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**Circulating Lipoproteins and Tissues Lipids :  
The Effects of Gemfibrozil on Lipid Metabolism of Rainbow Trout**

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**Circulating lipoproteins and tissue lipids:  
Effects of gemfibrozil on lipid metabolism in rainbow trout**

by

**John S. Prindiville**

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies  
In partial fulfillment of the requirements for the Master's of Science in Biology  
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## **Abstract**

Lipids support crucial functions in teleost fish, including the production of energy; lipoproteins support the movement of lipids through the vascular system. The objective of this thesis was to determine if gemfibrozil (GEM), a mammalian PPAR $\alpha$  agonist pharmaceutical, could alter the circulating lipoproteins and tissue lipid metabolism in the rainbow trout, *Oncorhynchus mykiss*.

Injections of GEM lowered the concentration of lipoproteins and changed their size distribution, lipid and fatty acid content. These changes were associated with an increased lipoprotein lipase transcript level but not activity. GEM increased liver size but did not affect its lipid content nor the activities or transcript levels of its PPARs and mitochondrial/peroxisomal enzymes.

This thesis provides evidence that the pharmacological effects of GEM are conserved across vertebrates. Furthermore, the decreased plasma lipids and altered lipoprotein composition demonstrated here may be relevant sublethal indicators of exposure to PPAR $\alpha$  agonists present in the aquatic environment.

## Résumé

Les lipides soutiennent un rôle crucial dans les poissons, incluant la production d'énergie; les lipoprotéines permettent le transport des lipides à travers le système vasculaire. L'objectif de cette thèse était de déterminer si le gemfibrozil (GEM), un médicament humain agoniste de PPAR $\alpha$ , change les lipoprotéines circulantes et le métabolisme des lipides dans les tissus de la truite arc-en-ciel, *Oncorhynchus mykiss*. Des injections de GEM ont abaissé la concentration des lipoprotéines et ont changé leur taille, et leurs contenus en lipides et en acides gras. Ces changements étaient associés à une augmentation du niveau des produits de transcription de la lipoprotéine lipase, mais pas de son activité. GEM a aussi augmenté significativement la taille du foie mais n'a pas changé le contenu de ses lipides. GEM n'avait pas d'effet sur les activités ou les niveaux de transcription des enzymes des mitochondries, des peroxysomes, ou de celui des PPARs. Cette thèse fournit des éléments prouvant que les effets de GEM sont conservés à travers les vertébrés. De plus, la diminution des lipides plasmatiques et le changement de composition des lipoprotéines démontrés ici pourraient être des indicateurs sublétaux de l'exposition aux agonistes des PPARs.

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## List of Abbreviations

<b>Abbreviation</b>	<b>Description</b>
ACO	acyl CoA Oxidase
APO	apopoprotien
CAD	coronary artery disease
CAT	catalase
CETP	cholesteryl ester transfer protein
CPT	carnitine palmitoyl transferase
CS	citrate synthase
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FA	fatty acid
Fibrate	fibric acid derivative
GEM	gemfibrozil
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
HL	hepatic lipase
HOAD	3-hydroxyacyl-CoA dehydrogenase
HSI	hepato-somatic index
LCAT	Lecithin-cholesterol acyl transferase
LDL	low-density lipoprotein
LPL	lipoprotein lipase
PhAC	pharmaceutically-active compound
PLTP	phospholipid transfer protein
PPAR	peroxisome proliferator activated receptor
PPRE	peroxisome proliferator receptor element
SOD	superoxide dismutase
VLDL	very-low density lipoprotein
WWTP	waste-water treatment plant

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Sometimes the easiest step to take after completing a bachelor's degree is to slip back into the familiar university setting and start a graduate program. While it was with this attitude I entered the M.Sc. Biology program, my perspective was quickly adjusted. Great colleagues and supervisors made all the difference. Now, the roughly two and a half years it has taken me to develop, write and submit this thesis seems like it has passed much more quickly.

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## Foreword

The lipid transport system of mammals and salmonids is highly elucidated, with both classes of organisms sharing broad similarities in their system's structure and function. Due to the conserved nature of the vertebrate lipid transport system, there is a possibility that pharmaceuticals designed to affect this system in mammals may also affect its functioning in fish, should those organisms become exposed. Fibric acid derivatives are a therapeutic class of pharmaceuticals designed to regulate the human lipid transport system by agonizing the transcriptional regulator PPAR $\alpha$ . Despite that they are well established as ubiquitous contaminants of the aquatic environment, there is a paucity of scientific literature that investigates the effects of PPAR $\alpha$  agonists on the lipid-transport system of non-mammalian aquatic vertebrates. Therefore, important information regarding if and how PPAR $\alpha$  agonists affect the lipid-transport systems of such organisms remains unavailable. Understanding the extent to which human pharmaceuticals can impact the physiology of a widely different species may yield interesting information into the evolution of the two species and may be a useful tool for conducting environmental risk assessments.

Studies for this thesis are designed to test the effects of the fibrate drug Gemfibrozil (GEM) on plasma lipid-transport and tissue lipid-metabolism in a model aquatic vertebrate, the rainbow trout (*Oncorhynchus mykiss*).

## **Chapter 1: General Introduction**

### **1.1 Lipids and lipoprotein transport in vertebrates**

#### **1.1.1 Lipids**

Lipids are a diverse class of biomolecules that, in fish, have fundamental roles in energy production, maintaining the integrity of cell membranes, and cytosolic signaling. Lipids that contain fatty acids (FA) include triglycerides and phospholipids, and these have different physiological functions. Triglycerides contain three FA and are mainly stored in adipose tissue or as intramuscular oil droplets that can be mobilized to sustain the energy requirements of the animal (Tocher, 2003). In this case, triglycerides provide FA chains to tissues for energy production. Phospholipids are mainly found in cell membranes and these lipids contain two FA chains. Phospholipids are incorporated with FA of varying chain length and number of double bonds to precisely adjust membrane fluidity in the face of environmental changes. Cholesterol is a sterol lipid that is an important molecule involved in cell signaling and steroidogenesis, as well as serving as an integral component of biological membranes.

The lipid transport system of vertebrates has evolved to carry lipids through the vascular system to the body, and it has two functionally different pathways to do so: the exogenous and endogenous pathways (Havel, 1987). The exogenous pathway transports lipids absorbed from the gut (i.e. dietary lipids) while the endogenous pathway shuttles stored or biosynthesized lipids. Since lipids represent

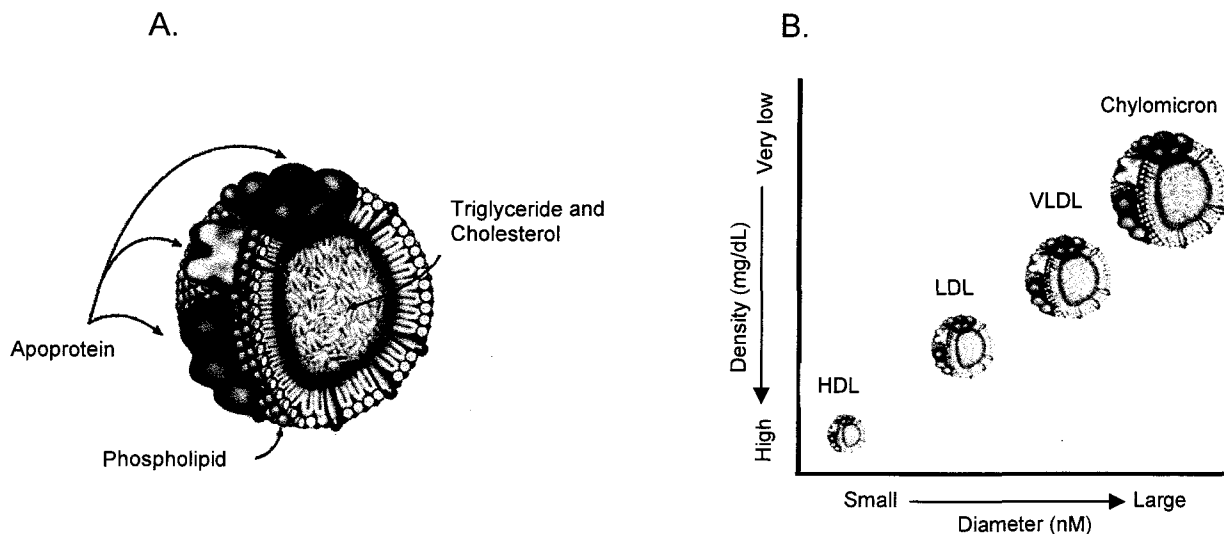
such fundamental components for sustaining energy production and cell membrane homeostasis, their provision to bodily tissues from the vascular system must be both sufficient and efficient. However, triglycerides and cholesterol are hydrophobic and are therefore inherently difficult to transport through an aqueous media, in this case blood plasma. As a way to circumvent this challenge and to render hydrophobic lipids 'soluble' for transport, they are packaged inside hydrophilic transport particles known as lipoproteins.

### 1.1.2 Lipoproteins

Insects, crustaceans, and vertebrates have all evolved the use of lipoproteins or lipoprotein-like particles as an efficient mechanism for moving large amounts of lipids through their circulating fluids (Chapman, 1980; Chapman, 1986).

Vertebrate lipoproteins are macromolecular complexes composed mainly of phospholipids, triglycerides, cholesterol and specialized proteins known as apoproteins (apo) (Havel, 1987; Havel et al., 1987). These proteins serve as ligands for lipoprotein receptors and regulate the activity of lipoprotein metabolizing enzymes. As depicted in Figure 1.1A, the core of the lipoprotein is formed primarily by hydrophobic triglycerides and esterified cholesterol while the surface envelope is an amphipathic monolayer consisting mainly of phospholipids and apos that allow the hydrophobic core to be miscible in, and transported through, blood plasma (Ginsberg, 1998). In vertebrates, there exist four main classes of lipoproteins that differ in their size, density (Figure 1.1B) and composition, yet that all share the same general structure. The lipoprotein classes possess characteristic apo compositions that determine their class-specific compositional and functional attributes (Table

1.2). Segregated based on the range of their densities, from largest and least dense to the smallest and most dense, the lipoprotein classes are: chylomicrons, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Babin and Vernier, 1989).



**Figure 1.1.** Illustrations of lipoprotein particles and classes. Panel A shows a representative schematic of a lipoprotein particle. Panel B shows an illustration of the different sizes and densities of the lipoprotein classes.

### 1.1.3 Lipoprotein metabolism

The metabolism of lipoproteins is exquisitely complex and involves multiple synthetic and catabolic pathways (Figure 1.2). The complete study of lipoprotein metabolism, including all metabolic pathways, cell membrane receptors and lipoprotein metabolism-related disease pathologies is the subject of numerous and extensive reviews (see, for example, Babin and Gibbons, 2009; Goldstein and Brown, 1977; Kontush and Chapman, 2006; Therond, 2009). Although there is some difference in detail, the metabolism of lipoproteins is comparable throughout the

vertebrates, including fish (Tocher, 2003). The focus of this section is to summarize reviews of vertebrate lipoprotein metabolism using the human/rodent system as a model, since it is the most thoroughly elucidated.

#### 1.1.3.1 Chylomicron

Chylomicrons are lipoproteins that form a principal component of the vertebrate dietary (i.e. exogenous) lipid transport system. Chylomicrons are assembled in the intestinal epithelium and are secreted into the blood primarily to transport dietary FA from the intestine to body tissues and to transport dietary cholesterol to the liver (Gotto et al., 1986). In the blood, chylomicrons are catabolised by an endothelium-bound enzyme called lipoprotein lipase (LPL) (Mamo and Proctor, 1999; Rye et al., 1999a). In this process, chylomicrons are converted into smaller particles known as chylomicron remnants. When apoE present on the chylomicron remnants binds to LDL receptors located on the liver, the remnants are endocytosed and removed from the blood (Kypreos and Zannis, 2006).

