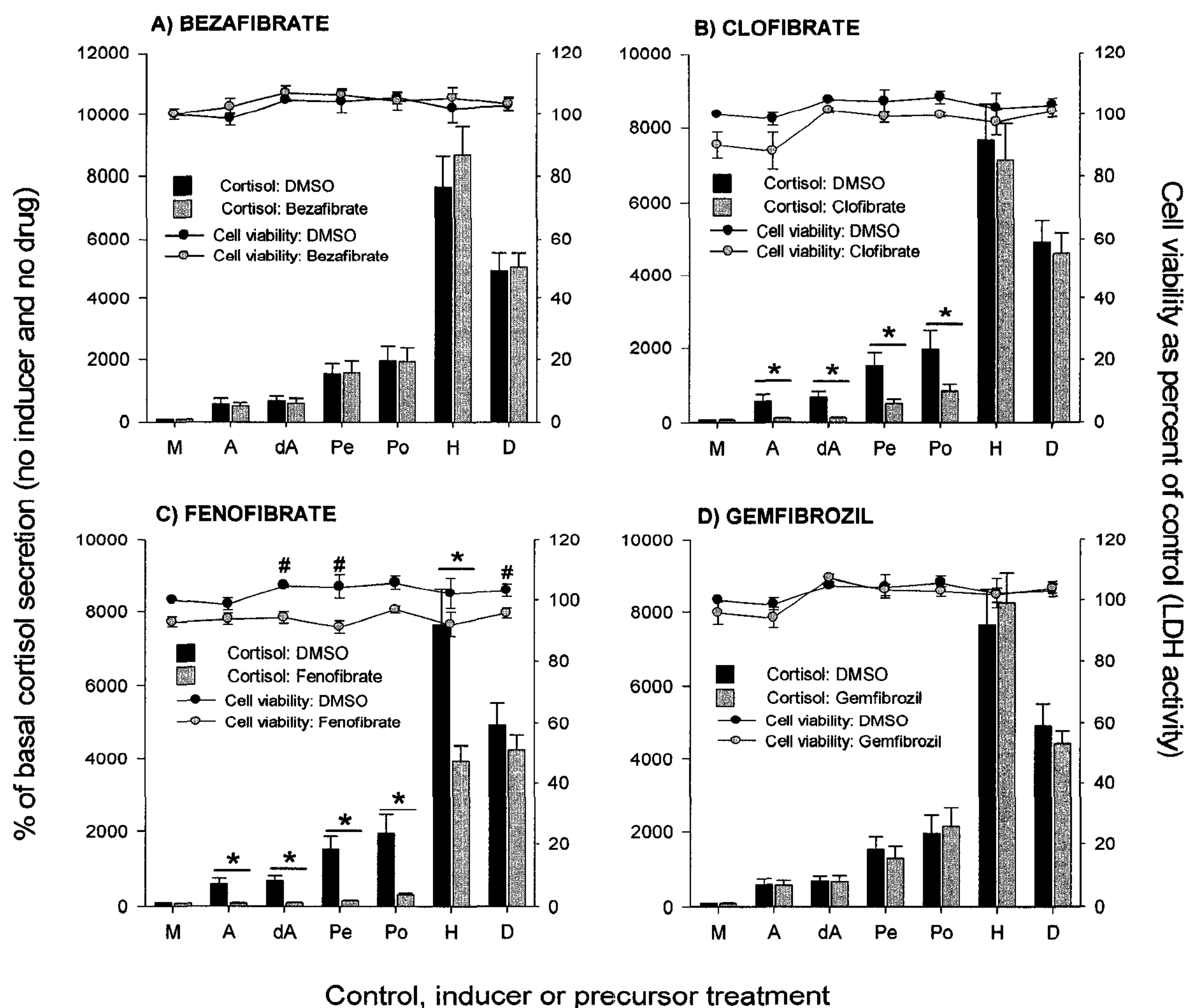

Figure 5.4. Cortisol secretion (vertical bars) and cell viability (circles) in dispersed trout head kidney cells exposed to medium (M, MEM), inducers (A, ACTH; dA, dbcAMP) or precursors (Pe, pregnenolone; Po, progesterone; H, 17-OH-progesterone; D, 11-deoxycortisol) and to different fibrate drugs A) BEZA, B) CLO, C) FENO and D) GEM. Data (means ± SEM, $n = 5$ to 6) are expressed as percent secretion compared with cells in the absence of drugs (black bars). Cell viability was determined with LDH enzyme activity. Statistical differences relative to control (no drug), were detected with non-parametric two-way ANOVAs followed by Bonferroni tests. Asterisks (*) for cortisol secretion and number sign (#) for cell viability indicate significant difference from control.

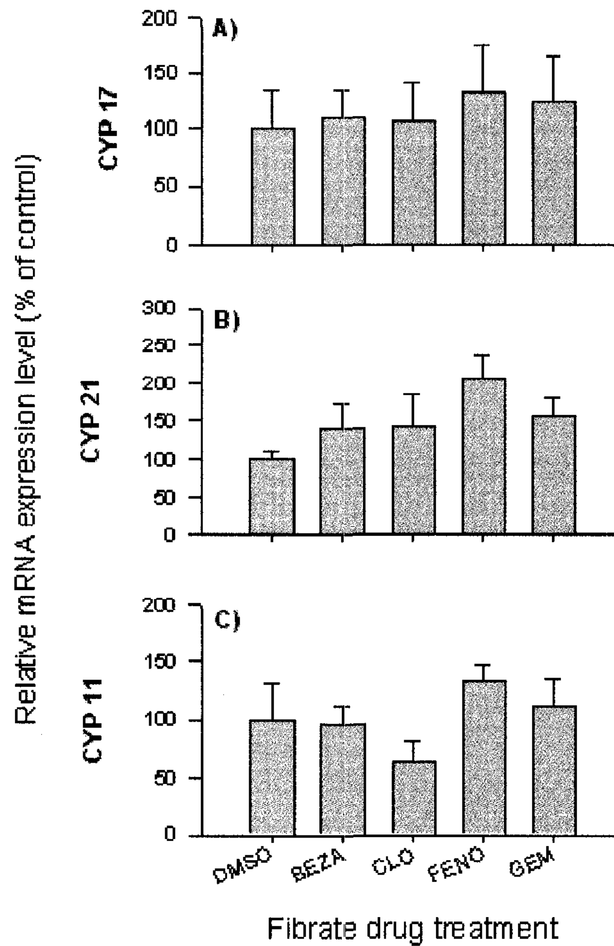

Figure 5.5. Relative mRNA expression levels expressed as percent of control for A) CYP17, B) CYP21 and C) CYP11 in trout head kidney cells following exposure to different fibrate drugs at 400 μM. Data represent means + SEM (n = 4). One-way ANOVA failed to detect any statistical differences among treatments.

5.4. Discussion

Results from this *in-vitro* study demonstrate for the first time that the human fibrate drugs CLO and FENO can compromise the normal stress response in rainbow trout. Cell density, incubation time and ACTH concentration were optimized for cortisol secretion prior to any toxicity testing. As reported by Leblond et al. (2001), cell density between 15 and 75 million cells/ml did not limit cortisol secretion and therefore density was adjusted to 50 million cells/ml to reduce the number of fish used for these experiments.

Two of the four selected fibrate drugs disrupted the stimulated interrenal endocrine response. Approximate effective concentrations that inhibited 50% (EC50) of ACTH induced-cortisol secretion were interpolated to be 200 μM and 80 μM for CLO and FENO, respectively. The observed impaired cortisol response is attributed to a loss of endocrine response rather than to cytotoxicity as cell viability was unaffected at these drug concentrations. CLO and FENO are therefore more potent at inhibiting ACTH-induced cortisol secretion than the anti-inflammatory and analgesic drugs salicylate, ibuprofen and acetaminophen which significantly affected *in-vitro* interrenal steroidogenesis with EC50 values all greater than 1 mM in rainbow trout (Gravel and Vijayan, 2006).

The next step was to identify the targets of CLO and FENO action in the head kidney cortisol response. The chosen approach to identify the compromised step(s) was to stimulate cortisol secretion with various inducers or precursors involved in cortisol synthesis (Bisson and Hontela, 2002). As with ACTH-induced cortisol secretion, 400 μM of CLO and FENO inhibited cortisol secretion stimulated by these inducers and precursors involved in cortisol biosynthesis. The observed decrease in cortisol secretion is attributed to an impaired steroidogenic pathway rather than to cytotoxicity as cell viability only decreased marginally

by 6% and 9% for CLO and FENO, respectively while the cortisol response was inhibited by 58 to 80% by CLO and 84 to 90% by FENO. In addition, endocrine disruption rather than cytotoxicity is further supported by the fact that the interpolated EC50s for both CLO and FENO are much lower than the expected LC50 based on results illustrated in Figure 5.2.