#### 1.1.3.2 VLDL

VLDL are synthesized in the liver and secreted into the plasma and are members of the endogenous lipid transport system as they transport both endogenously synthesized and diet-derived triglyceride and cholesterol (Gotto et al., 1986; Lee et al., 2003; Rye et al., 1999a). The initial step of VLDL metabolism essentially follows that of chylomicrons (i.e. hydrolysis by LPL), but the VLDL remnants can enter two different metabolic pathways (Havel, 1984). In one pathway, VLDL remnants are endocytosed by the liver after binding to LDL receptors (Babin

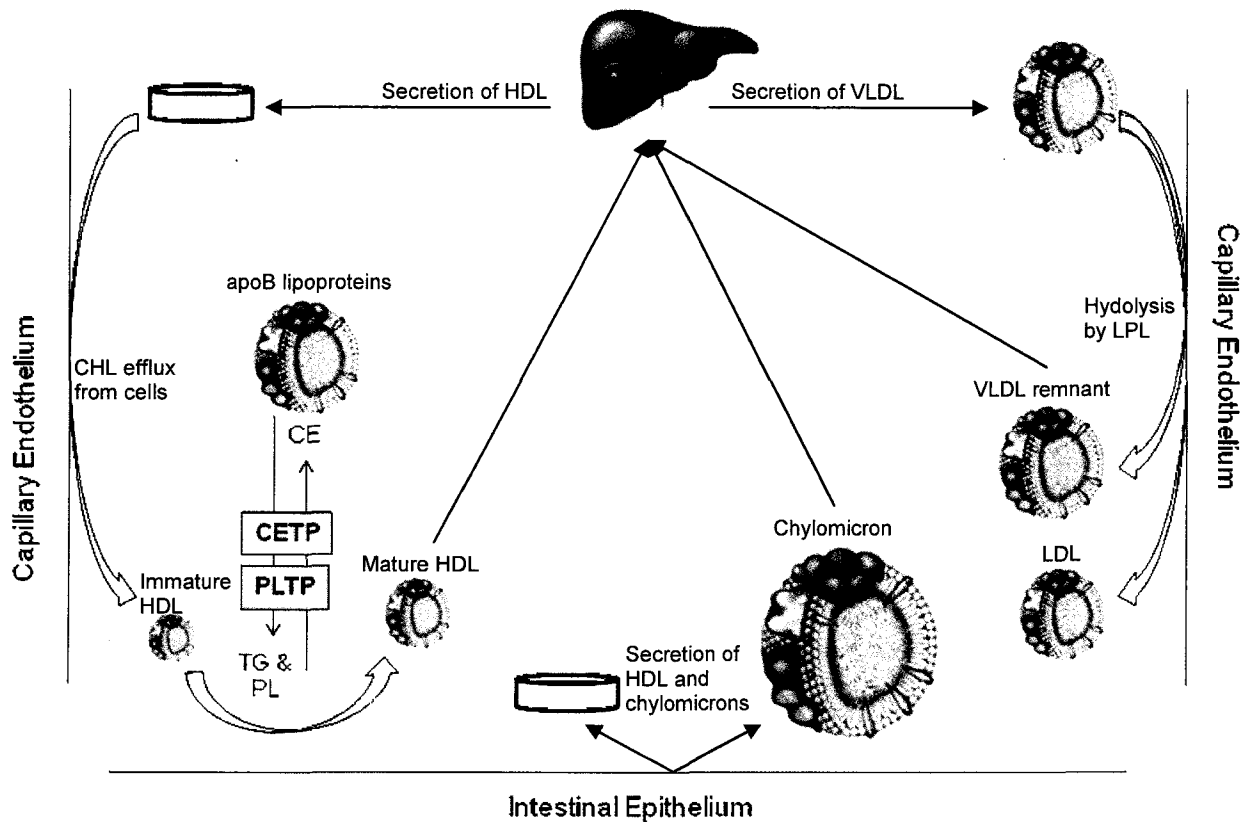
and Gibbons, 2009). In the other pathway, VLDL remnants interact with key lipoprotein remodeling enzymes, like cholesteryl-ester transfer protein (CETP) and phospholipid transfer protein (PLTP), and are converted into cholesterol-rich LDL (Babin and Gibbons, 2009; Rye et al., 1999b).

#### 1.1.3.3 LDL

LDL are the major carriers of cholesterol in human plasma. The major apo of LDL is apoB, which is needed for receptor-mediated clearance of LDL from the plasma (Ikonen, 2008; Law and Scott, 1990; Welty et al., 1995). It is this apo-mediated internalization of LDL that delivers circulating cholesterol to extra-hepatic tissues. Many extra-hepatic tissues that receive cholesterol cannot catabolize it or assemble it into lipoproteins, thus potentially over-accumulating cholesterol which can be a detriment to the tissue. Therefore, the removal of cholesterol from cells is a necessary and on-going process. The removal of excess cholesterol from cells is part of a pathway called reverse cholesterol transport; a pathway in which another class of lipoproteins, the HDL, are the principal actors.

#### 1.1.3.4 HDL

HDL is secreted into the blood by the liver and intestine as apo (apoAI) associated with phospholipids (Rye et al., 1999a). The immature HDL accumulates cholesterol from tissues that is then esterified by lecithin:cholesterol acyl transferase (LCAT). Other plasma lipid-transfer enzymes (i.e. CETP and PLTP) help the nascent HDL accumulate triglycerides and phospholipids by the reciprocal transfer of lipids between VLDL and HDL (Tall, 1995).



**Figure 1.2.** Overview of mammalian HDL, VLDL and chylomicron metabolism. Plasma HDL synthesis begins with the secretion of a phospholipid(PL)-apoAI complex from the liver and intestine. The PL-apoAI complex accumulates cholesterol from peripheral tissues to become immature HDL. The cholesterol is then esterified and exchanged for triglyceride from apoB-containing lipoproteins (see Table 1.2). HDL matures and deposits the CE at the liver. VLDL is secreted from the liver and is hydrolyzed by LPL. The product of this hydrolysis (i.e. VLDL remnants or LDL) can exchange lipids and CE with HDL or be endocytosed by apoB receptors on tissues. Chylomicrons are secreted from the intestine and are also hydrolyzed by endothelial lipases. Chylomicron remnants also bind hepatic apoB receptors and are endocytosed. A more detailed discussion of lipoprotein metabolism shown in this figure is found in section 1.1.3. Data for this figure are adapted from Linsel-Nitschke and Tall (2005), Mahley et al. (1984) and Rye et al. (1999b).

The major core component of human HDL is cholesterol, but unlike LDL, mature HDL is as a “reverse cholesterol transporter” as its accumulated cholesterol is quickly removed from the plasma by the liver (Fredenrich and Bayer, 2003). The HDL-mediated cholesterol efflux from peripheral tissues is the first key step in the reversal of cardiovascular diseases (Linsel-Nitschke and Tall, 2005; Singh et al., 2007).

#### 1.1.3.5 Lipoprotein lipases

Many lipases exist to hydrolyze plasma lipids and supply tissues with FA. The endothelial-bound lipases: hepatic lipase (HL) and lipoprotein lipase (LPL) are the most studied and their catalytic action exhibits lipoprotein class specificity and the ability to hydrolyze both triglycerides and phospholipids (Dugi et al., 1995). Of these enzymes, LPL is the most important for the hydrolysis and removal of triglycerides from lipoproteins.

LPL is an important regulator of whole organism lipoprotein metabolism as its main role is to hydrolyze the triglycerides from circulating chylomicrons and VLDL, thereby providing FA to tissues (Mead et al., 2002; Mead and Ramji, 2002) and yielding lipoprotein remnants that are removed from the circulation (Nilsson-Ehle et al., 1980). LPL is primarily found bound to the capillary endothelium by heparan-sulphate proteoglycans that create bridges allowing for the tissue uptake of the released FA (Merkel et al., 1998; Olivecrona and Bengtson-Olivecrona, 1999). When LPL is activated by the apoCII found on the lipoprotein surface, triglycerides are hydrolyzed and FA are subsequently released. LPL is most abundant in skeletal and heart muscles and in adipose tissue. LPL activity is also regulated in a tissue-

specific manner by both endogenous (e.g. fatty acids, apolipoproteins and hormones) and exogenous (e.g. nutrient availability) cues (Albaladejo et al., 2006; Auwerx et al., 1992; Howard et al., 1993; Ladu et al., 1991a; Ladu et al., 1991b; Ruge et al., 2005; Zechner, 1997).

#### 1.1.4 Lipoproteins of teleost fish

The structures, functions and metabolic fates of lipoproteins are thoroughly investigated in humans and rodents, likely because of the association of lipoproteins with cardiovascular diseases. Although considerably more work is needed to fully understand lipid transport and lipoprotein metabolism in fish, most research shows that the lipid transport systems of mammals and salmonid fishes share strong similarities, including the major lipoprotein classes, apo content, and endocytotic pathways (Ayrault-jarrier et al., 1988; Delcuve et al., 1992; Gjoen and Berg, 1992a; Gjoen and Berg, 1992b; Gjoen and Berg, 1993a; Gjoen and Berg, 1993b; Shen et al., 2000). Despite the similarities, differences do exist in the concentrations and in the physiological roles played by lipoproteins in the two animal groups. These differences are discussed below.

The difference in the concentration of lipoproteins between humans and teleost fish is substantial (Tables 1.1 and 1.2). The large concentrations of circulating lipoproteins in rainbow trout may be associated with at least two non-reproductive roles: exercise and thermal adaptation. Accumulating evidence supports that, unlike mammals that clearly utilize albumin-bound non-esterified FA (Nikolaidis and Mougios, 2004; Walker et al., 1991), endurance exercise in rainbow trout is primarily supported by oxidizing lipoprotein-bound triglycerides supplied to

red muscle (Kieffer et al., 1998; Lauff and Wood, 1996; Lauff and Wood, 1997; Magnoni et al., 2008; Magnoni and Weber, 2007; Rome, 1998). The HDL of rainbow trout accumulate highly unsaturated FA in phospholipids during low-temperature acclimation experiments (Wallaert and Babin, 1994b), suggesting that HDL supplies highly unsaturated FA to tissues, possibly for membrane reorganization during homeoviscous adaptation. The concentration of vertebrate lipoproteins fluctuates circannually and these fluctuations are under hormonal regulation (Duggan et al., 2001; Laplaud et al., 1980; Manttari et al., 1993; Wallaert and Babin, 1993). For rainbow trout, increases in LDL and HDL are correlated with yearly reproductive events and coincide with elevated concentrations of circulating testosterone and estradiol (Wallaert and Babin, 1994a). This suggests that lipoproteins have functions related to reproduction, at least in rainbow trout. However, unlike in oviparous vertebrates, the physiological basis for the circannual variation in lipoprotein concentrations in mammals are not established (Laplaud et al., 1986; Seed, 1991; Vitale et al., 2009).

By supporting reproduction, homeoviscous adaptation, and sustained exercise, functions of lipoproteins in teleost fish differ substantially from their functions in mammals. These functional differences may partially account for the three-fold greater concentration of circulating lipoproteins in rainbow trout (Table 1.1). Because of their unique roles, the physiological pathways that regulate lipoprotein concentration in teleost fish may be sensitive to contaminants present in the aquatic environment.

**Table 1.1.** The density, concentration and profile (percent total) of lipoproteins<sup>a</sup> in the plasma of rainbow trout and humans.

	Density (d) (g/ml)		Concentration (mg/100 ml)		Percent total (%)	
	<u>Rainbow trout</u>	<u>human</u>	<u>rainbow trout</u>	<u>human</u>	<u>rainbow trout</u>	<u>human</u>
VLDL	1.015<d<1.040	0.93<d<1.019	259.1	132	15.5	17.9
LDL	1.040<d<1.085	1.019<d<1.063	631.7	374	22	50.8
HDL	1.085<d<1.210	1.063<d<1.125	1229	230	62.5	31.3
Total			2119.8	736	100	100

a- Data adapted from Babin and Vernier (1989)

**Table 1.2.** The composition<sup>a</sup> (% weight) of lipoproteins in rainbow trout and humans including the major apoproteins (apo)<sup>a,b</sup>.

	<u>VLDL</u>		<u>LDL</u>		<u>HDL</u>		<u>VTG</u>
	Trout	Human	Trout	Human	Trout	Human	Trout
Composition							
Core							
TG	52	55	22.3	6	11.1	4.5	4
CE	11	19	14.9	47.5	9.1	17.5	2
Surface							
Free CHL	5.7	6	6.3	7	3.4	4	n/a
PL	18.5	17.5	27	21	31.7	29	11
Protein	12.8	8	29.5	20	44.7	50	82
apo <sup>a,b</sup>	AI, AII, B, C	B-100, E, CI, CII, CIII	B, AII, C	B-100	AI, AII	AI, AII, E, CI, CII, CIII	

a- Data adapted from Babin and Vernier (1989)

b- Data adapted from Mahley et al. (1984)

### 1.1.5 Lipoproteins and disease

Coronary artery disease is a cardiovascular disease characterized by the accumulation of lipid-laden cells beneath the vascular endothelium of the coronary artery that block free passage through the arterial lumen (Hansson, 2005; Libby and Theroux, 2005). Individuals with the greatest risk of developing this disease suffer from chronically elevated plasma triglyceride and LDL concentrations, and depressed concentrations of high-density lipoprotein-cholesterol (HDL-C) (Wilson and Meigs, 2008). Fibric acid derivatives (fibrates) are a class of pharmaceutical used to regulate the concentration and composition of lipoproteins to effectively mitigate the development of cardiovascular diseases.

### 1.2 Physiological effects and mechanism of action of fibrates

Fibrate drugs have a multifaceted effect on plasma lipid transport that includes altering lipid uptake and release by peripheral tissues, changing patterns of hepatic lipoprotein uptake and release and, lastly, by boosting hepatic lipid metabolism while inhibiting lipid synthesis. There are a number of fibrate pharmaceuticals, including clofibrate, bezafibrate, ciprofibrate, fenofibrate and gemfibrozil (GEM), all sharing similar structures and functions (Chuang et al., 2009). This section will discuss the major physiological effects of fibrates using GEM as the model compound. When considering the effects of GEM on lipid and lipoprotein metabolism, it is important to recognize that fibrate drugs show inconsistent therapeutic outcomes in clinical trials (Backes et al., 2007), have large ranges in their clinical effectiveness (Robins and Bloomfield, 2006), and that there are

considerable species-differences in the outcomes of fibrate exposure (Alegret et al., 1994; Vazquez et al., 1996).

### 1.2.1 Physiological effects of GEM

The primary physiological effects of GEM therapy in humans are reductions in total plasma triglycerides and increases in plasma HDL and HDL-C, thereby mitigating the risk of developing coronary artery disease. The reduction in plasma triglycerides is likely the result of a combination of enhanced LPL activity on VLDL (Fruchart and Duriez, 2006), increased receptor-mediated hepatic uptake of LDL (Yang et al., 1996) and decreased synthesis and secretion of both triglycerides (Musanti et al., 1996; Ozansoy et al., 2001; Zhu et al., 2002). By increasing hepatic FA metabolism (Fruchart and Duriez, 2006) and altering the rate of lipoprotein secretion/uptake, GEM changes the FA profile of circulating lipids; increasing the poly-unsaturated FA (PUFA) while decreasing saturated and mono-unsaturated FA (Smith et al., 2002). In keeping with the multiple species-specific responses to fibrate exposure, studies with GEM-fed rats conflict with these human data; the rats showed increased plasma saturated FA and decreased PUFA (Alegret et al., 1994; Vazquez et al., 1996).

### 1.2.2 Mechanism of action of GEM

The biological effects of GEM are exerted through the activation of nuclear receptors and transcription regulators called Peroxisome Proliferator-Activated Receptors (PPARs) (Backes et al., 2007; Steiner, 2005). Three PPAR subtypes (PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$ ), each with distinct tissue distributions and functional

properties, are identified in mammals, amphibians, birds and teleost fish (Barbier et al., 2002). Studies from PPAR $\alpha$ -null mice were crucial for elucidating the role of PPAR $\alpha$  in mitochondrial/peroxisomal FA oxidation and the assembly, secretion and uptake of lipoproteins (Lefebvre et al., 2006). Mammalian PPAR $\alpha$  is highly expressed in the liver where it acts as a physiological sensor of lipid levels by binding particular non-esterified and unsaturated FA and eicosanoids (Kliwer et al., 1997; Murakami et al., 1999). Yet, PPAR $\alpha$  is also activated by a variety of synthetic compounds, including fibrates (Forman et al., 1997). GEM preferentially activates PPAR $\alpha$ , but also binds to the  $\beta$  and  $\gamma$  subtypes, albeit with a weaker affinity (Barbier et al., 2002; Duez et al., 2005; Hossain et al., 2008). Upon ligand binding, the PPAR heterodimerizes with the retinoid X receptor (RXR) (Desvergne et al., 2006). The heterodimer complex subsequently binds to PPAR response elements (PPREs) in the promoter region of target genes, thereby regulating their expression. A number of mitochondrial and peroxisomal  $\beta$ -oxidation enzymes, including LPL, are identified as containing a PPRE in the promoter regions of their genes (Mandard et al., 2004).

#### **1.4 Pharmaceuticals in the environment**

Pharmaceutically-active compounds (PhACs) are a diverse group of biologically active compounds that affect the physiology of both humans and animals. Traditionally, PhACs were not viewed as environmental contaminants and, as a result of this neglect, PhACs have experienced several decades of unrestricted discharge into the aquatic environment (Hua et al., 2006). PhACs are introduced into the environment through many routes, but the principal pathway is through release

from waste water treatment plants (WWTP's) (Daughton and Ternes, 1999; Lishman et al., 2006). WWTP's filter PhACs with variable efficacy and can also facilitate the transformation of metabolized drugs back into their original parent compounds, consequently flushing bioactive PhACs into the aquatic environment (Drewes et al., 2002; Heberer et al., 2002; Isidori et al., 2007; Richardson and Ternes, 2005). Despite their existence in the aquatic environment, most PhACs have not been tested for any special ecological effects, yet their likelihood of affecting non-target organisms is high as many of their physiological targets are evolutionarily conserved (Bendz et al., 2005; Daughton and Ternes, 1999; Trudeau et al., 2005). The PhACs that most typically appear in WWTP effluent include oral contraceptives, cytostatic drugs, contrast media,  $\beta$ -blockers, anti-epileptics, antibacterial drugs, analgesics and blood-lipid regulators, including fibrates (Heberer et al., 2002).

#### 1.4.1 GEM: occurrence in the environment

GEM is administered in twice daily doses of 600 mg. Together, these large daily doses and inadequate treatment methods used in WWTPs permit residual amounts of GEM to reach water bodies that are recipients of the WWTP effluent (see Table 1.3). When in water, GEM preferentially partitions into the organic phases ( $\log k_{ow} = 4.77$ ) (Sanderson et al., 2003) and recent studies found that GEM bioconcentrates from the aquatic medium into the plasma of teleost fish (Brown et al., 2007; Mimeault et al., 2005). Therefore, attention to the potential biological effects of GEM to fish should to be examined.

**Table 1.3.** The concentration of gemfibrozil in wastewater treatment plant (WWTP) effluent or surface water at locations in Canada and Western Europe.

Country/ continent	Region	Source	Concentration (Median or range)	Reference
Canada	Halifax	WWTP effluent	300 ng/L	Comeau et al., 2008
Canada	Pictou	Watershed	6 ng/L	Comeau et al., 2008
Italy	Lake Maggiore	Surface water	0.2-1.7 ng/L	Loos et al., 2007
Canada	Ontario	WWTP effluent	0.25-0.43 µg/L	Lishman et al., 2006
Canada	Ontario	WWTP effluent	0.08-2.09 µg/L	Lee et al., 2005
Europe	Western	WWTP effluent	0.56 µg/L	Paxeus et al., 2004
Canada	Ontario	Surface water	0.012 µg/L	Metcalfe et al., 2003a
Canada	Ontario	Surface water	0.06 µg/L	Metcalfe et al., 2003b
Canada	Various	WWTP effluent	1.3 µg/L	Metcalfe et al., 2003b

#### 1.4.2 Fibrates: Exposure and Effects in Non-Target Organisms

The effects of both pharmacological and environmentally-relevant concentrations of fibrate drugs, including GEM, have been investigated in many non-target species including plants, invertebrates and vertebrates.

Of the effects on plants, D'Abrosca et al. (2008) demonstrated negative effects of GEM on the germination and root elongation of a variety of crop species. GEM also induces physiological changes in aquatic invertebrates. For example, waterborne GEM showed teratogenic effects in *Hydra attenuate*, inhibited the population growth of rotifers and crustaceans and altered immunological response in freshwater mussels (Gagne et al., 2006b; Isidori et al., 2007; Quinn et al., 2008a;

Quinn et al., 2008b). The mechanisms that account for these effects have not been investigated, but the ability of a vertebrate-targeting pharmaceutical to alter the physiology of both plants and invertebrates highlights some of the unexpected consequences that may occur when PhACs enter the environment.

Fish are often used to investigate the effects of aquatic pollutants on physiological systems, and a number of studies report the varied bioactivity of fibrates in these organisms. For instance, recent studies show that fibrates interfere with the reproductive axis of teleost fish (Mimeault et al., 2005; Runnalls et al., 2007; Weston et al., 2009). Studies of their effects on fish PPAR expression show that GEM reduces hepatic PPAR $\beta$  mRNA transcript levels in goldfish (*Carassius auratus*) (Mimeault et al., 2006) and that clofibrate induces the expression of both PPAR $\alpha$  and PPAR $\gamma$  in zebrafish hepatocyte cultures (Ibabe et al., 2005). Likewise, hepatocyte cultures from Atlantic salmon (*Salmo salar*) exposed to bezafibrate and clofibrate showed increased expression of PPAR $\gamma$  and a complimentary increase in ACO activity (Ruyter et al., 1997). These studies suggest that PPARs are important for mediating an enzymatic response of fibrates in fish and that fibrates regulate the expression of PPARs in teleosts differently than in mammals.

Most studies that test the ability of fibrates to disrupt the lipid metabolism of fish do so indirectly by measuring peroxisomal proliferation and oxidative stress (Donohue et al., 1993; Mimeault et al., 2006; Weston et al., 2009). Only a few studies have directly evaluated the effects of fibrates on lipid metabolism. In one of these studies, zebrafish (*Danio rerio*) embryos exposed to micromolar concentrations of both clofibrate and GEM showed decreased consumption of the embryonic yolk sac; this correlated with decreased size at the larval stage (Raldua

et al., 2008). Another study reported that fenofibrate-fed rainbow trout had a decreased tissue content of two major fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and provided evidence for the up-regulation of both the peroxisomal and mitochondrial  $\beta$ -oxidation pathways in the liver (Du et al., 2004). In a subsequent study, fenofibrate fed to grass carp (*Ctenopharyngodon idella*) on a high-fat diet decreased plasma triglycerides and cholesterol content, total lipid content of the whole body and the liver, and, again, decreased the EPA and DHA content in tissues (Du et al., 2008). There is also very limited evidence that fibrates increase the HDL-C of rainbow trout after feeding with fenofibrate (Du et al., 2008), or by decreasing the total amount of lipoprotein-bound cholesterol after exposure to micromolar concentrations of clofibrate (Runnalls et al., 2007).

Taken as a group, these studies show that at both pharmacologically and environmentally-relevant concentrations, fibrates, including GEM, have varied biological effects in a wide collection of organisms. Moreover, fibrates are capable of interfering with the lipid metabolism in multiple species of teleost fish by increasing the activities of both peroxisomal and mitochondrial enzymes; these possibly change the composition of lipoproteins. The effects are likely mediated through interactions with PPARs, but the weight of evidence hints toward a different pattern of fibrate-PPAR interaction in fish than in mammals. Given the varied effects of fibrates on lipid metabolism in teleost fish, a more detailed evaluation of the capacity of fibrates to change the composition of lipoproteins in teleost fish is clearly needed.

## **1.5 Thesis Rationale, Objectives and Hypothesis**

### **1.5.1 Rationale**

Lipids and lipoproteins are central to the ability to mobilize energy stores for both exercise and homeoviscous adaptation as well as to the reproductive potential of at least salmonids, if not all teleost fish. Since regulatory aspects of lipid metabolism appear to be conserved (Chapman, 1980; Gunnarsson et al., 2008; Hihi et al., 2008) there is the possibility that the lipoprotein metabolism of salmonid fish will be affected by fibrates.

It is important to recognize that the evolutionary conservation of drug targets (e.g. PPARs) and physiological systems (e.g. lipid transport) do not exclude the existence of evolutionary divergences. Hence, the pharmacological effects of a drug may be quite different in species sharing the same or similar drug target. The differential effect of fibrates on the FA profile of humans and rats was already cited in as an example of this phenomenon (see 1.2.1). Another and perhaps more pertinent example of the divergent response to fibrates across species is that fibrates are potent hepatocarcinogens in most rodents, while humans remain immune to this toxicity (Klaunig et al., 2003). Therefore, how the lipid transport system of salmonid fish may respond to the pharmacological action of fibrates is inherently unpredictable.

### **1.5.2 Objective**

The main objective of this thesis is to characterize how exposure to a PhAC and known mammalian PPAR $\alpha$  agonist affects lipid transport and use in the rainbow

trout. This objective will extend the previous research (see 1.4.2) by possibly exposing a novel effect of PPAR $\alpha$  agonists in fish. This objective will therefore be useful in determining whether changes to the lipid transport system should be investigated as a possible sublethal indicator of the biological effects of environmental exposure to fibrates. Furthermore, by comparing the response of rainbow trout with that of humans and rodents, this objective may reveal features of lipoprotein metabolism that are uniquely present in fish.

### 1.5.3 Hypothesis and Outline

To achieve the objective, studies for this thesis are designed to test the hypothesis that the pharmacological effects of Fibrates on lipid metabolism are conserved across vertebrates. To test the hypothesis, I will expose rainbow trout by intraperitoneal injections to GEM at a concentration commonly used to demonstrate its pharmacological effects in rodents (i.e. 100 mg GEM/kg). Furthermore, I will measure three pharmacologically-relevant endpoints in rainbow trout after exposure to GEM. These are: 1) the concentration and composition of lipids and lipoproteins, 2) the activity of enzymes associated with lipid and lipoprotein metabolism, and 3) the gene expression of enzymes and transcription regulators. The results of these measurements are presented in two chapters; Chapter 2 examines changes to lipoproteins and Chapter 3 examines changes to tissue lipid metabolism. Chapter 4 will summarize the key findings of this thesis and discuss whether any of the endpoints measured should be evaluated using an environmentally-relevant concentration of fibrates, since fish are continually exposed to these compounds in their environment.

## **Chapter 2: Gemfibrozil changes the quality and quantity of lipoproteins in the rainbow trout**

### **2.1. Introduction**

Fibrates are pharmaceuticals prescribed to reduce circulating lipid levels for preventing coronary artery disease (Backes et al., 2007). For humans, these drugs change the composition of plasma lipoproteins by increasing high-density lipoprotein cholesterol (HDL-C), decreasing very-low-density lipoprotein triglyceride (VLDL-TG) (Gnasso et al., 1986; Manttari et al., 1990) and increasing the content of poly-unsaturated fatty acids (PUFA) in plasma (Nyalala et al., 2008; Smith et al., 2002). Fibrate drugs act by binding to the ligand-activated transcriptional regulator called Peroxisome Proliferator-Activated Receptor  $\alpha$  (PPAR $\alpha$ ) (Forman et al., 1997). When bound by a ligand, PPAR $\alpha$  regulates the expression of an array of genes involved in lipid and lipoprotein metabolism (Mandard et al., 2004). PPAR $\alpha$  agonists enhance the expression of lipoprotein lipase (LPL) (Schoonjans et al., 1996; Yamazaki et al., 2002) and its increased activity is a principal mechanism by which fibrates exert their hypolipidemic effects and modify lipoprotein composition (Boberg et al., 1977; Grundy and Vega, 1987; Remick et al., 2008; Saku et al., 1985). LPL is bound to the capillary endothelium and its lipolytic activity is dependent on the surface apoprotein (apo) moiety of the passing lipoproteins, with apoCII activating and apoCIII inhibiting activity (Mead et al., 1999; Merkel et al., 1998). Fibrates suppress hepatic apoCIII gene expression (Staels et al., 1995) and decrease the apoCIII:apoCII ratio in both

rats and humans (Gnasso et al., 1986; McConathy et al., 1992; Saku et al., 1985; Wang et al., 1992). However, these physiological responses to fibrates are variable even among human patients (Backes et al., 2007), and because the battery of genes regulated by PPARs is different across mammals (Mukherjee et al., 1994), predicting the hypolipidemic effects of these drugs in non-mammalian species is challenging at best.

The varied bioactivity of fibrates in aquatic vertebrates is well established. For instance, by decreasing testosterone concentration and the production of eggs and sperm (Mimeault et al., 2005; Runnalls et al., 2007; Weston et al., 2009), fibrates have deleterious effects on the reproductive axis of teleosts. Fibrates also challenge the hepatic antioxidant systems in teleosts by downregulating the activity of cytochrome P450 enzymes and upregulating the activity antioxidant enzymes (Mimeault et al., 2006; Thibaut et al., 2006). Direct effects on lipid utilization in fish include inhibition of yolk absorption in zebrafish (*Danio rerio*) (Raldua et al., 2008), alteration of mitochondrial and peroxisomal lipid metabolism in the liver of rainbow trout (Du et al., 2004), and changes in tissue fatty acid (FA) profiles in the grass carp (*Ctenopharyngodon idella*) (Du et al., 2008). Despite recent attempts to characterize the effects of fibrates on lipoprotein composition (Du et al., 2008; Runnalls et al., 2007), a comprehensive assessment of the effects of these PPAR $\alpha$  agonists on lipoprotein metabolism, blood-lipid classes and their fatty acid composition has not been carried out in fish.