The identification of the target step of a fibrate drug within corticosteroidogenesis is based on the concept that an impaired response due to a fibrate drug is most likely due to an effect downstream from the compound used to induce cortisol secretion. Conversely, when the cortisol response is restored in the presence of the fibrate drug, the target step is most likely located upstream from the compound used to induce cortisol secretion (Bisson et al., 2002). As CLO compromised induced-cortisol secretion in the presence of ACTH, dbcAMP, pregnenolone and progesterone but not with 17α-hydroprogesterone and 11-deoxycortisol, the potential target step is the conversion of progesterone into 17α-hydroprogesterone which is catalyzed by the CYP17 enzyme. Similarly, FENO impaired ACTH, dbcAMP, pregnenolone and progesterone and 17α-hydroprogesterone-induced cortisol production but not secretion induced with 11-deoxycortisol. Thus, the potential target step of FENO in the cortisol biosynthesis pathway would most likely be between 17α-hydroprogesterone and 11-deoxycortisol, a step catalyzed by the CYP21A2 enzyme.

To determine if the effects of CLO and FENO resulted from potential effects on transcription, mRNA levels for CYP17 and CYP21A2 enzymes were assessed. CYP11 mRNA levels were assessed as a negative control since induction of cortisol with 11-deoxycortisol was not impaired and this enzyme has also been shown not to be a limiting factor in the normal production of cortisol in rainbow trout (Aluru and Vijayan, 2006). Relative mRNA quantification demonstrated that none of the fibrate drugs affected mRNA

levels of the three enzymes. Therefore, our current data suggest that inhibition of cortisol production does not involve changes in the steady-state mRNA levels of enzymes downstream from pregnenolone in the interrenal steroidogenic pathway of trout. This finding does not exclude potential post-transcriptional or translational effects on these enzymes and further work is required to elucidate the intracellular mode of action of CLO and FENO on CYP17 and CYP21A2 enzymes, respectively in interrenal cells. *In-vivo* investigation could also reveal if other sites along the HPI axis may be affected by CLO and FENO.

The explanation for the diverse abilities to inhibit induced-cortisol secretion by the different fibrate drugs remains unknown at this point. All four fibrates are reported to be ligands of the mammalian peroxisome proliferator activated-receptor alpha (PPARα) but can also bind to PPARβ and γ (Desvergne and Wahli, 1999) and GEM was demonstrated to affect PPARβ in goldfish (Mimeault et al., 2006) and PPARγ in rainbow trout (Chapter 4). Further work will be needed to elucidate if the relative affinities of the different trout PPARs for the fibrate drugs play a role in their mode of action.

The *in-vivo* significance and the environmental relevance of these findings need to be addressed. The lowest concentration used in this study (0.004 μM) corresponds to the levels of GEM detected in Canadian surface waters (1 μg/L) (Daughton and Ternes, 1999; Kolpin et al., 2002; Metcalfe et al., 2003a; 2003b). The high concentrations of the interpolated EC50s for CLO (200 μM) and FENO (80 μM) are theoretically possible as GEM was detected in goldfish plasma at concentrations as high as 400 μM when exposed to a high waterborne GEM concentration unlikely to occur in the environment (100,000 μg/L) (Mimeault et al., 2005). Nevertheless, GEM was also reported to bioconcentrate by a factor up to 500 when

exposed to environmentally relevant concentration (Mimeault et al., 2005). Assuming similar bioconcentration patterns for all fibrate drugs in rainbow trout, a waterborne concentration of 0.40 μM CLO (97 μg/L) could achieve the 200 μM EC50 concentration in the blood; however, the maximum environmental concentration so far reported for CLO was from a sewage treatment plant effluent and only reached 0.8 μg/L (Andreozzi et al., 2003). Similarly, a FENO water concentration of 0.16 μM (58 μg/L) would be sufficient to bioconcentrate FENO in blood up to 80 μM but the maximum concentration reported in the environment for FENO is 1.4 μg/L in surface water (Pederson et al., 2005). Thus, environmental levels of both CLO and FENO are well below the estimated levels that could bioconcentrate in the plasma to achieve the EC50s reported here. Current results from this study therefore suggest that the endocrine disrupting properties of CLO and FENO in interrenal cells may not occur in the environment. Nevertheless, considering the short exposure period and the rapid action of CLO and FENO on corticosteroidogenesis, further research is required to assess the *in-vivo* effects of long-term exposure of the fibrate drugs on the stress response.

5.5. Conclusions

The two human pharmaceuticals CLO and FENO impair induced-cortisol secretion in the rainbow trout *in-vitro* head kidney preparation. Potential target steps within corticosteroidogenesis were identified to be the steps catalyzed by CYP17 and/or CYP21A2 enzymes, suggesting for the first time, steps downstream from pregnenolone as potential target steps for the impaired stress response. Further research is required in order to

determine the environmental relevance and the *in-vivo* effects of CLO and FENO on cortisol secretion.

CHAPTER 6

General conclusions and perspectives

The overall objective of this thesis was to examine the potential effects of human pharmaceuticals found in the aquatic environment on non-target fish species. To do so, non-traditional endpoints were used as environmental concentrations of human drugs are much lower than the pharmacological concentrations eliciting physiological or traditional toxicological effects. Fibrate drugs, principally gemfibrozil (GEM) but also bezafibrate (BEZA), clofibrate (CLO) and fenofibrate (FENO), were selected based on their occurrence and apparent environmental persistence to assess their effects in the goldfish, *Carassius auratus* and/or the rainbow trout, *Oncorhynchus mykiss*.

The research presented in this thesis was designed to test the hypotheses that human drugs are taken up by fish from the aquatic medium and that exposure to fibrate drugs lead to unfavourable effects in non-target species. The effects of GEM on PPAR mRNA levels were investigated as potential biomarkers of exposure as fibrates bind these nuclear receptors which are responsible for their mode of action. In addition, the tissue distribution of the different PPAR isotypes was established in both fish species for receptor localization characterisation. To facilitate the overall interpretation of results reported in this thesis, environmental concentrations for BEZA, CLO, FENO and GEM, measured BCFs, and concentrations at which effects were observed in the experiments are plotted together (Fig. 6.1).

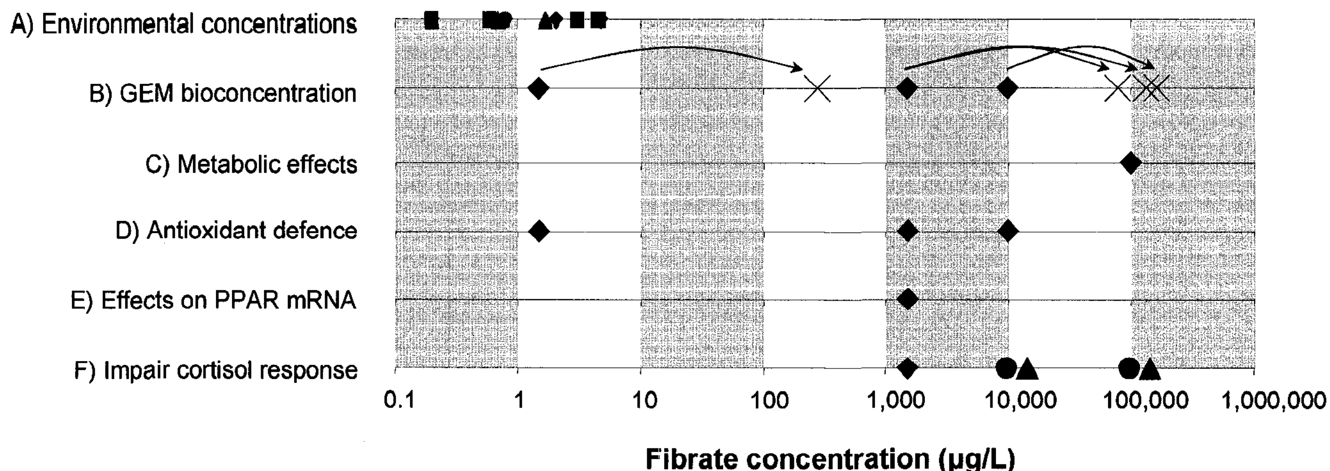

Figure 6.1. Overall perspective of statistically significant effects of fibrate drugs in goldfish and rainbow trout. The different endpoints are listed on the left of the figure against the log of the fibrate concentration at which fish were exposed. Different drugs are represented by different symbols (squares for BEZA, circles for CLO, triangles for FENO and rhombus for GEM) and X represent plasma concentrations as opposed other symbols which represent exposure.

The ability of the fibrate drug GEM to bioconcentrate in goldfish plasma demonstrated that GEM is taken up by fish from the aquatic environment and suggested that it is bioavailable in aquatic systems (Chapter 2; Mimeault et al. 2005). A later field study confirmed the bioavailability of GEM and reported bioconcentration factors (BCFs) comparable to those presented in this thesis (Brown et al., 2007). However, BCFs for a given drug vary with environmental conditions and factors responsible are not fully understood (Brown et al., 2007). Assessing the bioconcentration potential of human pharmaceuticals under laboratory conditions was the initial step in determining their potential toxicological

impact and further research should aim to quantify the bioavailability and bioaccumulation of human drugs in fish species under field conditions. Variables such as sorbtion to organic matter may introduce an additional exposure route that would contribute to the bioaccumulation of human drugs in fish (Maskaoui et al., 2007). Preliminary diet experiments, not included in this thesis, suggested that GEM is also taken up through the digestive track in fish as in human. The concurrent demonstration of the bioavailability and bioconcentration of different human drugs calls for more attention to their potential effects and must be considered in risk assessment as their low environmental concentrations are not indicative of their potential toxicity (Fig. 6.1.A and B). In addition, environmental factors such as temperature may also increase the significance of human drug bioconcentration as uptake rates of contaminants have been shown to vary with temperature in rainbow trout (Sijm et al., 1993).