The lipid transport system of rainbow trout resembles that of humans. Both have similar lipoprotein classes (Babin and Vernier, 1989), exhibit LPL activity (Albalat et al., 2006), and express PPARs. However, rainbow trout possess multiple

PPAR paralogs (Leaver et al., 2005; Leaver et al., 2007) and considerable interspecies differences in the concentration and physiological uses of lipoproteins do exist (see Chapman, 1980; Weber, 2009). Such differences suggest an evolutionary divergence in the regulation of lipoprotein metabolism to match structure with function. Hence, even though the pharmacological targets of fibrates are conserved (i.e. PPARs), the physiological responses elicited through these targets may not be.

This study is designed to characterize how lipoprotein metabolism of trout responds to gemfibrozil (GEM), a fibrate drug, and whether these responses highlight features of lipoprotein metabolism that are unique to these organisms. These objectives may be useful in identifying novel physiological effects of GEM- a ubiquitous aquatic contaminant- in the rainbow trout. The features of lipoprotein metabolism investigated in this chapter include lipoprotein concentration, lipoprotein composition (i.e. their lipid and FA content), and estimated changes in lipoprotein size. This chapter will also investigate whether trout respond to fibrates by changing the activity and gene expression of LPL.

## **2.2 Materials and Methods**

### **2.2.1 Animals and Experimental Design**

#### **2.2.1.1 Animals**

Juvenile female rainbow trout, *Oncorhynchus mykiss*, were purchased from Linwood Acres Trout Farm (Campbellcroft, ON, Canada) and were kept in a 1300 L

flow-through holding tank in dechloraminated, well-oxygenated City of Ottawa tapwater at 13°C under 12·h L:12·h D photoperiod. Fish were acclimated to these conditions for at least 1 month before experiments. Prior to GEM exposure, trout were fed floating fish pellets (Martins Mills, Elmira, ON, Canada) five times per week until satiated. During the exposure period, the fish were fasted and kept in 115 L fiberglass tanks at a density of 8 fish per tank. All experiments were approved by the Animal Care Committee of the University of Ottawa and adhere to guidelines established by the Canadian Council on Animal Care for the use of animals in teaching and research.

#### 2.2.1.2 Experimental design

Rainbow trout ( $210.6 \pm 3.5$  g) were randomly divided into experimental (n=12) and control (n=12) groups. Groups were exposed to dimethylsulfoxide (DMSO; Sigma) (control) or GEM dissolved in DMSO by intraperitoneal injections (i.p.) every third day for 15 days (between February and March). The experimental group received a dose of 100 mg GEM/kg fish, a dose known to alter circulating lipids in rodents (Ozansoy et al., 2001), and injections for both groups were given at a volume was 0.5  $\mu$ L/g fish. Three day injection intervals were chosen based on the Mimeault et al. (2005) study that showed the majority (~90%) of GEM injected i.p. into goldfish (5 mg GEM/kg fish) was nearly completely cleared from the plasma after three days. DMSO was confirmed as an appropriate control as its effects on plasma lipid concentration did not differ from 0.9% saline when injected i.p. (see Appendix A). After 15 days, fish were anesthetized with benzocaine (100 mg/L; Sigma) prior to blood sampling from the caudal vessels using a 1 mL syringe fitted

with a 23-gauge needle. Fish were then sacrificed by a swift blow to the head followed by cervical dislocation and the liver, adipose tissue (~200 mg), red muscle (~800 mg), white muscle (1.0 g) and heart were removed, weighed, snap frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  until used. Plasma was separated from blood by a 2 min centrifugation at 12,000 g and aliquots were stored at  $-80^{\circ}\text{C}$  until analyses of plasma lipids and cholesterol. One aliquot of fresh plasma was used immediately for lipoprotein determination.

### 2.2.2 Plasma Biochemistry

#### 2.2.2.1 Lipoprotein separation from whole plasma

*Density gradient set-up:* Lipoprotein classes were separated from whole plasma by density-gradient ultracentrifugation using Optiprep (60% Iodixanol; Axis-Shield, Oslo, Norway), a product that self-generates density gradients. The density gradient was developed and optimized using plasma from non-injected control fish according to Yee et al. (2008) and Graham et al. (1996), with necessary adjustments in rotor speed to account for differences in equipment specifications (ultracentrifuge/rotor types). To set-up the density gradient, a 20% iodixanol-plasma solution was prepared by diluting 0.5 ml Optiprep with 1 ml fresh, chylomicron-free plasma; chylomicrons are absent in fasted fish (Kjaer et al., 2009). Iodixanol solutions of 9% and 12% were prepared by diluting 3 mL Optiprep with 17 mL HEPES-buffered saline (0.85% (w/v) NaCl, 10 mM HEPES-NaOH, pH7.4) and 3 mL Optiprep with 12 mL HEPES-buffered saline, respectively. The three-step gradient was prepared by carefully under-layering each successive concentration using a 5

mL disposable syringe fitted with a long, wide, metal cannula. Briefly, 1.3 mL of the 9% solution was placed in the Optiseal ultracentrifugation tube (capacity 4.7 mL) (Beckman-Coulter, Palo Alto, CA, USA); this was then under-layered with 1.3 mL of the 12% solution which was then under-layered with a final 1.3 mL of the 20% iodixanol/plasma solution. The tube was then carefully filled to the top with HEPES-buffered saline (0.8 mL). The tube was capped and placed in an TLA 100.4 rotor (Beckman-Coulter) and centrifuged at 60,000 rpm (195,552 g) for 3.75 h at 13 °C in an Optima XL-100K ultracentrifuge (Beckman-Coulter) set at slow acceleration and deceleration. Samples were fractionated within 1 h of centrifugation.

*Lipoprotein collection:* The centrifuged plasma was fractionated by puncturing the base of the Optiseal tube with a 23 gauge needle and collecting the drips, from the most dense to the least dense, into approximately 32 collection tubes (~150 µL per tube). The density of each fraction was determined by measuring absorbance at 420 nm using a quartz microplate and a microplate reader (Molecular Device Spectramax Plus 384, Sunneyvale, CA). Profiles of the density and the triglyceride and cholesterol content of the fractionated plasma are shown in Appendix B. Groups of collection tubes were pooled, based on their density, to obtain the three main lipoprotein categories; e.g. HDL = 1-7, LDL = 8-24, VLDL = 25-32, where densities in mg/dL are  $d > 1.080$  for HDL,  $1.080 > d > 1.040$  for LDL and  $d < 1.040$  for VLDL according to (Babin and Vernier, 1989). Lipoproteins were stored at  $-80^{\circ}\text{C}$  for subsequent analysis of lipid and cholesterol content. The concentration of lipoproteins was calculated as the sum of phospholipids + triglycerides + cholesterol.

#### 2.2.2.2 Lipid composition of whole plasma and lipoprotein classes

*Spectrophotometric assays:* Triglyceride, phospholipid and cholesterol concentrations from both whole and separated (i.e. lipoprotein classes) plasma were assessed using commercial kits (triglyceride and cholesterol, TECO Diagnostics, Anaheim, California, USA; phospholipid, WAKO Diagnostics, Richmond, Virginia, USA) and protein using the Bicinchoninic Acid determination assay (Sigma) with bovine serum albumin (BSA) as standard. Whole and separated protein concentrations are shown in Appendix C.

*Gas chromatography:* The FA content of both whole plasma and the three lipoprotein categories were measured by gas chromatography after acid transesterification (Chapelle and Zwingelstein, 1984). Briefly, plasma lipids were extracted with a 2:1 (v/v) mixture of chloroform and methanol (Folch et al., 1957) and centrifuged (3000 g for 10 min). The pellet was discarded and the supernatant was filtered before adding KCl (0.25%) to help eliminate water-soluble compounds. After shaking, the mixture was heated for 3 min at 72 °C to separate aqueous and organic phases. The aqueous phase was discarded and the organic phase was dried at 40 °C under a stream of N<sub>2</sub> gas. Total lipids were immediately resuspended in 3:2 (v/v) hexane:isopropanol and stored at -20 °C before separating them into two lipid classes. Total plasma lipids were dried and resuspended in a small volume of chloroform and applied to a Supelclean solid-phase extraction column (100 mg LC NH<sub>2</sub>; Sigma) used to separate triglycerides and phospholipids by sequential elution as described previously by Bernard et al. (1999). Heptadecanoic acid (17:0) was used as an internal standard as preliminary experiments showed the absence of this acid from the plasma of rainbow trout. The internal standard was added as a free acid to the triglyceride and phospholipid fractions after solid-phase column

separation, but before trans-esterification. The fatty acid methyl esters obtained from each lipid fraction were analyzed on an Agilent Technologies 6890N gas chromatograph equipped with a fused silica capillary column (Supelco DB-23, 60 mX0.25 mm i.d., 0.25  $\mu\text{m}$  film thickness) using hydrogen as the carrier gas at constant pressure and at a linear flow of  $2580 \text{ cm}\cdot\text{min}^{-1}$ . The chromatograph was equipped with an automatic injection system (Agilent Technologies 7683B Series). The following conditions were used during analysis: a) oven temperature was programmed for 1 min at  $130 \text{ }^\circ\text{C}$ , up to  $170 \text{ }^\circ\text{C}$  at  $6.5 \text{ }^\circ\text{C}\cdot\text{min}^{-1}$ , up to  $215 \text{ }^\circ\text{C}$  at  $2.75 \text{ }^\circ\text{C}\cdot\text{min}^{-1}$ , held at  $215 \text{ }^\circ\text{C}$  for 12 min, up to  $230 \text{ }^\circ\text{C}$  at  $40 \text{ }^\circ\text{C}\cdot\text{min}^{-1}$ , and held at  $230 \text{ }^\circ\text{C}$  for 3 min; b) injector temperature was  $270 \text{ }^\circ\text{C}$  using a 50:1 split ratio; and, c) detector temperature was  $280 \text{ }^\circ\text{C}$ . Each methyl ester was identified specifically by determining its exact retention time using authentic standards (Supelco, Bellefonte, PA, USA). Concentrations of individual fatty acids were calculated by comparing the areas under their respective peaks with that of the internal standard peak.

### 2.2.3 Enzyme assays

#### 2.2.3.1 Lipoprotein lipase

LPL (E.C. 3.1.1.34) activity was assessed in frozen tissue samples within 4 weeks of sampling according to Magnoni and Weber (2007). Liver, red muscle, white muscle and adipose tissue ( $\sim 0.5 \text{ g}$ ) were homogenized in 9 vol buffer (0.2 M sucrose, 25 mM Tris-HCl, 1 mM EDTA, adjusted to pH 7.4) using a ground glass homogenizer on ice. Homogenates were centrifuged ( $20,000 \text{ g}$ , 20 min at  $4 \text{ }^\circ\text{C}$ ) and

the clear phase between the top, solid layer and the pellet was used for LPL analysis.

The substrate used to measure LPL activity was a mixture containing 2 mL 20% lipid solution (Intralipid, Sigma) and 8 mL distilled water that is emulsified with 45  $\mu\text{Ci}$  tri[9,10(n)- $^3\text{H}$ ]oleate (Amersham, Buckinghamshire, UK). This emulsion is commonly used to measure LPL in a variety of vertebrate tissues (Karpe and Hultin, 1995; Lindberg and Olivecrona, 1995) and its suitability as an artificial substrate was specifically demonstrated for rainbow trout plasma and tissues (Albalat et al., 2005; Albalat et al., 2006; Lindberg and Olivecrona, 2002). This mixture was sonicated for 5 min at 70% pulse mode and low setting (Branson Sonifier 450; Danbury, CT, USA). Each assay was carried out using a 50  $\mu\text{L}$  aliquot of the emulsion as substrate, mixed with 50  $\mu\text{L}$  preheated rat serum, 250  $\mu\text{L}$  assay medium (0.3 M Tris, 0.2 M NaCl, 5 mL/L of 100 U/ml heparin, 12% BSA, adjusted to pH 8.5), and 100  $\mu\text{L}$  tissue homogenate. The reaction was stopped after a 1 h incubation at 20 °C by adding 3 mL methanol:chloroform:heptane (1.41:1.25:1 v/v/v) and 100  $\mu\text{L}$  0.1 M NaOH and vortexing. After centrifugation (1500 g, 10 min at 20 °C), 500  $\mu\text{L}$  of the upper phase was counted in 10 ml Safety Solve cocktail (Research Products, Mount Prospect, IL, USA) using a liquid scintillation counter (Beckman-Coulter CS6500). All LPL determinations were performed in triplicate and values averaged.

#### 2.2.4 Gene expression

Hepatic transcript levels of LPL, apoCII and elongation factor 1  $\alpha$  (EF1 $\alpha$ ) were assessed using real-time RT-PCR (QRT-PCR). Total RNA was extracted and DNase treated using the RNeasy® Micro Kit (Qiagen, Mississauga, ON). Total RNA

quantity was measured using a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) and only RNA with a 260/280 ratio of >1.9 was used for cDNA synthesis. First strand cDNA synthesis was prepared from 2 µg total RNA and 200 ng random hexamer primers (Invitrogen, Burlington, ON) using Superscript II RNase H–reverse transcriptase (SSII). Each PCR reaction contained the following final concentrations: 20 ng first-strand cDNA template, 1X QPCR buffer, 5 mM MgCl<sub>2</sub>, 150–600 nM gene specific primer, 0.5X SYBR green (Invitrogen), 200 µM dNTPs, 1.25U HotStarTaq (Invitrogen), and 20 µM ROX reference dye, in a 25 µL reaction volume. The thermal cycling parameters and primer sequences are described in Kolditz et al. (2008) and Shen et al. (2000) for LPL and apoCII, respectively. Dilutions of cDNA pooled from all individual samples (both control and treated) were used to construct a relative standard curve for each primer set. After the reaction was completed, a dissociation curve was produced starting at 55 °C (+1 °C/30 s). QRT-PCR used a MX4000® Multiplex Quantitative PCR system (StrataGene, La Jolla, CA, USA) and data were analyzed using the MX4000 Software Package. Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using the amplified PCR product from a pool of all individual samples (n=16) as a template, and were used to calculate mRNA copy number in each sample. Genes were normalized to the expression of the control gene EF1α (elongation factor 1 α) since its expression did not change between experimental groups. In all cases, only standard curves with slopes between –3.2 and –3.5 with R<sup>2</sup> values of >0.98 were used. After each run, a random subset of PCR products (total n=4) from control and treatment groups were pooled and purified using a Minelute Reaction Cleanup kit

(Qiagen) and sequenced with a CEQ™8000 Genetic Analysis System(Beckman-Coulter) to confirm specificity of the assays.

### 2.2.5 Statistical analyses

Experimental results are presented as means  $\pm$  standard error of the mean (s.e.m.). All statistical analyses were conducted using SigmaPlot™ 11.0 software (SPSS Corp., Chicago, IL). Statistical significance was tested with One-Way ANOVA on raw data, or in the case of percentages and ratios, on transformed data (arcsin\* $\sqrt{\text{data}}$ ), followed by a Bonferroni correction for multiple comparisons. A value of  $P < 0.05$  was considered significant.

## 2.3 Results

### 2.3.1 Total circulating lipids

The total concentration of circulating lipid classes is presented in Figure 2.1A. All measured circulating lipids in the GEM-treated group (100 mg GEM/kg) changed significantly compared with the control group (100% DMSO): phospholipids (-34%,  $P=0.002$ ), triglycerides (-47%,  $P=0.004$ ), and cholesterol (-22%,  $P=0.041$ ). Despite major differences to the concentration of circulating lipids after GEM exposure, changes in the relative concentration (i.e. the lipid profile) were small and significant only in triglycerides (-2.7%,  $P=0.022$ ) and in cholesterol (+3.4%,  $P < 0.001$ ) (Fig. 2.1B).

### 2.3.2 Lipoprotein classes

The concentrations of total circulating lipoprotein classes in the GEM-treated group were significantly lower than those of the control group (Fig. 2.2A). As noted in section 2.2.2.1, the concentration of lipoproteins in this study is calculated as the sum of the concentrations of phospholipids, triglycerides, and cholesterol contained in the plasma fractionated by density-gradient ultracentrifugation. The largest difference in concentration occurred in the VLDL fraction (-58%,  $P=0.02$ ) but the decrease in total lipoprotein concentration (-29%,  $P=0.001$ ) reflected concentration decreases in the two most abundant lipoprotein classes, HDL (-27%,  $P=0.002$ ) and LDL (-34%,  $P=0.001$ ). The profile, or relative concentration of plasma lipoproteins, calculated here (Fig. 2.2B) is in good agreement with other studies in rainbow trout (Babin and Vernier, 1989). Unlike lipoprotein concentration, the plasma lipoprotein profile was unchanged after exposure to GEM.

To investigate possible changes in lipoprotein composition, the percent abundance of phospholipids, triglycerides and cholesterol in each of the three lipoprotein classes was measured and plotted in Figure 2.3. Cholesterol is the only lipid that did not change in any of the lipoprotein classes after treatment with GEM (Fig. 2.3C). Significant changes to phospholipids and triglycerides were detected and these changes were shown to be lipoprotein class specific (Fig. 2.3A, B). For example, the percent abundance of phospholipids was higher only in the VLDL fraction (+17%,  $P=0.006$ ), while that of triglycerides was lower across all lipoprotein classes (HDL: -2%,  $P=0.012$ ; LDL: -3%,  $P=0.007$ ), with the greatest difference occurring in VLDL (-15%,  $P=0.004$ ), possibly reflecting the specificity of GEM for triglyceride metabolism in this species.

Differential changes to the surface and core of lipoprotein particles results in changes to lipoprotein size, so the phospholipid:triglyceride (PL: TG) ratio was calculated for each lipoprotein class as a proxy measurement of this change. Figure 2.4 shows that in each lipoprotein class the ratio increased (HDL: +1.6 fold,  $P=0.020$ ; LDL: +1.6 fold,  $P=0.009$ ; VLDL: +2.2 fold,  $P=0.027$ ), supporting an average decrease of lipoprotein size in each lipoprotein class after exposure to GEM. However, no difference in the fold-change in the PL: TG ratio between lipoprotein classes was detected.

### 2.3.3 Fatty acids

Changes of the individual FA of triglycerides and phospholipids were measured within each lipoprotein class (Tables 2.1 and 2.2). The major differences in FA content occurred in the content of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). The effect of GEM on the content of EPA and DHA varied across both lipid (i.e. phospholipid and triglyceride) and lipoprotein class (i.e. HDL, LDL and VLDL). The content EPA in phospholipids differed only in the VLDL fraction (-1.5%  $P=0.009$ ) (Table 2.1) while the EPA of triglycerides differed in all three lipoprotein classes (HDL: -2%,  $P=0.008$ ; LDL: -3%,  $P=0.001$ ; VLDL: -3.5%,  $P<0.001$ ) (Table 2.2). DHA in phospholipids decreased only in the VLDL fraction (-8%  $P<0.001$ ) (Table 2.1) and the DHA of triglycerides did not change after exposure to GEM (Table 2.2). These changes contributed to a significant difference in the composition of PUFA of VLDL (-11%,  $P=0.001$  for triglycerides and -8%,  $P=0.035$  for phospholipids). To more clearly illustrate that the effect of GEM on EPA and DHA varies across both lipid and lipoprotein class, the PL: TG ratio for EPA and

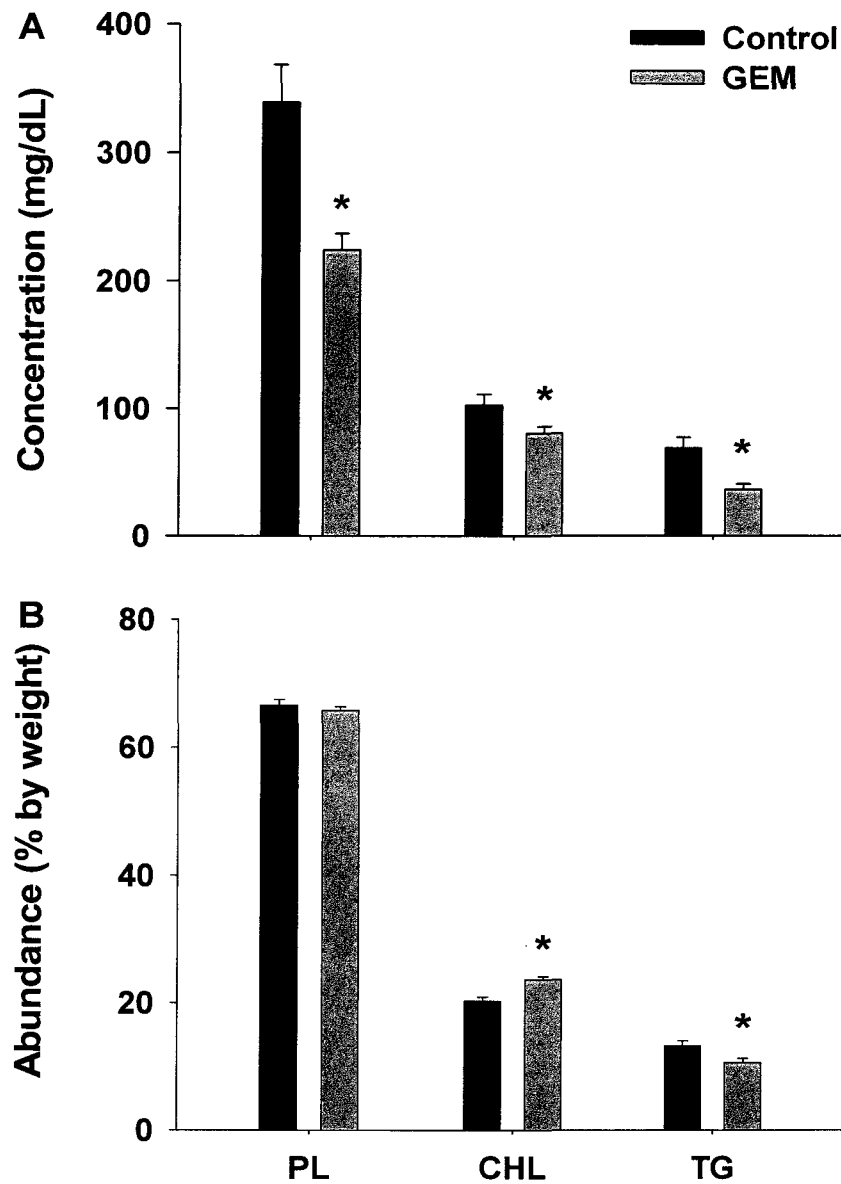
DHA in each of the three lipoprotein classes was plotted (Fig. 2.5). The PL:TG ratio of EPA differed significantly in the LDL (+0.6,  $P<0.001$ ) and VLDL(+0.5,  $P<0.009$ ) fractions, but not in HDL (Fig. 2.5A). The PL:TG ratio for DHA (Fig. 2.5B) followed that of EPA (+0.8 in LDL,  $P=0.026$ ), although differences in the VLDL fraction were not large enough to reach statistical significance ( $P>0.05$ ).

#### 2.3.4 LPL activity

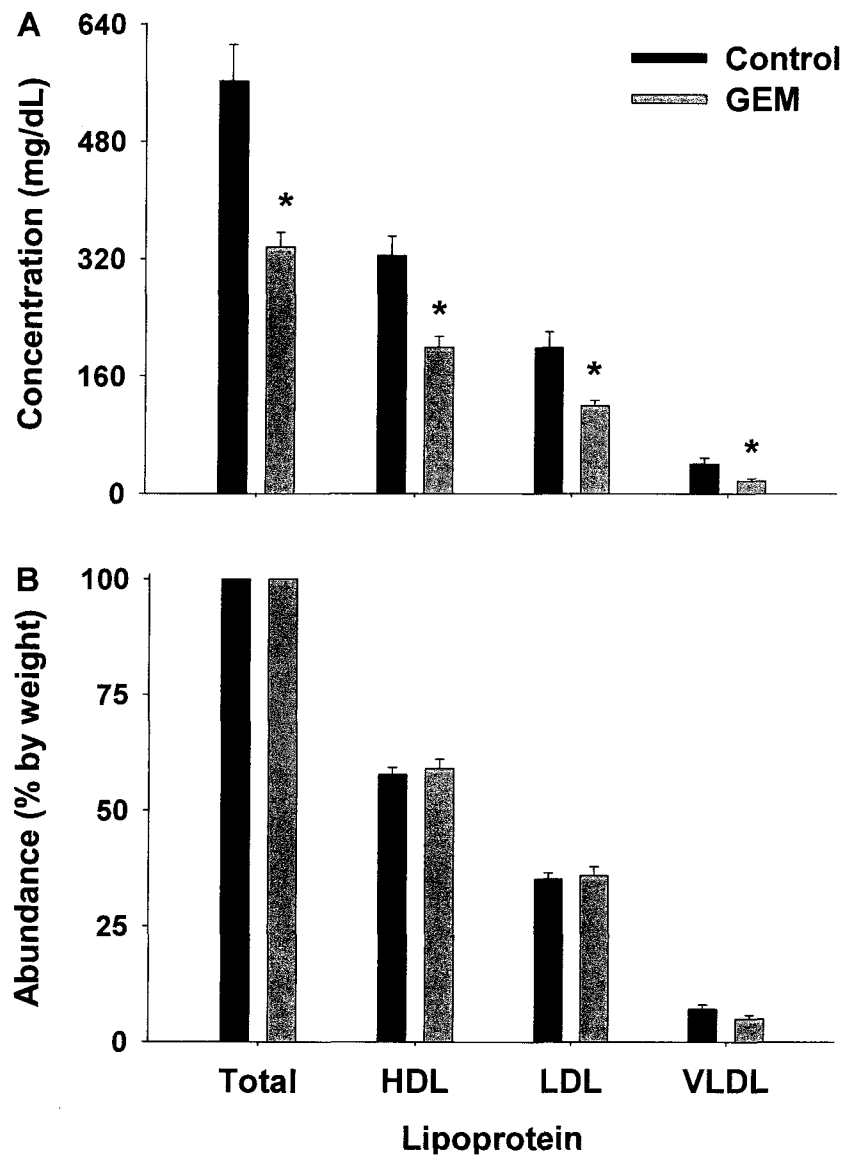
The activity of LPL in white muscle, red muscle, liver and adipose tissue were estimated to determine whether lipolysis by LPL was involved in changes to the plasma lipid and FA contents (Fig. 2.7A). No changes were detected in LPL activity between the treatment and control groups for any tissue assessed (liver,  $P=0.071$ ; red muscle,  $P=0.066$ ; adipose tissue,  $P=0.241$ ; white muscle,  $P=0.685$ ).