The work presented in this thesis also provides evidence that *in-vivo* exposure to GEM can lead to unfavorable effects in fish. First, the bioactivity of GEM in goldfish was established by demonstrating that a pharmacological dose led to the same therapeutic effects in fish as in humans within 7 days of exposure (Fig. 6.1.C) (Appendix B). Such a response was expected knowing that fish and mammals share many of the same enzyme and receptor systems (Huggett et al., 2003). Nevertheless, while it provides evidence of physiological effects of human drugs in fish species, it remains irrelevant to the wildlife and the Canadian aquatic system due to the dose and the exposure route used which are both unlikely to occur in nature. The environmental relevance of this work was first addressed by exposing fish to waterborne GEM rather than injections. As no previous experiments had been done on GEM at the onset of these studies, very high waterborne concentrations were initially used. The

effects of GEM on the antioxidant defense system were studied as this system is essential to resist oxidative stress damage on lipid, proteins and DNA and to maintain the integrity and function of cells (Dorval et al., 2003; Bagnyukova et al., 2005). Exposure of goldfish to 1,500 and 10,000 μg GEM/L for 4 days only resulted in a modest antioxidant response and did not lead to oxidative stress, at least in the form of lipid peroxidation (Appendix C). To make these studies more environmentally-relevant a subsequent experiment exposed goldfish to an environmentally relevant concentration of 1.5 μg GEM/L for an exposure period of 14 and 28 days (Fig. 6.1.D) (Chapter 3; Mimeault et al. 2006). Results from this study provided evidence that exposure to this and a supra-environmental (1,500 μg/L) waterborne levels of GEM challenged the hepatic oxidative status in goldfish without leading to oxidative damage in the form of lipid peroxidation. Conversely, in rainbow trout, exposure for 1, 7 and 14 days to 1,500 μg GEM/L did not affect the hepatic antioxidant defense status (Chapter 4). The different responses of the antioxidant defense system to GEM exposure between goldfish and trout provide evidence that fish species as mammalian species respond differently to PP; at this time what is responsible for these differences are unknown although gender and levels of PPAR may be involved as in mammals (Amacher et al., 1997; Holden and Tugwood, 1999; Lambe et al., 1999). The mode of action of GEM on the antioxidant defense system in goldfish remains to be identified as no evidence of peroxisome proliferation was demonstrated. Further research should assess the ability of an environmental concentration of GEM to induce peroxisome proliferation in goldfish at the onset of exposure. In addition, the role of the cytochrome P-450 4A family known to generate ROS in mammals, should be further examined in fish (O'Brien et al., 2005).

In an attempt to identify a nuclear biomarker of GEM exposure, levels of PPARα mRNA were determined. Results from this thesis demonstrated that exposure to GEM does not regulate the expression of PPARα mRNA levels in either goldfish or rainbow trout, under different exposure regimes, different concentrations and different durations (Chapter 3, Chapter 4 and Appendix D). As GEM does not mediate changes in PPARα mRNA in these two fish species, the monitoring of hepatic PPAR mRNA levels is not a good toxicological biomarker of GEM exposure in either freshwater fish species studied. However, an unexpected modulation of similar amplitude of PPARβ mRNA levels was observed in goldfish and rainbow trout exposed to high concentration of GEM (1,500 μg/L) for 14 days (Fig. 6.1.E). In addition, preliminary experiments not included in this thesis, using rainbow trout head kidney cells exposed to FENO also demonstrated a reduction of PPARβ mRNA levels after a 2 h drug exposure. Taken together, the consistent reductions in PPARβ mRNA levels in different fish species, in different cell types and using different fibrate drugs lead to the conclusion that the monitoring of PPARβ, rather than PPARα, mRNA may be an appropriate indicator of fibrate drug exposure. Finally, the reported reduction in PPARγ mRNA levels after 7 days of exposure in rainbow trout appears to be transient as levels recovered to control after 14 days. Whether this modulation is species specific, only a function of the high GEM concentration used, or PPARγ mRNA is modulated at the onset of GEM exposure and followed by PPARβ mRNA variations, needs to be addressed in future research.

Investigations of the effects of GEM on PPARs prompted an analysis of their tissue distribution in both rainbow trout and goldfish. All three PPAR mRNAs were detected in all tested tissues in both species. Contrarily to mammals and marine fish species, PPARα

mRNA was not predominantly expressed in liver which may partially explain the lack of effect of GEM on PPARα mRNA levels. The ubiquitous expression of PPARβ in all tissues was consistent to that previously reported for this subtype in other vertebrate species (Desvergne and Wahli, 1999). Finally, PPARγ has the most distinct distribution but even in this case remains relatively less restricted in fish than in mammals (Braissant et al., 1996; Leaver et al., 2005).

A series of *in-vivo* and *in-vitro* experiments were also designed to assess the effects of GEM on the stress axis. The ACTH-induced stress response in intact goldfish was not affected after 14 or 28 days of waterborne GEM exposure (1,500 μg/L) (Appendix E). Experiments with rainbow trout resulted in highly variable and thus difficult to interpret response (Appendix F) and prompted the use of the *in-vitro* head kidney cell experiments to reduce the stress induced by handling (Chapter 5). The endocrine disrupting properties of some human fibrate drugs were demonstrated by the ability of high doses of CLO and FENO, as opposed to BEZA and GEM, to compromise the ACTH-induced cortisol response in rainbow trout head kidney cells (Fig. 6.1.F).

At this stage, what seems to be the main concern for aquatic species exposed to human pharmaceuticals are effects on the antioxidant defense system being the only endpoint impaired at an environmental concentration (Fig. 6.1.). This suggests that the antioxidant defense system is a sensitive function and other species could be affected at environmental exposure. Injections of GEM and FENO in the bivalve mollusk, *Mytilus galloprovincialis*, were shown to increase catalase activities and to challenge the antioxidant defense system at concentrations slightly higher that the ones reported in the aquatic system (Canesi et al.,

2007). In addition, the antioxidant defense system of the crustacean *Artemia parthenogenetica* is challenged by a 48 h exposure to high concentrations of clofibrate but does not result in oxidative damage in the form of lipid peroxidation. Finally, FENO and CLO were also shown to increase basal ROS production in rainbow trout hepatocytes (Laville et al., 2004). All together, evidence demonstrates that fibrate drugs have the ability to induce ROS and/or the antioxidant defense system in aquatic species over a broad range of concentrations and should therefore be considered an important parameter to assess the effects of fibrate drugs on non-target aquatic species.

Although Figure 6.1 seems to indicate that endocrine disruption is not a concern for human pharmaceuticals at environmental concentrations, several factors should be considered before reaching such as conclusion. First, longer exposures are required to determine the potential of fibrate drugs to disrupt corticosteroidogenesis over time. Second, the effect of *in-vivo* exposure to these fibrate drugs on *in-vitro* ACTH-stimulated cortisol response should be determined. And finally, other studies have already established the potency of environmental concentrations of human pharmaceuticals to act as an endocrine disruptors. GEM was shown to reduce plasma testosterone concentrations by 49% in goldfish exposed 1.5 μg GEM/L for 14 days, hence raising the potential for GEM to affect reproduction (Mimeault et al. 2005). Similarly, decreased testosterone levels were reported in medaka (*Oryias latipes*) exposed to a beta-blocker, propanolol, albeit at a concentration at the highest end of those reported in the environment (Huggett et al., 2002). Finally, waterborne exposure to 10 μg/L of clofibric acid, a clofibrate metabolite, resulted in a marked reduction of sperm count in fathead minnows (Runnalls et al., 2007). Evidence

therefore demonstrates the endocrine disrupting property of human drugs in non-target aquatic species on two endocrine axes, the stress and the reproductive axes.

The results presented in this thesis demonstrate that both oxidative stress and endocrine parameters are relevant endpoints in the assessment of the effects of human pharmaceutical in the environment. In addition, the comparison of clofibric acid studies further confirms the relevance of non-traditional endpoints. While no effects on mortality was reported in invertebrate and fish species exposed to increasing concentrations of clofibric acid up to 1,000 μg/L (Nunes et al., 2005; Emblidge and Delorenzo, 2006), sperm counts were reduced in fathead minnows exposed to 10 μg/L of clofibric acid (Runnalls et al., 2007).