#### 2.3.5 LPL and apoCII gene expression

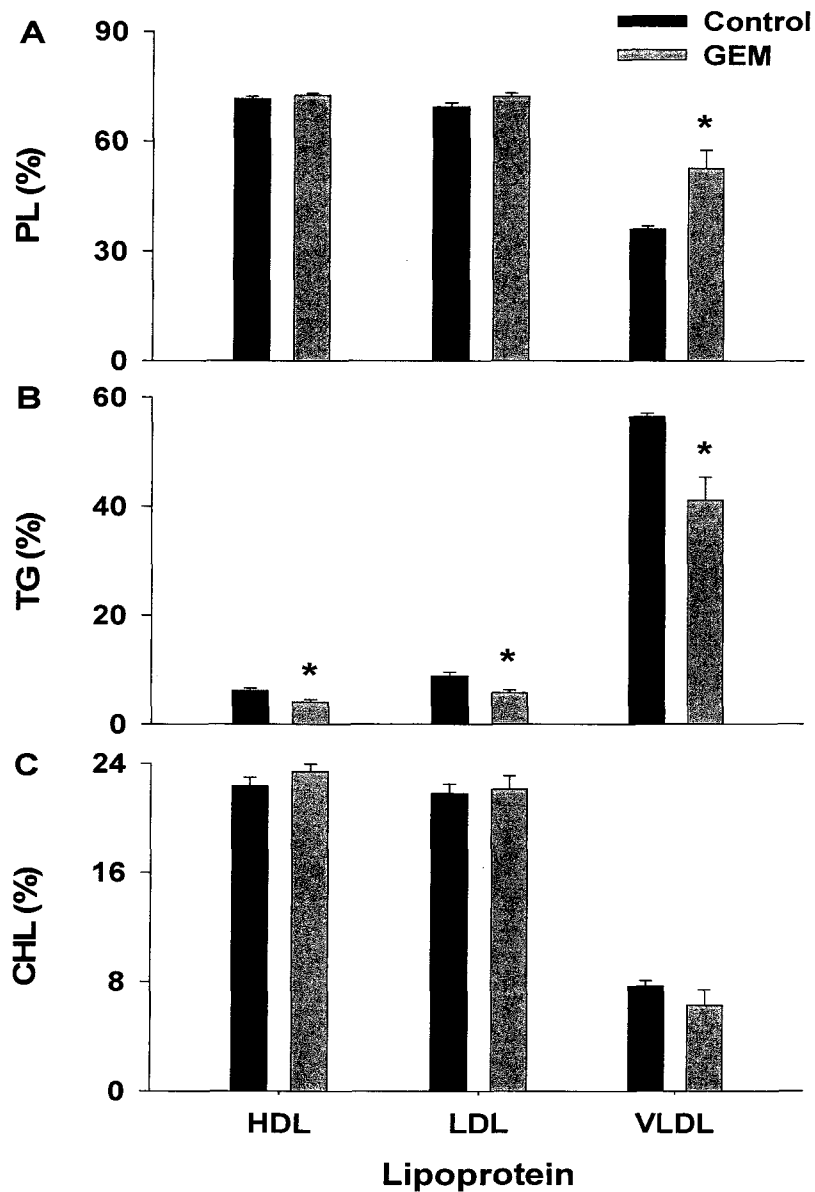
Hepatic transcript levels of LPL and its cofactor, apoCII, were estimated to establish a possible link between GEM and the expression of the LPL gene. Hepatic LPL transcripts in the GEM-treated group were increased significantly compared with the control (+2.4 fold,  $P<0.05$ ) while there were no changes in apoCII transcripts.



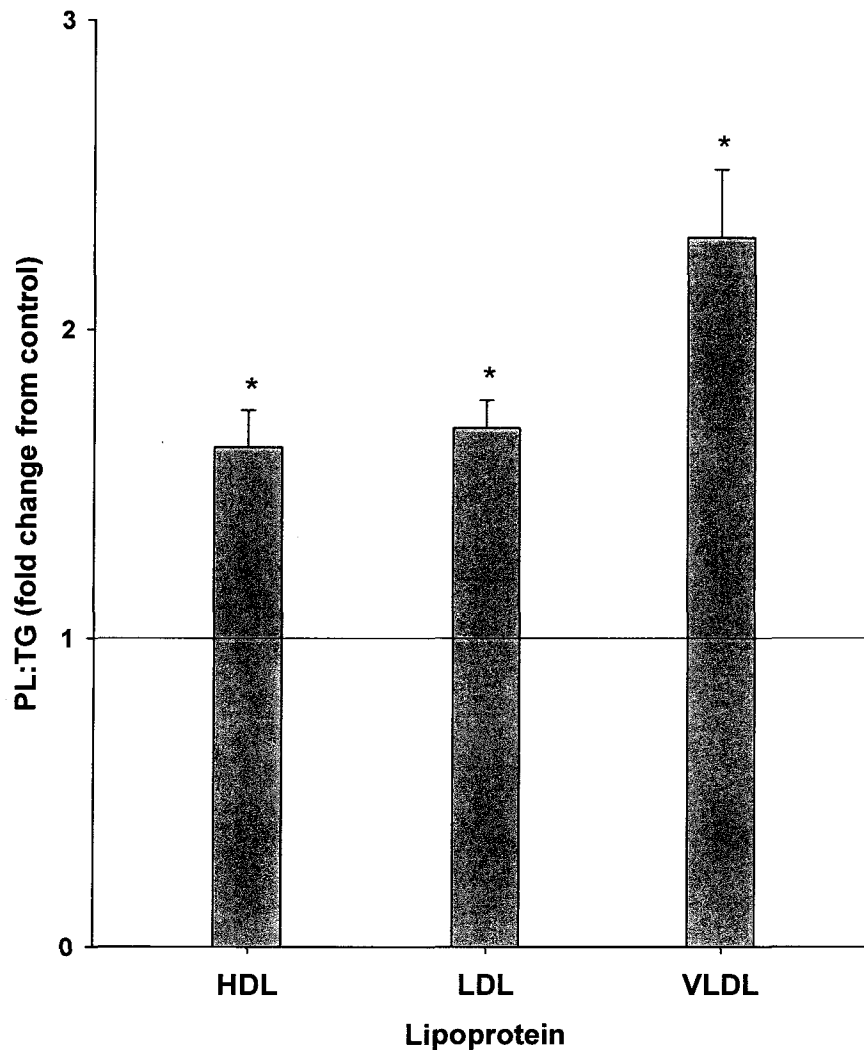
**Figure 2.1.** The concentration (A) and profile (B) of phospholipids (PL), triglycerides (TG) and cholesterol (CHL) in the plasma of rainbow trout. Fish were injected with either 100% DMSO (Control) or 100 mg/kg Gemfibrozil (GEM) over a 15 day period. Data represent means + s.e.m. (n=12). A significant difference between groups is indicated with an asterisk ( $P < 0.05$ ).



**Figure 2.2.** The concentration (A) and profile (B) of lipoproteins in the plasma of rainbow trout. Total lipoprotein concentrations are calculated as the sum of phospholipids + triglycerides + cholesterol in each lipoprotein class. Data represents means + s.e.m. (n=7). A significant difference between treatment groups is indicated with an asterisk ( $P \leq 0.02$ ). HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.



**Figure 2.3.** The composition of phospholipid (A), triglyceride (B), and cholesterol (C) in the lipoproteins of rainbow trout. Total lipoprotein concentrations were calculated as the sum of PL + TG + CHL in each lipoprotein class. See Figures 2.1 for details. Data represents means + s.e.m. (n=7). A significant difference between treatment groups is indicated with an asterisk ( $P < 0.05$ ). See Figure 2.2 for abbreviations.



**Figure 2.4.** Effect of GEM on the average size of lipoprotein particles. Ratio values (PL: TG) are presented as means + s.e.m (n=7); the horizontal line represents control values (n=7). Actual control ratios are  $12.2 \pm 0.9$ ,  $8.3 \pm 0.9$ ,  $0.6 \pm 0.02$  for HDL, LDL and VLDL, respectively. A significant difference between treatment groups is indicated with an asterisk ( $P < 0.03$ ). No difference in the fold change of PL: TG between lipoprotein classes was detected ( $P > 0.05$ ). See Figure 2.2 for abbreviations.

**Table 2.1.** The fatty acid composition of phospholipids in the lipoproteins of control and GEM-treated rainbow trout. Data represent means ( $\pm$  s.e.m.) of the percent of total fatty acids by concentration ( $\mu$ mol fatty acids/ml plasma). A significant difference between treatment (n=11) and control groups (n=12) is indicated with an asterisk ( $P<0.05$ ).

Fatty acids	HDL <sup>1</sup>		LDL <sup>1</sup>		VLDL <sup>1</sup>	
	Control	GEM	Control	GEM	Control	GEM
EPA <sup>2</sup>	6.9 $\pm$ 0.2	5.6 $\pm$ 1.0	6.2 $\pm$ 0.3	6.5 $\pm$ 0.3	5.1 $\pm$ 0.3	3.9 $\pm$ 0.2*
DHA <sup>2</sup>	40.7 $\pm$ 1.0	38.2 $\pm$ 0.9	37.5 $\pm$ 1.5	37.9 $\pm$ 1.6	30.2 $\pm$ 1.7	21.7 $\pm$ 1.0*
$\Sigma$ n-3 <sup>3</sup>	54.5 $\pm$ 1.3	51.0 $\pm$ 1.1	49.6 $\pm$ 1.8	51.1 $\pm$ 1.9	39.4 $\pm$ 2.3	28.4 $\pm$ 1.5*
$\Sigma$ n-6 <sup>3</sup>	1.0 $\pm$ 0.02	1.1 $\pm$ 0.03	1.1 $\pm$ 0.1	1.0 $\pm$ 0.1	0.5 $\pm$ 0.2	0.3 $\pm$ 0.1
$\Sigma$ SFA <sup>4</sup>	28.7 $\pm$ 1.1	30.7 $\pm$ 0.9	32.2 $\pm$ 1.4	33.7 $\pm$ 1.0	46.8 $\pm$ 6.5	53.3 $\pm$ 1.8
$\Sigma$ MUFA <sup>4</sup>	15.6 $\pm$ 0.4	17.0 $\pm$ 0.9	16.9 $\pm$ 1.8	14.0 $\pm$ 1.4	11.7 $\pm$ 1.4	16.6 $\pm$ 1.7
$\Sigma$ PUFA <sup>4</sup>	55.5 $\pm$ 1.4	52.2 $\pm$ 1.1	50.8 $\pm$ 1.7	52.2 $\pm$ 1.9	41.3 $\pm$ 2.1	30.0 $\pm$ 1.3*

1- HDL, high density; LDL, low density; VLDL, very low density lipoproteins.

2- Eicosapentaenoic acid (EPA, 20:5 n-3) and Docosapentaenoic acid (DHA, 22:6 n-3)

3- Omega 3 (n-3) and Omega 6 (n-6) fatty acids

4- Saturated (SFA); Mono-unsaturated (MUFA) ; Poly-unsaturated (PUFA) fatty acids

**Table 2.2.** The fatty acid composition of triglycerides in the lipoproteins of control and GEM-treated rainbow trout. Data represent means ( $\pm$  s.e.m.) of the percent of total fatty acids by concentration ( $\mu$ mol fatty acids/ml plasma). A significant difference between treatment (n=11) and control groups (n=12) is indicated with an asterisk ( $P<0.05$ ).

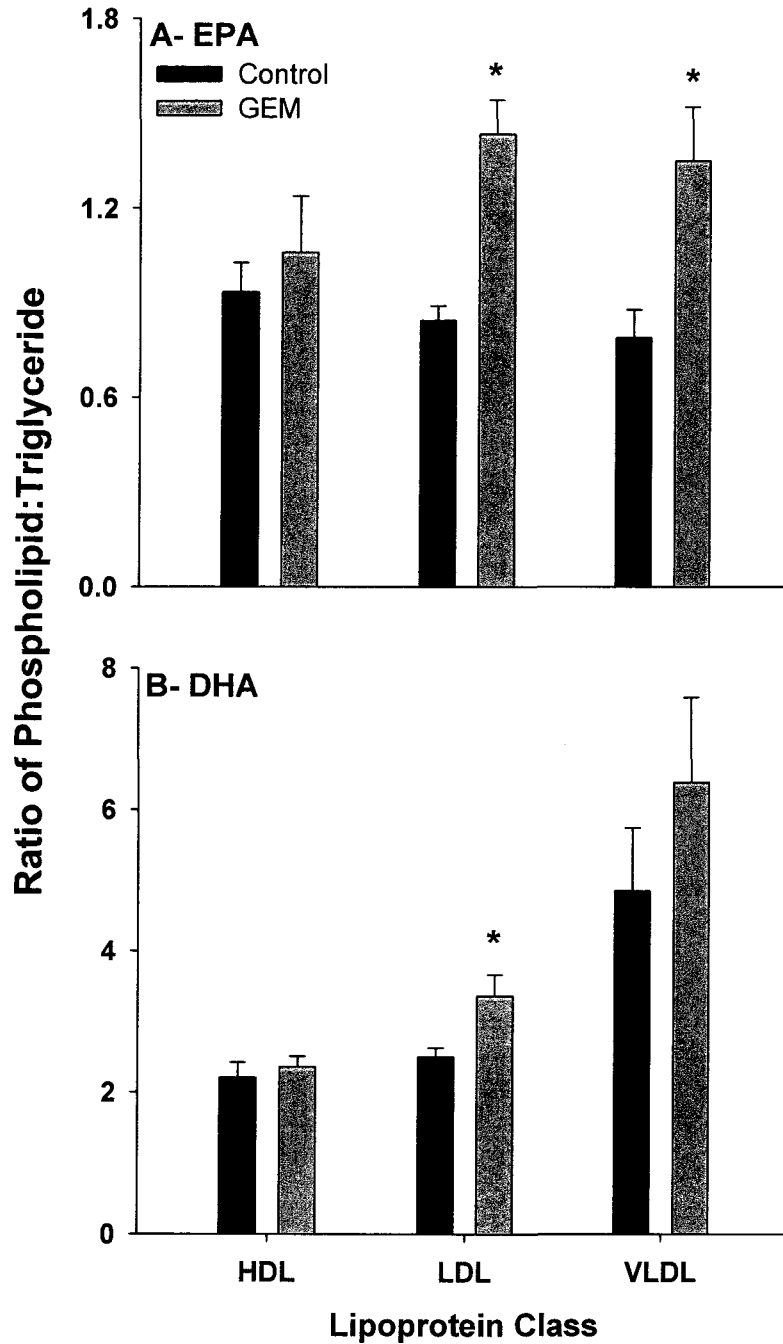
Fatty acids	HDL <sup>1</sup>		LDL <sup>1</sup>		VLDL <sup>1</sup>	
	Control	GEM	Control	GEM	Control	GEM
EPA <sup>2</sup>	7.8 $\pm$ 0.7	5.7 $\pm$ 0.4*	7.5 $\pm$ 0.5	4.7 $\pm$ 0.4*	6.7 $\pm$ 0.6	3.2 $\pm$ 0.4*
DHA <sup>2</sup>	19.7 $\pm$ 1.7	16.8 $\pm$ 1.3	15.5 $\pm$ 1.3	11.9 $\pm$ 1.2	7.0 $\pm$ 0.9	4.4 $\pm$ 1.0
$\Sigma$ n-3 <sup>3</sup>	37.0 $\pm$ 2.5	31.5 $\pm$ 1.6	33.2 $\pm$ 2.2	28.8 $\pm$ 3.3	27.9 $\pm$ 1.1	21.0 $\pm$ 3.0
$\Sigma$ n-6 <sup>3</sup>	0.7 $\pm$ 0.2	0.6 $\pm$ 0.2	1.4 $\pm$ 0.1	0.6 $\pm$ 0.2*	2.0 $\pm$ 0.1	0.8 $\pm$ 0.3*
$\Sigma$ SFA <sup>4</sup>	30.1 $\pm$ 3.1	34.3 $\pm$ 2.4	29.2 $\pm$ 2.8	33.8 $\pm$ 3.9	25.3 $\pm$ 2.3	36.1 $\pm$ 5.3
$\Sigma$ MUFA <sup>4</sup>	31.6 $\pm$ 1.5	33.3 $\pm$ 1.9	35.9 $\pm$ 2.2	36.6 $\pm$ 2.6	44.7 $\pm$ 1.8	41.9 $\pm$ 3.5
$\Sigma$ PUFA <sup>4</sup>	38.2 $\pm$ 2.9	32.3 $\pm$ 1.8	34.8 $\pm$ 2.2	29.5 $\pm$ 3.3	29.9 $\pm$ 1.3	21.9 $\pm$ 3.2*

1- HDL, high density; LDL, low density; VLDL, very low density lipoproteins

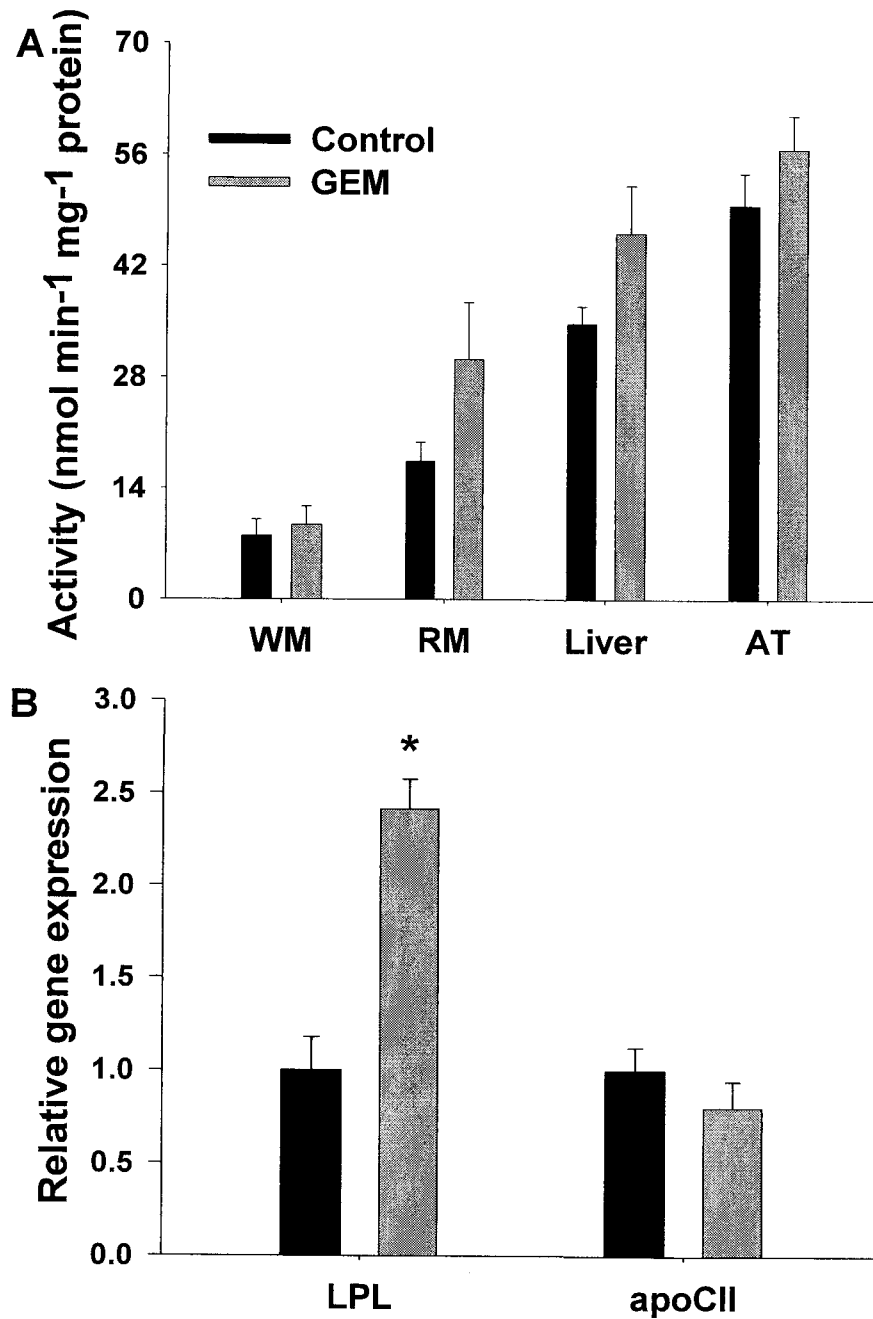
2- Eicosapentaenoic acid (EPA, 20:5 n-3) and Docosapentaenoic acid (DHA, 22:6 n-3)

3- Omega 3 (n-3) and Omega 6 (n-6) fatty acids

4- Saturated (SFA); Mono-unsaturated (MUFA) ; Poly-unsaturated (PUFA) fatty acids



**Figure 2.5.** The ratio of phospholipid:triglyceride (PL: TG) for individual fatty acids in the plasma lipoproteins of rainbow trout. Panel A) shows the ratio of PL-EPA: TG-EPA and B) shows the ratio of PL-DHA: TG-DHA. Data are presented as means + s.e.m, n=7. Statistical differences between the experimental groups is indicated with an asterisk ( $P < 0.005$ ). See Figures 2.2 and 2.5 for abbreviations.



**Figure 2.6.** The activity of lipoprotein lipase (LPL) (A) and relative (to EF1 $\alpha$ ) hepatic LPL and apoCII transcripts (B) in rainbow trout. Data are presented as means + s.e.m, n=8. Statistical differences between the experimental groups is indicated with an asterisk ( $P<0.005$ ). WM= white muscle; RM= red muscle; AT= adipose tissue.

## **2.4. Discussion**

This study shows that GEM, a mammalian PPAR $\alpha$  agonist, has a hypolipidemic effect in trout and changes the quantity and quality of their lipoproteins. This study is the first to characterize the effects of GEM, a human blood-lipid regulator, on the lipoprotein profile of a non-mammalian vertebrate. Including studies of humans and rodents, this study is one of only a few that demonstrates changes to the FA content in both lipid (i.e. phospholipid and triglyceride) and lipoprotein classes (i.e. HDL, LDL, VLDL) after exposure to a mammalian PPAR $\alpha$  agonist.

### **2.4.1 Concentration of lipids and lipoprotein classes**

GEM is prescribed to adjust circulating lipid and lipoprotein classes in hyperlipidemic humans. This study shows that GEM strongly hinders lipid availability in rainbow trout by decreasing the concentrations of all plasma lipids and, by consequence, lipoproteins. What is surprising is that while the concentration of both lipids and lipoproteins decreased, their profiles in the plasma were largely unchanged. The absence of shifts in the lipoprotein profile after GEM-treatment is unusual and highlights the possibility that the lipoprotein profile of trout is tightly regulated to reflect its pivotal roles in energy supply (Magnoni and Weber, 2007; Magnoni et al., 2006), homeoviscous adaptation (Wallaert and Babin, 1994b), and, maturation of oocytes (Wallaert and Babin, 1994a). Estimating the protein content of the lipoprotein classes could help to reveal changes to the profile that could not be distinguished in this study. Here, the total lipoprotein concentration (i.e.

phospholipid+triglyceride+cholesterol) excluded protein because the density gradient used to separate the lipoprotein classes unequally distributed plasma proteins across the lipoprotein classes; changes to protein content across classes are therefore are not comparable (see Appendix C for details). In humans, GEM can change the lipoprotein pool in three ways: it can change the lipoprotein profile (Otvos et al., 2006), change lipoprotein size (Asztalos et al., 2008; Yang et al., 1996), and change lipoprotein composition (Gnasso et al., 1986; Manttari et al., 1990), but it is important to note that these adjustments do not always occur together after treatment with GEM. In this study, GEM changed lipoprotein composition, mainly by decreasing triglycerides (Fig. 2.3). Not only does this change suggest impairment of normal lipoprotein metabolism, it implies that the lipoprotein size decreased because of the increased ratio of PL: TG (Fig. 2.4). The structure of lipoproteins consists of a surface envelope of polar phospholipids and a non-polar core of triglycerides and esterified cholesterol. One benefit of this architecture is that average size estimates of lipoproteins can be based on ratios of their components. For example, the ratio of PL: TG is inversely related to lipoprotein size with VLDL having the lowest ratio and HDL the highest (see Fig. 2.3). It is unusual to see the decrease of lipoprotein concentration and size occurring proportionally across all three lipoprotein classes, as is reported in this study. Nevertheless, it is clear that GEM and therefore perhaps other PPAR $\alpha$  agonists can both quantitatively and qualitatively change the lipoproteins of rainbow trout.

#### 2.4.2 Lipid Composition

The FA composition of circulating lipids is affected by fibrate therapy, however there are major qualitative differences in this response across species, even just across mammals. For example, in humans, GEM-therapy increases circulating PUFA content and increases susceptibility of lipoproteins to oxidation (Nyalala et al., 2008; Smith et al., 2002); the opposite is true for rats (Vazquez et al., 1996). The response of rainbow trout to GEM is more similar to that of rats as there is a marked decrease in the percent of circulating PUFA (Table 2.1 and 2.2). Here, the decrease in the percent circulating PUFA occurred in both lipid classes (i.e. phospholipids and triglycerides), mostly due to a decrease in EPA and DHA, but the magnitude of the decreases differed depending on the the lipid-class and the lipoprotein-class (Fig. 2.6). The selective removal of FA from lipids and lipoproteins shows that GEM alters lipoproteins at every level of their structural organization and does so differently in each lipoprotein class, thus solidifying its role a blood-lipid and lipoprotein regulator in rainbow trout.

#### 2.4.3 Lipoprotein Lipase (LPL)

A primary mechanism for mediating the hypolipidemic effects of fibrates in both humans and rats is the increased clearance of triglyceride-rich lipoproteins by LPL. The action of human LPL on the hydrolysis of VLDL is shown to change the FA content of this class of lipoproteins (Agren et al., 2002). This is not surprising since human LPL shows specificity toward hydrolysis of FA located at the *sn*-1 and -3 positions of the triglyceride molecule, which are primarily occupied by mono-unsaturated FA (MUFA) (Nilsson-Ehle et al., 1973). Human LPL is also known to

have phospholipase activity and therefore it may be responsible for at least some of the effects of GEM on the FA composition of phospholipids in humans (Dugi et al., 1995). Although human LPL has little effect on the hydrolysis of circulating HDL, it is reported that both rat and chicken LPL exhibit lipase activity in the presence of both HDL and LDL (Sato et al., 1998). A similar situation may exist in fish since fish LPL is reported to carry out the function of mammalian hepatic lipase, which is involved in the hydrolysis of HDL these species (Lindberg and Olivecrona, 2002). To investigate whether LPL was associated with the change in FA content of the lipids and lipoproteins measured in this study, the transcript and activity levels of LPL, together with the transcript level of apoCII, the cofactor of LPL, were estimated. GEM-treatment significantly increased LPL transcript levels but this did not translate into an increased LPL activity in any of the tissues assayed. Furthermore, apoCII transcript levels were unchanged between treatment and control. Although these results do support that the transcription of trout LPL is regulated by PPARs, they do not support LPL being responsible for the hypolipidemic effects shown in this study. However, the lack of change in LPL activity, as it is assayed in this study, does not necessarily mean that its ability to hydrolyze lipids has not changes *in vivo*. For example, the ability of trout LPL to bind lipoproteins may be independent of its enzymatic activity, as is the case with endothelial lipases in humans, thus masking possible increases in lipolytic action *in vivo* (Fuki et al., 2003). Another possible explanation for the fixed LPL activity is that the assay used in this study is not appropriate to estimate changes in whole-body LPL activity. Heparin releases LPL from all endothelia and, therefore, post-heparin LPL activity may be a more suitable assay as the activity will reflect changes to whole-body LPL activity, not just activity

changes in the select tissues assayed here (Bisgaier, 1998; Kahri et al., 1995). Lastly, although trout LPL serves an essentially similar lipolytic role as the mammalian enzyme (Fremont et al., 1987), trout LPL does differ from its human homolog in its amino acid sequence, its tissue expression/distribution and in its basal activity (Lindberg and Olivecrona, 2002). These differences may make trout LPL unresponsive or less responsive to GEM. Thus, further studies are required to detect which lipases, if any, are involved in the FA changes seen in both phospholipids and triglycerides in this study. However, it is possible that changes to circulating FA are independent of the lipolytic activity of any enzyme. HDL and VLDL are secreted from the liver and hence changes to the FA composition of the lipoprotein lipids may be a reflection of metabolic changes within the liver itself rather than outside it. Fibrates induce hepatic peroxisomal (Gagne et al., 2006a; Yang et al., 1990) and mitochondrial proliferation (Du et al., 2008; Du et al., 2004) in fish, so perhaps these effects are contributing to the decrease in lipoprotein PUFA content in this study, but additional experiments are needed to support this contention.