Another significant contribution of this thesis to the field of assessing the effects of human drugs in aquatic species relies on the identification of novel target sites for human drugs in steroidogenesis. A potential role for StAR had been previously reported to explain testosterone plasma reduction in goldfish, nevertheless, results also suggested that GEM would potentially exerts its effects at more than one step within the steroidogenesis pathway (Mimeault et al., 2005). Impaired cortisol response in rainbow trout head kidney cells exposed to salicylate corresponded to a significant drop in the gene expression of StAR and peripheral-type benzodiazepine receptor, two key regulatory proteins involved at the beginning of the steroid biosynthethic cascade (Gravel and Vijayan, 2006). In this thesis, a mechanistic approach was used to identify potential target steps of CLO and FENO in corticosteroidogenesis. For the first time, steps downstream from P450ssc in corticosteroidogenesis were suggested as potential drug targets. CLO and FENO exposure affected the CYP17 and CYP21A2 enzymes, respectively. The effects CLO and FENO are

thought to be post-transcriptional as the mRNA levels of these potential target enzymes were not affected. Results from this thesis therefore confirmed that fibrate drugs can impact multiple point in steroidogenesis and provided key information on the mechanism(s) through which fibrate drugs impair corticosteroidogenenesis.

The discovery of new contaminants in the environment prompts research to assess their potential toxicological impacts on non-target species. This thesis contributes to the emerging field of pharmaceuticals in the aquatic environment by leading research in the bioconcentration potential of human drugs in aquatic species thereby providing evidence that environmental concentrations are not indicative of their potential toxicity. In addition, the work presented in this thesis demonstrates that exposure to human drugs might challenge or impair essential functions in at least two fish species and highlights the endocrine disrupting properties of fibrate drugs. Future research should focus on assessing effects over longer periods of time and at lower concentrations.

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APPENDIX A

List of publications, presentations and contributions

Peer-reviewed publications:

Mimeault C, Trudeau, VL, and TW Moon. 2006. Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-activated receptor beta (PPARβ) mRNA levels in male goldfish (*Carassius auratus*). Toxicology 228: 140-50.

Mimeault C, Woodhouse AJ, Miao XS, Metcalfe CD, Moon TW, Trudeau VL. 2005. The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, *Carassius auratus*. Aquatic Toxicology 73 (1): 44-54.

Trudeau VL, Metcalfe CD, **Mimeault C**, Moon TW. 2005. Pharmaceuticals in the environment: Drugged fish? Biochemistry and Molecular Biology of Fishes. Vol 6 (Chapter 17): 475-93.

Manuscript in preparation from completed experiments:

Mimeault C, Chen X, Trudeau VL and Moon TW. 2008. In-vitro corticosteroidogenesis is impaired by fibrate drugs in rainbow trout (*Oncorhynchus mykiss*) head kidney cells. *To be submitted to Aquatic Toxicology.*

Oral communications at scientific meetings:

Moon TW, **Mimeault C**, Woodhouse A, Cameron C, Estey C, Bullock L, Mennigen J and Trudeau VL. Lipid and cholesterol lowering drugs and an anti-depressant modulate multiple pathways within steroidogenesis and reproduction in fish. Aquatic Toxicology Workshop (ATW), Halifax, NS, Canada. October 2007.

Trudeau VL, Martyniuk C, Meningen J, Gerry E, Zhang D, Cameron C, Woodhouse M, **Mimeault C**, Moon TW, Ekker M, and Metcalfe C. Pharmaceuticals in the aquatic environment: Drugged fish? 23rd Conference of European Comparative Endocrinologists. Manchetser, UK. August 2006.

Mimeault C, Trudeau VL and Moon TW. The human pharmaceutical gemfibrozil challenges the antioxidant defense system in goldfish (*Carassius auratus*). International Congress on the Biology of Fish (ICBF). St-John's, Newfoundland, Canada. July 2006. Awarded a student travel grant.

Moon TW, **Mimeault C**, Cameron C, Estey C, Marlatt V, and Trudeau VL. Fish swimming in pharmaceuticals – is there a problem? International Congress on the Biology of Fish, St. John's, Newfoundland, Canada. July 2006.

Trudeau VL, Martyniuk CJ, Marlatt V, Werry K, Cahn J, Lui G, Woodhouse M, **Mimeault C**, Moon T, Turque N, Demeneix B. Expression profiling EDC actions in the brains of aquatic vertebrates: approaches and challenges. International Symposium on Environmental Endocrine Disruptors. Nagoya, Japan. December 2004.

Mimeault C, Woodhouse AJ, Leclair J, Metcalfe CD, Trudeau VL and Moon, TW. Swimming in pharmaceuticals: the effects of gemfibrozil in goldfish. Society of Environmental Toxicology and Chemistry (SETAC). Portland, OR, USA. November 2004.

Mimeault C, Trudeau VL, Metcalfe CD and Moon TW. Do pharmaceuticals in the environment get into non-target aquatic species? Aquatic Toxicology Workshop (ATW). Ottawa, ON, Canada. October 2003. Selected in best student presentations.

Metcalfe CD, **Mimeault C**, Moon TW and Trudeau VL. Pharmaceuticals in the aquatic environment - Drugged fish? Envirpharma, European Conference on Human and Veterinarian Pharmaceuticals in the Environment. Lyon, France. April 2003.

Moon TW, Trudeau VL, **Mimeault C**, Woodhouse A, Liu G, Van Der Kraak G and Metcalfe C. The impact of an environmentally relevant fibrate drug on lipid dynamics and steroid production in the goldfish, *Carassius auratus*. CNTC Annual Research Symposium, Ottawa, Ontario, Canada, March 2003.

Moon TW, **Mimeault C**, Trudeau V and Metcalfe CD. Pharmaceuticals as EDCs: the metabolic impact of Gemfibrozil in goldfish. International Congress of the Biology of Fish. Vancouver, British-Columbia, Canada. July 2002.

Mimeault C, Trudeau VL and Moon TW. Effects of gemfibrozil, a pharmaceutical in the Canadian environment, on PPAR expression levels in goldfish. Aquatic Toxicology Workshop (ATW). Whistler, BC, Canada. October 2002.

Mimeault C, Moon TW and Trudeau VL. Piscine evaluation of PPAR expression and oxidative stress following exposure to the peroxisome proliferator gemfibrozil. Canadian Society of Zoologists (CSZ). Sudbury, ON, Canada. May 2001.

Posters at scientific meetings:

Moon TW, Estey C, Bullock L, Woodhouse A, Cameron C, **Mimeault C** and Trudeau VL. Do human pharmaceuticals found in the aquatic environment affect fish species? Annual Aquatic Toxicology (ATW) Workshop, Halifax, NS, Canada. October 2007.

Moon TW, Estey C, Bullock L, Woodhouse A, Cameron C, **Mimeault C** and Trudeau VL. Do human pharmaceuticals found in the aquatic environment affect fish species? Canadian Society of Zoology. McGill University, Montreal, Quebec, Canada. May 2007.

Woodhouse A, **Mimeault C**, Moon TW and Trudeau VL. Pharmaceuticals in Canadian sewage treatment plant effluents: can they lead to reproductive impairment in non-target aquatic species? International Symposium on Fish Endocrinology. Castellón, Spain. September 2004.

Woodhouse A, **Mimeault C**, Moon TW and Trudeau VL. Swimming in pharmaceuticals: Gemfibrozil bioconcentration and testosterone reduction. Laurentian SETAC, Ottawa, Ontario, Canada. June 2004.

Mimeault C, Trudeau VL, Metcalfe CD and Moon TW. Les produits pharmaceutiques se retrouvent-ils dans les poissons? Centre de recherche en toxicologie de l'environnement (TOXEN). Montréal, QUE, Canada. Mars 2003.

Mimeault C, Trudeau VL and Moon TW. Pharmaceuticals in the aquatic environment: Is there a problem? Canadian Network of Toxicology Centres (CNTC). Ottawa, ON, Canada. March 2003.

Mimeault C, Trudeau VL and Moon TW. Pharmaceuticals in the aquatic environment - is there a problem? Canadian Society of Zoology, Lethbridge, Alberta, Canada. May 2002.

Mimeault C, Moon TW, Metcalfe C and Trudeau VL. Effects of the peroxisome proliferator Gemfibrozil in goldfish. TSRI National Conference, Ottawa, Ontario, Canada. March 2002.

Mimeault C, Moon TW, Metcalfe CD and Trudeau VL. Effects of the peroxisome proliferator gemfibrozil in goldfish. Society of Environmental Toxicology and Chemistry (SETAC). Baltimore, MA, USA. November 2001.

Mimeault C, Moon TW and Trudeau VL. Piscine evaluation of PPAR expression and oxidative stress following exposure to the peroxisome proliferator Gemfibrozil. Canadian Society of Zoology. Sudbury, Ontario, Canada. May 2001.

APPENDIX B

Bioactivity of pharmacological dose of GEM in goldfish

Objective: This experiment was a preliminary analysis of the effect of GEM on serum triglyceride and glucose concentrations at a human equivalent dose (100 μg GEM/g of fish) to determine if the reported biological effects in human can also occur in non-target species.

Methods: Male goldfish (24.9 ± 1.0, n = 24) were intraperitoneal (I.P.) injected at day 0, 2, 4 and 6 with saline, DMSO, 10 or 100 μg GEM/g of fish. GEM was initially dissolved in DMSO and injection volumes were 0.5 μl/g fish. On day 7, fish were sacrificed by trans-spinal sectioning after blood sampling. Plasma was separated from red cells by a 10 min centrifugation at 12,000 *g* and aliquots stored at –80 °C until analyzed for triglycerides and glucose. Log-transformed data were analyzed with one-way ANOVA followed by Bonferonni tests for multiple comparisons. A value of $P < 0.05$ was considered significant.

Results: The human equivalent dose (100 μg GEM/g of fish) caused a significant 42% reduction in serum triglyceride (TGs) levels compared to the DMSO control ($P = 0.003$) (Fig. B1). GEM caused serum glucose concentration to increase by 89 and 72% compared with the DMSO control when exposed to 10 and 100 μg GEM/g of fish, respectively ($P < 0.001$) (Fig. B1).

Interpretation: TG levels are the principal target for which humans are prescribed GEM. The observed 42% TG reduction in goldfish is comparable to the 35% reduction reported in humans (Spencer and Barradell, 1996) and the increase in plasma glucose suggests an exposure to a stress. Together, these effects demonstrate the bioactivity of GEM in a non-target aquatic species.

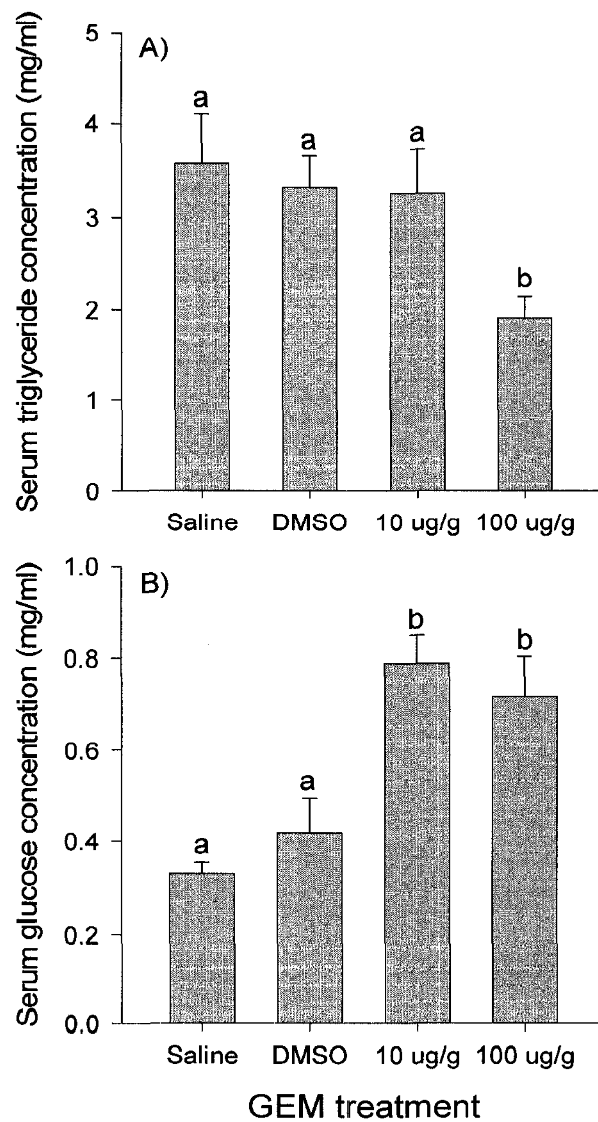

Figure B1. Serum concentrations of A) triglyceride and B) glucose in goldfish exposed to GEM through injections. Data represent mean + SEM (n = 8). Letters denote statistical difference detected with an ANOVA followed by a Bonferroni test.

APPENDIX C

Oxidative stress in goldfish exposed to high doses of waterborne GEM

Objective: To determine the effects of high waterborne GEM concentrations (1,500 and 10,000 μg/L) on hepatic antioxidant defense systems in goldfish.

Methods: Waterborne exposed male goldfish from the exposure route experiment (Chapter 2; Mimeault et al., 2005) were used for these analyses. 96 h after GEM addition to the water, livers were collected, snap frozen in liquid nitrogen and stored at -80 °C until analyses for antioxidant enzymes and TBARS as described in Chapter 3 were undertaken. A value of $P < 0.05$ was considered significant.

Results: Exposure to waterborne GEM for 96 h did not affect GPx ($P = 0.970$), GST ($P = 0.152$) or CAT ($P = 0.698$) activities in goldfish livers but 10,000 μg GEM/L significantly increased GR ($P = 0.020$) activity by 49% (Fig. D1). TBARS were not affected by GEM exposure ($P = 0.093$) (Fig. D2).

Interpretation: Very high exposure to waterborne GEM is required to induce hepatic antioxidant challenge in goldfish liver but oxidative stress is not likely to occur in goldfish liver as TBARS levels were not affected.

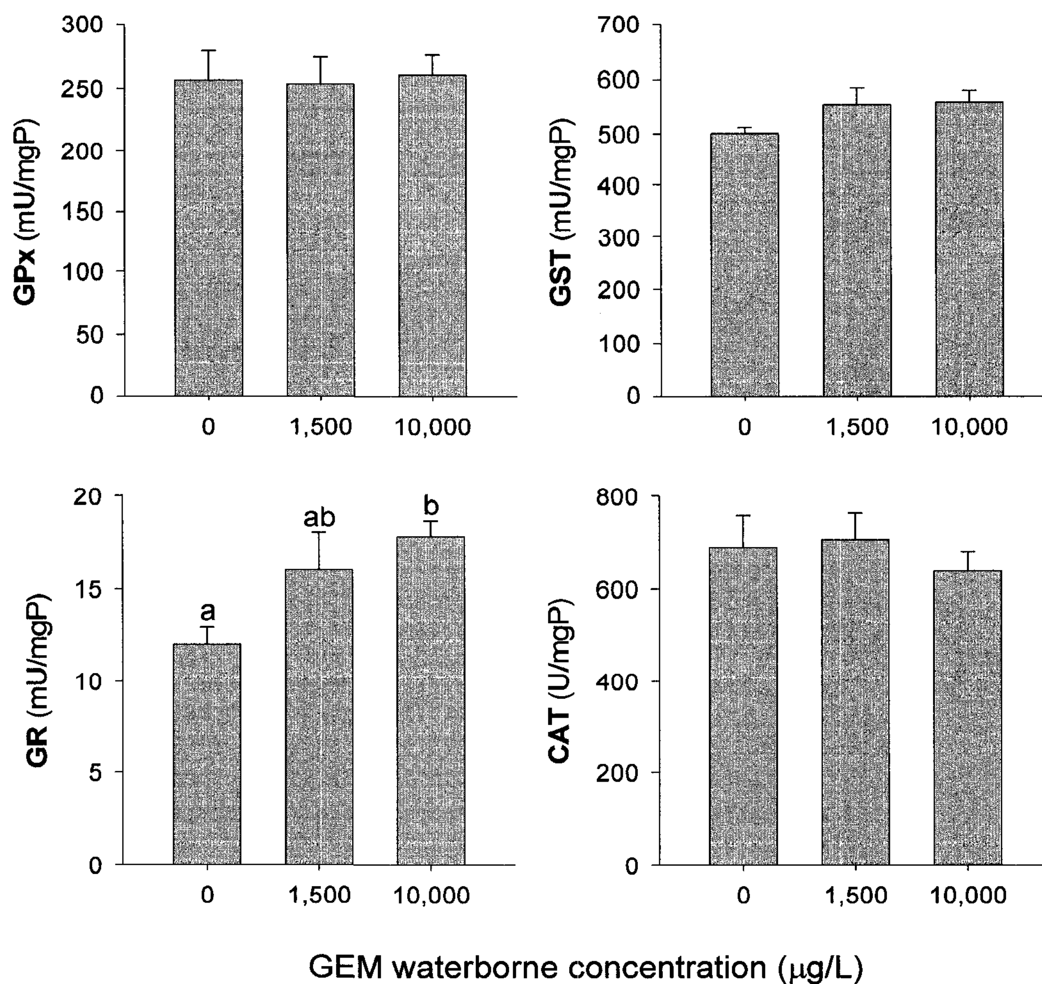

Figure C1. Hepatic antioxidant enzyme specific activities in goldfish following waterborne GEM exposures for 96 h: (A) glutathione peroxidase (GPx), (B) glutathione-S-tranferase (GST), (C) glutathione reductase (GR), and (D) catalase (CAT). Data represent means + S.E.M. (n = 8). One-way ANOVA failed to detect any effect of GEM exposure for GPx (P = 0.970), GST (P = 0.152) and CAT (P = 0.698) but revealed an effect of GEM on GR (P = 0.020). Bonferroni's multiple comparison tests were used to detect differences between means, which are indicated by different letters ($P < 0.05$).

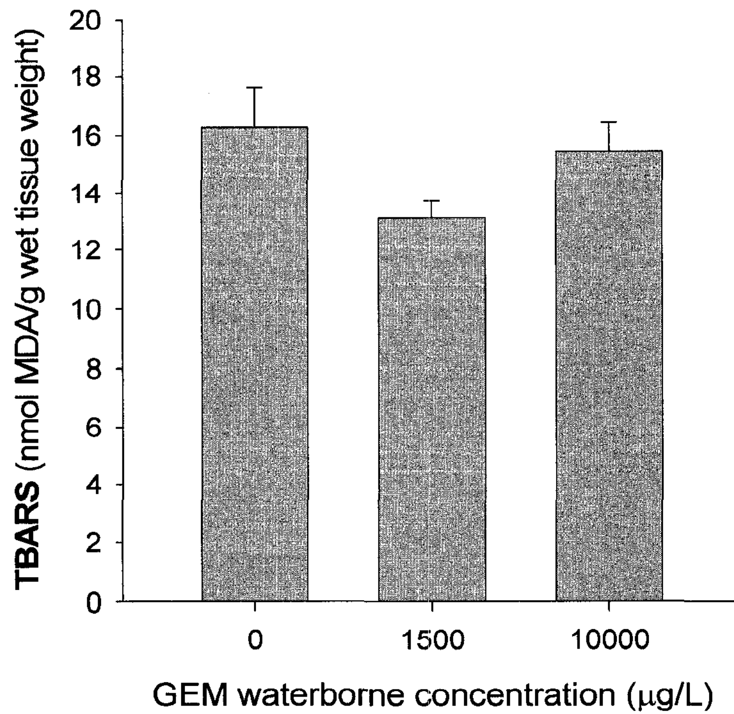

Figure C2. Thiobarbituric acid reactive substances (TBARS) expressed as malonaldehyde levels (nmol MDA/g wet tissue weight) in goldfish livers exposed to waterborne GEM for 96 h. One-way ANOVA failed to detect any effect of GEM on TBARS (P = 0.093). Data represent means + S.E.M. (n = 8).

APPENDIX D

PPARs mRNA levels in GEM-injected and waterborne-exposed goldfish

Objective: To determine the effects of GEM on hepatic PPAR mRNA levels through injections and water exposure.

Methods: I.P. injected and waterborne exposed male goldfish from the exposure route experiment (Chapter 2) were used for these analyses. 96 h after injection or GEM addition to the water, livers were collected, snap frozen in liquid nitrogen and stored at -80 °C until RNA isolation, cDNA synthesis and semi-quantitative mRNA reverse-transcriptase PCR was performed as in Chapter 3. Optimal cycle numbers for PPARα, -β, -γ and β-actin were 31, 32, 30 and 29, respectively. Hybridization temperatures were 60 °C for PPARs and 55 °C for β-actin.

Results: Goldfish hepatic PPARα ($P = 0.888$), PPARβ ($P = 0.282$) and PPARγ ($P = 0.051$) mRNA levels were not affected 96 h after a GEM (10,000 μg GEM/L) injection (Fig. C1). Waterborne exposure (1,500 and 10,000 μg GEM/) did not affect hepatic PPARα ($P = 0.263$), PPARβ ($P = 0.793$) and PPARγ ($P = 0.664$) mRNA levels (Fig. C2).

Interpretation: GEM does not modulate any PPAR mRNA levels, hence their monitoring does not seem to be a good indicator when exposure is of short duration.

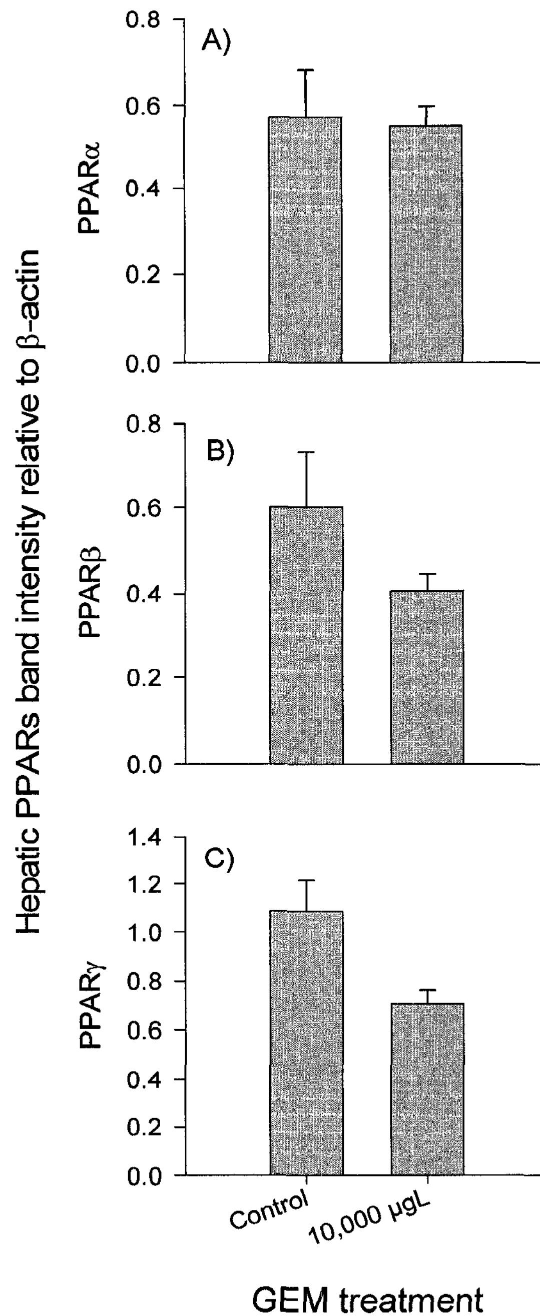

Figure D1. Relative transcript levels of (A) PPARα, (B) PPARβ and (C) PPARγ in goldfish liver exposed to GEM through I.P. injections. Liver samples were collected 96 h after injections. Data represent means + SEM (n = 4 to 6). Student t-tests failed to detect any effect of GEM on PPARα (P = 0.888), PPARβ (P = 0.282) or PPARγ (P = 0.051) mRNA levels.

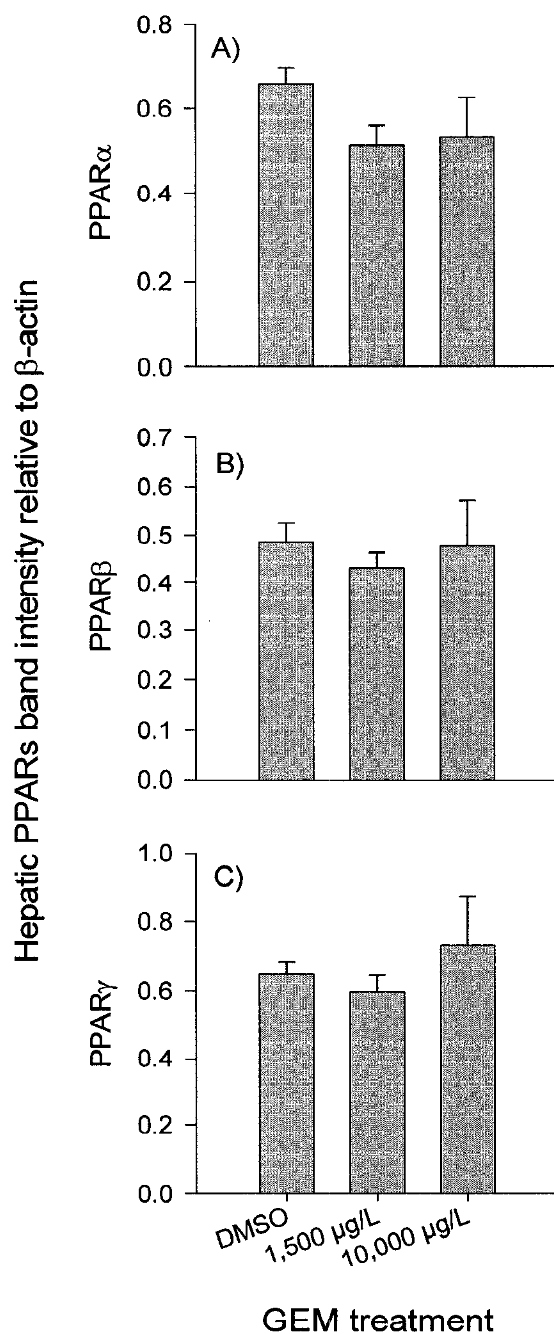

Figure D2. Relative transcript levels of (A) PPARα, (B) PPARβ and (C) PPARγ in goldfish liver exposed to GEM through the water. Liver samples were collected 96 h after GEM was added to the water. Data represent means + SEM (n = 6). One-way ANOVA failed to detect any effect of GEM on PPARα (P = 0.263), PPARβ (P = 0.793) or PPARγ (P = 0.664) mRNA levels.

APPENDIX E

***In-vivo* cortisol response in goldfish exposed to waterborne GEM**

Objective: This experiment was designed to determine the effect of waterborne GEM on the goldfish *in-vivo* cortisol response following saline or ACTH injections.

Methods: Optimal ACTH concentration were determined by stimulating cortisol release in male goldfish injected with increasing ACTH concentrations (0, 2.5, 5, 10, 40 and 60 IU/ml at 100 µl/100 g) and blood sampled one hour after injection. Optimal time lapse to achieve maximum cortisol concentration were determined by injecting male goldfish with 15 IU ACTH/ml and blood sampling at different time points (0, 1, 2 and 4 h) after injection. Males and females goldfish were kept separated for GEM exposure. Goldfish were either exposed to DMSO or waterborne GEM (1,500 µg/L). Water was dosed every 3rd day and fish were feed daily. After 14 and 28 days of exposure, fish were quickly anesthetized (100 mg MS-222/L of water) and injected with either saline or 15 IU ACTH/ml at 100 µl/100 g and returned to the tank. One hour after injection, fish were quickly anesthetized and blood samples collected from the caudal vessels. Plasma was separated from red cells by a 10 min centrifugation at 12,000 *g* and aliquots stored at –20 °C until analyzed. Cortisol was assessed as reported in Chapter 5.

Results: Selected ACTH concentration (15 IU/ml) and time after ACTH injection (1 h) were determined based on preliminary experimental results (Fig. E1). Stimulated cortisol levels with either saline or ACTH injections in male goldfish were not affected by waterborne GEM exposure (P = 0.342) after 14 or 28 days (P = 0.342) (Fig. E2). Stimulated cortisol levels with either saline or ACTH injections in female goldfish were not affected by waterborne GEM exposure (P = 0.440) after 14 or 28 days (P = 0.339) (Fig. E3).

Interpretation: Waterborne exposure to 1,500 µg GEM/L for up to 28 days does not impair *in-vivo* cortisol stress response in goldfish.

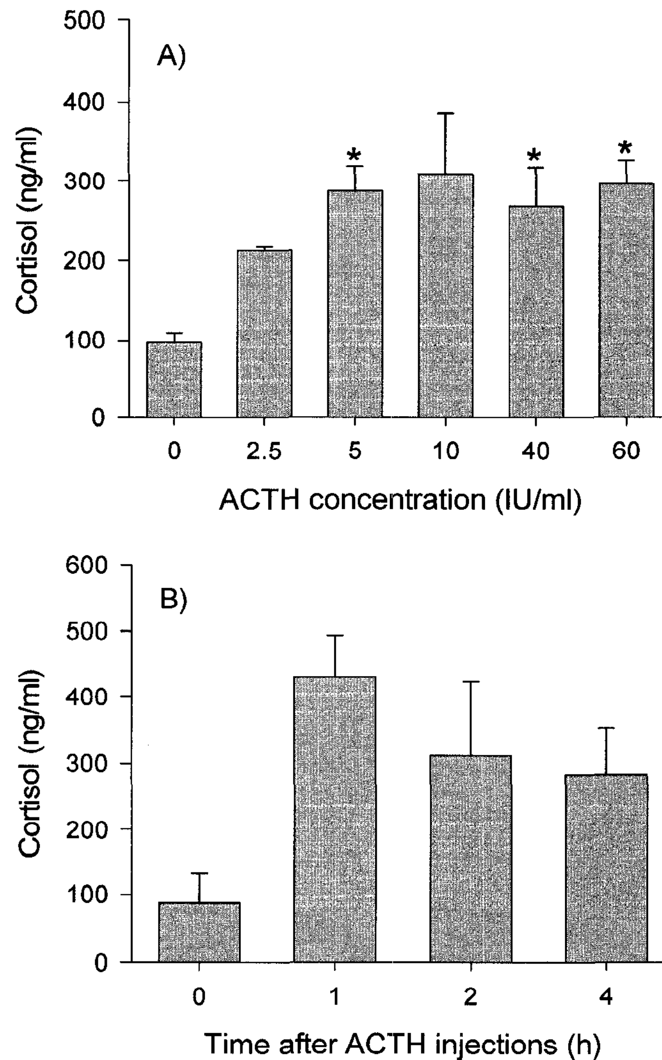

Figure E1. Preliminary experiments to determine A) ACTH concentration and B) time after ACTH injection yielding highest plasma cortisol concentration in goldfish. Data represent means + SEM, n = 3 to 4. One-way ANOVAs revealed significant induction of cortisol with increasing ACTH concentrations (P = 0.028) but failed to detect significant increases in cortisol as a function of time after injection (P = 0.091). Differences detected with a Bonferroni test are indicated by an asterisk (*).

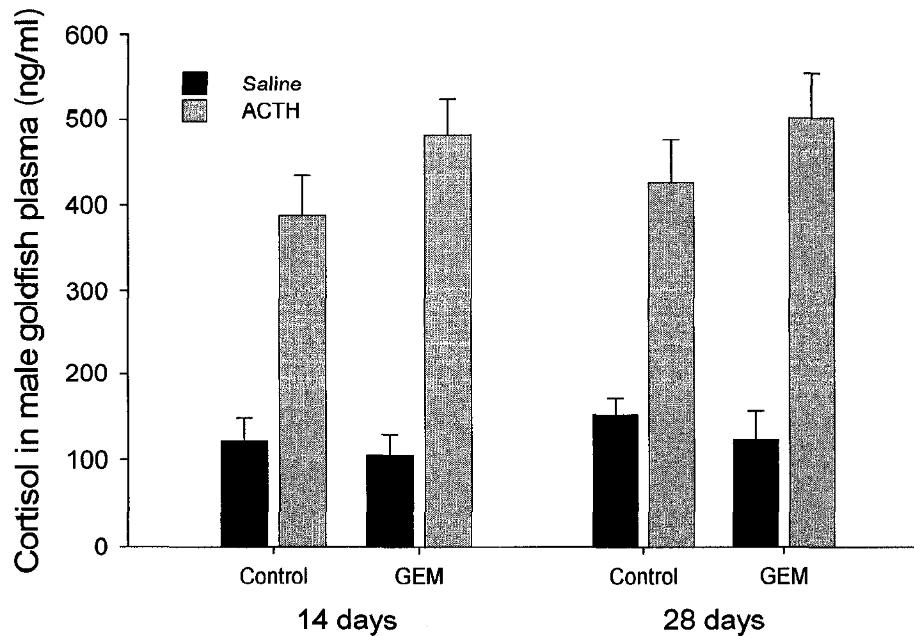

Figure E2. Cortisol levels in male goldfish plasma stimulated either with saline or ACTH (15 IU/ml) injections after 14 and 28 days of exposure to DMSO (Control) or waterborne GEM (1,500 µg/L). Data represent means + SEM, n = 11 to 12. A three-way ANOVA revealed no effect of duration of exposure ($P = 0.342$), presence of drug ($P = 0.274$) or any of the interactions but detected a significant effect of ACTH ($P < 0.001$).

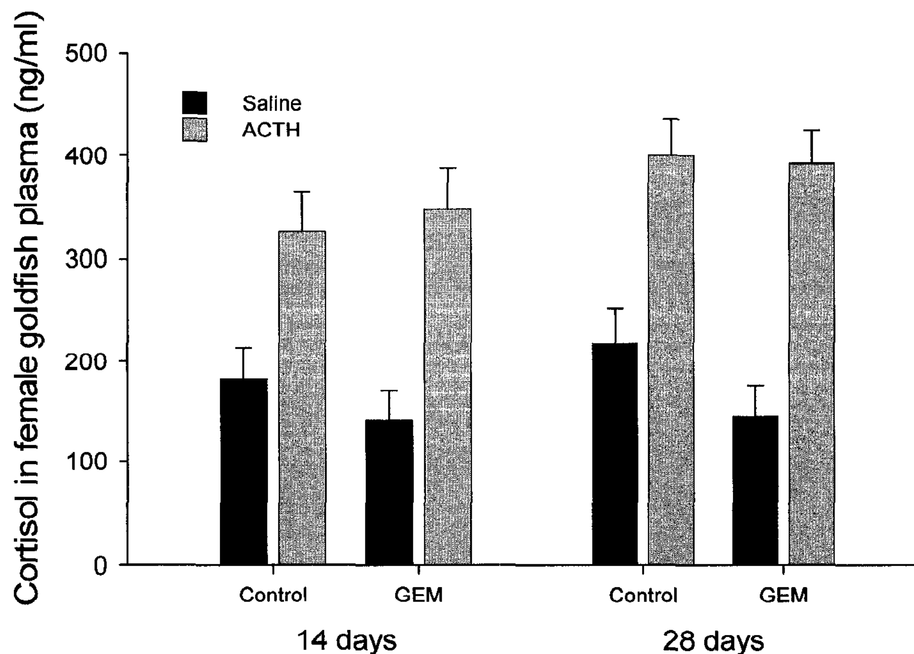

Figure E3. Cortisol levels in female goldfish plasma stimulated either with saline or ACTH (15 IU/ml) injections after 14 and 28 days of exposure to DMSO (Control) or waterborne GEM (1,500 μg/L). Data represent means + SEM, n = 11 to 12. A three-way ANOVA revealed no effect of duration of exposure ($P = 0.339$), presence of drug ($P = 0.440$) or any of the interactions but detected a significant of ACTH ($P < 0.001$).

APPENDIX F

In-vivo cortisol response in rainbow trout exposed to waterborne GEM

Objective: This experiment was designed to determine the effect of waterborne GEM on the rainbow trout *in-vivo* cortisol response following exposure to an acute handling stress.

Methods: For the preliminary air exposure experiment, 2 rainbow trout were sampled for blood to determine basal cortisol levels. An additional 8 rainbow trout were exposed to air in nets for 30 sec and returned to water. Blood samples were collected at different post-stress time points. Fish exposed to waterborne GEM as described in Chapter 4 were used to assess the effect of GEM on the *in-vivo* cortisol response. In addition to the six groups of fish previously reported (1, 7 and 14 day exposures to nominal waterborne GEM concentrations of 0 or 1,500 μg/L) another identical six groups were exposed to air for 30 sec to induce a cortisol response. Blood was sampled from anesthetized fish (benzocaine) on days 1, 7 and 14 for both unstressed and air-stressed fish (30 min after stress). Cortisol was analyzed as described in Chapter 5.

Results: The air exposure test revealed 30 min post-stress to be a good time to sample blood to detect optimal induced plasma cortisol levels (Fig. F1). Percent increase in plasma cortisol in rainbow trout stimulated with a 30 sec air exposure compared to basal cortisol levels were significantly lower in fish exposed to waterborne GEM than in control fish after 7 and 14 days ($P = 0.003$ for duration of exposure and $P = 0.007$ for the interaction) (Fig. F2). The biological significance of these results remain to be explored as the basal cortisol levels in fish exposed to GEM were higher than in control fish ($P = 0.022$) (Fig. F3).

Interpretation: While 1,500 μg GEM/L seems to impair the *in-vivo* cortisol response in rainbow trout, the basal cortisol levels were higher in GEM-exposed fish than in the control fish. Results were difficult to interpret and *in-vitro* experiments were therefore undertaken to evaluate the effect of fibrate on cortisol response (see Chapter 5).

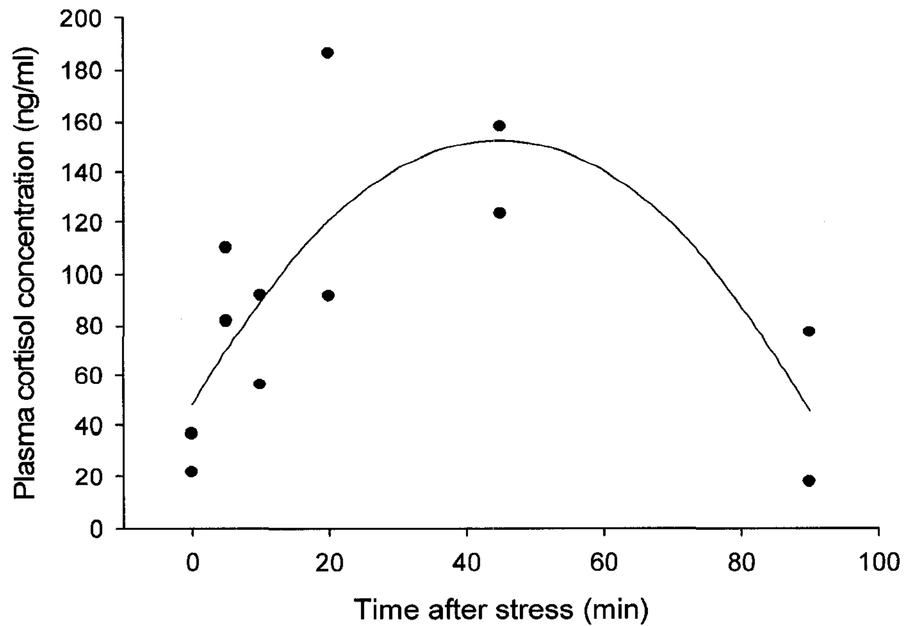

Figure F1. Effects over time of a 30 sec air exposure on rainbow trout plasma cortisol concentration. Trout were stimulated once at time zero and blood samples were collected at different post-stress time points. Different fish were used at each time point. Data represent single cortisol measurements, total n = 10. Non-linear regression analysis revealed a significant relation ($P = 0.014$) and $R^2 = 0.610$.

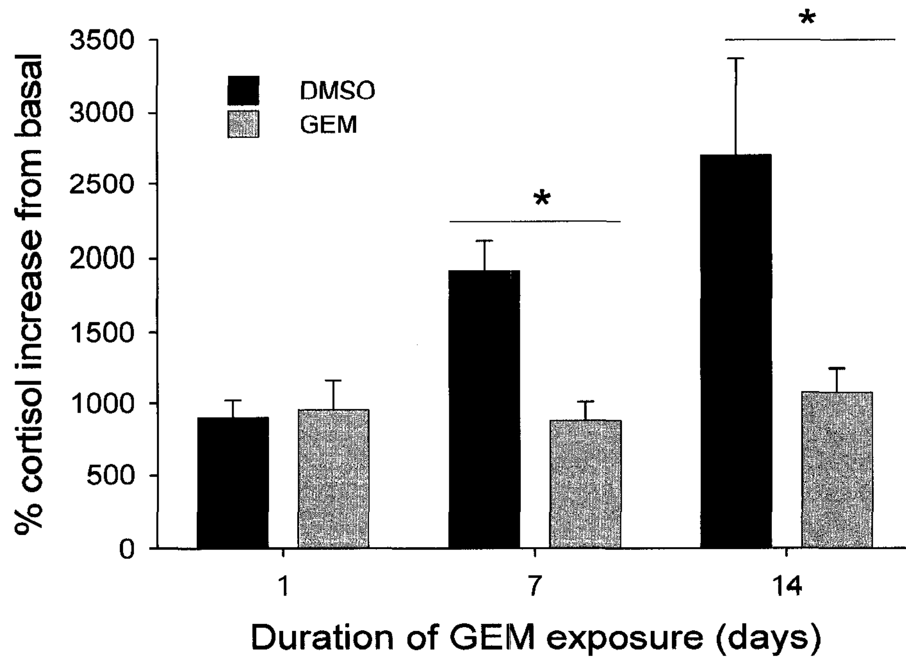

Figure F2. Percent increase in plasma cortisol levels in rainbow trout stimulated with a 30 sec air exposure compared to basal cortisol levels. Black and grey bars represent cortisol induction in the absence and the presence of GEM (1,500 μg/L), respectively. Data represent means + SEM, n = 5 to 6. A two-way ANOVA failed to detect an individual effect of the drug ($P = 0.869$), nevertheless a time ($P = 0.003$) and an interaction ($P = 0.007$) effect were detected. Multiple comparison tests were done using Bonferroni tests and differences are indicated by an asterisk (*).

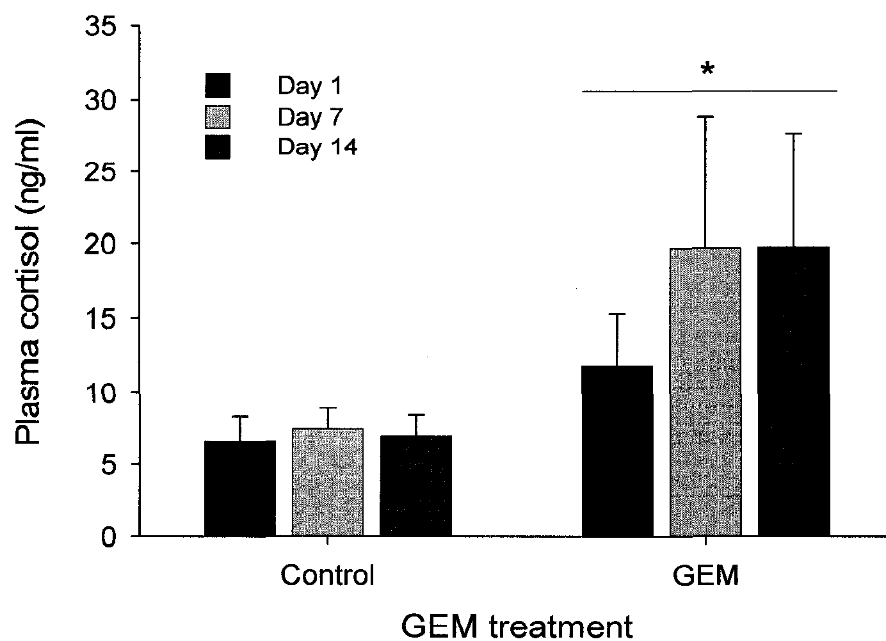

Figure F3. Basal cortisol levels in rainbow trout plasma in the absence or the presence of GEM (1,500 μg/L) in water as a function of treatment length (in days). Data represent means + SEM, $n = 5$ to 6. A two-way ANOVA on log-transformed data revealed no effect of duration of exposure ($P = 0.783$) or interaction ($P = 0.971$) but an effect of the drug ($P = 0.022$). Differences detected with a Bonferroni test are indicated by an asterisk (*).