#### 2.4.4 Conclusions

The major hypolipidemic effects described in this study support a similar regulatory scheme for lipoprotein metabolism in trout and mammals and the evolutionary conservation of the function of PPARs, which are the pharmacological target of fibrates. This study supports two others that report exposure to both pharmacologically and environmentally-relevant concentrations of fibrates have little or no effect on the cholesterol content of fish lipoproteins (Du et al., 2008; Weston et

al., 2009). However, this study clearly shows that while cholesterol content remains static, exposure to fibrates causes a disruption to the content of lipoprotein triglyceride, phospholipid and FA. Therefore, while the change in the cholesterol content of lipoproteins is used as a benchmark for the effectiveness of fibrates in humans, this study suggests that changes in lipoprotein triglyceride or phospholipid may be more appropriate biomarkers of the biological effects of fibrates in fish. Considering that a pharmacological concentration of GEM severely impairs fatty acid availability in rainbow trout, future studies should investigate whether any of the changes to the triglyceride, phospholipid or fatty acid content of lipoproteins reported here persist after exposures to environmentally-relevant concentrations of fibrates.

In this discussion it was suggested that peroxisomal and mitochondrial proliferation, along with changes in tissue lipids and fatty acids may be a cause or consequence of the hypolipidemic effects of GEM in plasma. These possibilities will be investigated in the next chapter.

## **Chapter 3: Gemfibrozil does not disrupt lipid metabolism in the oxidative tissues of rainbow trout**

### **3.1 Introduction**

Fibric acid derivatives (fibrates) are drugs used to treat hypertriglyceridemia. Fibrates evoke their hypolipidemic effects by binding to the transcriptional regulator, peroxisome proliferator activated receptor  $\alpha$  (PPAR $\alpha$ ) (Forman et al., 1997), although a recent in vitro gene knockdown study indicates the effects of some fibrates, including gemfibrozil (GEM), are also dependent on the presence of both PPAR $\beta$  and PPAR $\gamma$  (Hossain et al., 2008). The primary target genes of mammalian PPAR $\alpha$  are those involved in the regulation of both mitochondrial and peroxisomal lipid metabolism (Gulick et al., 1994; Mandard et al., 2004). Rainbow trout (*Onchorhynchus mykiss*) is a species susceptible to hepatic peroxisomal proliferation, induction of acyl-CoA oxidase (ACO) and lipid peroxidation after exposure to fibrates (Donohue et al., 1993; Gagne et al., 2006a; Scarano et al., 1994; Yang et al., 1990). The effects of fibrates on tissue fatty acid (FA) content and metabolism are well characterized in rodents (Alegret et al., 1995; Sanchez et al., 1993; Vazquez et al., 1995a; Vazquez et al., 1995b), but much less information is available for non-mammalian and especially aquatic vertebrates. There are only two studies that investigate changes in tissue FA content in fish after exposure to fibrates. In these studies (Du et al., 2008; Du et al., 2004), fenofibrate was found to

activate enzymes of both peroxisomal and mitochondrial  $\beta$ -oxidation, and to decrease the content of poly-unsaturated fatty acids (PUFA) in the liver and white muscle of rainbow trout and juvenile grass carp (*Ctenopharyngodon idella*). However, these studies did not identify in which lipid classes (i.e. triglyceride and phospholipid) the decrease in FA occurred, nor did they investigate a possible role for PPAR's in mediating the observed perturbations in lipid metabolism. The FA content of different lipid classes has critical physiological functions; e.g. in the case of phospholipids, maintaining properties of membrane fluidity (Tocher, 2003) and the activity of membrane-associated enzymes (Hulbert et al., 2005; Nagahuedi et al., 2009), and, in the case of triglycerides, providing substrates for aerobic metabolism. The present work was undertaken to examine if exposure to the fibrate, GEM, alters the FA content of phospholipids and triglycerides in the oxidative tissues of rainbow trout, and whether there are changes in the activity of enzymes of peroxisomal/mitochondrial  $\beta$ -oxidation and antioxidant defence. As lipoproteins are secreted and endocytized by the liver, hepatic lipid content may reflect the changes in lipoprotein composition that were demonstrated in Chapter 2. Because the array of genes regulated by PPARs differs between species (Mukherjee et al., 1994), the effects of GEM on the gene expression of these enzymes and the three PPAR subtypes was also investigated.

### **3.2 Materials and Methods**

### 3.2.1 Animals and Experimental Design

#### 3.2.1.1 Animals

Juvenile female rainbow trout, *Oncorhynchus mykiss*, were purchased from Linwood Acres Trout Farm (Campbellcroft, ON, Canada). They were kept in a 1300-L flow-through holding tank in dechloraminated, well-oxygenated water at 13°C under a 12-h:12-h L:D photoperiod. The animals were acclimated to these conditions for at least 1 month before experiments. Prior to GEM exposure, the trout were fed floating fish pellets (Martins Mills, Elmira, ON, Canada) five times per week until satiated. During the exposure period, the fish were fasted and kept in 115 L fiberglass tanks at a density of 8 fish per tank. All experiments were approved by the Animal Care Committee at the University of Ottawa and adhere to guidelines established by the Canadian Council on Animal Care for the use of animals in teaching and research.

#### 3.2.1.2 Experimental design

Rainbow trout ( $210.6 \pm 3.5$  g) were randomly divided into experimental (n=12) and control (n=12) groups. Groups were exposed to dimethylsulfoxide (DMSO; Sigma) (Control) or GEM dissolved in DMSO by intraperitoneal injections (i.p.) every third day for 15 days (between February and March). The experimental group received a dose of 100 mg GEM/kg fish and injections for both groups were given at a volume of 0.5  $\mu$ L/g fish. After 15 days, fish were anaesthetized with benzocaine (100 mg/L; Sigma) and then sacrificed by a swift blow to the head followed by cervical dislocation and the liver, red muscle (~800 mg) and heart were removed,

weighed, snap frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  until analysis. One aliquot of fresh tissue was used immediately for mitochondrial isolation. Hepatosomatic index (HSI) and body weight was assessed for each fish upon termination of the experiment.

### 3.2.2 Glycogen assay

Liver glycogen was measured using the enzymatic method described by Perry et al. (1988). Briefly, standards were prepared using type II oyster glycogen at 0, 2.5, 5, 7.5, 10, 15, and 20 mg/mL. Frozen liver was sonicated in 4:1 vol/wt of ice cold 6% perchloric acid (PCA). The homogenate was left on ice for 20 min and then centrifuged for 5 min at 10,000 x g. The supernatant (100  $\mu\text{L}$ ) was transferred to a glass test-tube containing 50  $\mu\text{l}$  1M  $\text{NaHCO}_3$  and 1 ml amyloglucosidase solution (in 30 ml of ddH<sub>2</sub>O add: 0.144 mL glacial acetic acid, 0.292 g sodium acetate, and 30 mg amyloglucosidase). The test tubes were incubated at  $37^{\circ}\text{C}$  for 2 h. The incubation was terminated by adding 25  $\mu\text{L}$  70% PCA. The samples were centrifuged at 7000 xg for 2 min and assayed for glucose concentration. Briefly, 200  $\mu\text{L}$  of assay mix (in mM: 60 trizma base, 40 tris-HCl, 1  $\text{MgSO}_4$ , 2  $\text{NAD}^+$ , 1 ATP, and 0.1 units/mL G6PH) was added to each sample and glycogen standard and incubated for 5 min at room temperature in a 96-well plate. The plate was read at 340 nm after which 10  $\mu\text{L}$  hexokinase solution (0.3 units hexokinase/mL) was added to each sample and standard and incubated for an additional 30 min at room temperature. Glucose concentration in each sample and standard well was determined by absorbance at 340 nm. Glycogen concentration was calculated from

the glucose content in the glycogen standards which yields units of  $\mu\text{mol}$  glycosyl units per gram tissue wet weight. To correct for glycogen in the amyloglucosidase, blank samples were run in parallel and glycogen values in the blanks were subtracted from the sample values.

### 3.2.3 Lipid extraction, separation and fatty acid composition

The concentrations of plasma triglyceride and phospholipid, as well as their FA contents, from the liver and red muscle were measured by gas chromatography after acid trans-esterification. Briefly, frozen tissues ( $\sim 0.25$  g) were homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) in 15 ml of a 2:1 (v/v) mixture of chloroform and methanol (Folch et al., 1957) and centrifuged (3000 g for 10 min). The supernatant was filtered before adding KCl (0.25%) to help eliminate water-soluble compounds. After shaking, the mixture was heated for 3 min at 65 °C to separate aqueous and organic phases. The aqueous phase was discarded and the organic phase was dried under a continuous vacuum at 65 °C (Büchi Rotavapor, Flawil, Switzerland). Phospholipids and triglycerides were then separated from total lipids by sequential elution and analyzed by gas chromatography. Details of these procedures are described in section 2.2.2.2. Individual FA representing less than 0.5% of the total were omitted from analysis.

### 3.2.4 Enzyme Activities

#### 3.2.4.1 Carnitine Palmitoyl Transferase 1 (CPT1)

*Mitochondrial isolation:* Mitochondria were isolated from fresh red muscle (RM) and liver of rainbow trout according to Morash et al. (2008). Briefly, each tissue

was immediately excised (RM ~1 g and liver ~2 g) and placed in mitochondrial isolation buffer (MIB) consisting of (in mM) 140 KCl, 10 EDTA, 5 MgCl<sub>2</sub>, 20 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 0.5% BSA (adjusted to pH 7.0) for RM and 250 sucrose, 1 EDTA, 20 HEPES and 0.5% BSA (adjusted to pH 7.4) for liver, on ice. Tissues were diced, washed with fresh chilled MIB, and then homogenized using a Teflon pestle in a chilled glass homogenizer. Homogenates were centrifuged at 800 g for 10 min at 4 °C. The supernatant was centrifuged at 9000 g for 10 min at 4 °C. Supernatants for each tissue were then discarded and pellets were resuspended in a small volume of the appropriate MIB lacking BSA. The resuspended homogenate was collected into a 15 mL centrifuge tube and centrifuged again at 9000 g for 10 min at 4 °C. The supernatant was discarded and the mitochondrial pellet was resuspended in an appropriate volume of MIB lacking BSA and kept on ice.

Assay: Radioactive CPT I (E.C. 2.3.1.21) assay followed Morash et al. (2008). The assay buffer (adjusted to pH 7.0) contained (in mM) 20 HEPES, 40 KCl, 1 EGTA, 220 sucrose, 0.1 DTT (dithiothreitol), 0.04 palmitoyl-CoA, 1 carnitine and 1.3 mg/mL BSA. 1 µCi/sample of L-[methyl-<sup>3</sup>H] carnitine hydrochloride (specific activity 82.0 Ci/mmol) (Amersham Biosciences, QC, Canada) was added and 70 µL of the assay mixture was placed in a 1.5 mL conical centrifuge tube. The reaction was started by the addition of 20 µL mitochondria diluted 5 times with MIB (~2 mg protein/mL), and incubated at room temperature for 8 min. The reaction was stopped by the addition of 60 µL 1 M HCl followed by 200 µL saturated butanol. The upper (butanol) phase containing the palmitoyl-[<sup>3</sup>H]-carnitine was collected and added to another 1.5 mL conical centrifuge tube containing 200 µL distilled water and 200 µL

water-saturated butanol. Saturated butanol was made by mixing 40ml of butanol with 10 ml of water in a separating funnel, shaking vigorously for 30 seconds and then removing the bottom (water) layer. The tube was vortexed and quickly centrifuged. The upper butanol layer was collected and the procedure was repeated two more times with centrifuge tubes containing first 200  $\mu$ L distilled water and second 100  $\mu$ L distilled water. The final upper butanol layer was collected and counted in 5 ml Safety Solve cocktail (Research Products, Mount Prospect, IL, USA) using a liquid scintillation counter (Beckman-Coulter CS6500). Twenty  $\mu$ l assay buffer with L-[methyl- $^3$ H]carnitine hydrochloride was also counted in duplicate for determination of individual specific activity. All CPT1 determinations were performed in duplicate and values averaged.

#### 3.2.4.2 Citrate Synthase, total CPT, HOAD, Catalase and Superoxide Dismutase

The activity of citrate synthase (CS; E.C. 2.3.3.1), CPT (CPT 1 + CPT 2; E.C. 2.3.1.21), 3-hydroxyacyl-CoA dehydrogenase (HOAD; E.C. 1.1.1.35) were measured according to Maillet and Weber (2007), catalase activity (CAT; E.C.1.11.1.6) according to Luschak and Luschak (2001) and superoxide dismutase activity (SOD; E.C. 1.15.1.1) using a commercially available kit (Sigma, 19160-1KT-F). Enzyme activities were estimated in the liver and red muscle using a Spectramax spectrophotometer (Gemini XS, Molecular devices, Sunnyvale, CA, USA) and 96-well flat-bottom plastic microplates (Thermo Fisher Scientific, Nepean, ON, Canada), except that a quartz cuvette was required for CAT. Enzyme activities were estimated in triplicate and mean values were used for calculations. Preliminary measurements

were performed to determine homogenate concentrations yielding maximum reaction velocities.

*Homogenate preparation:* Briefly, tissues were homogenized in an appropriate buffer (1:10 w/v) (40 mM Hepes, pH7.3 for CS, total CPT, HOAD, SOD and 50 mM potassium-phosphate containing 0.5 mM EDTA, pH7.0 for CAT) by first grinding the tissues in liquid nitrogen using a mortar and pestle and then using a narrow clearance Teflon homogenizer in a chilled glass homogenizer tube to lyse the cells (CS, total CPT, HOAD), or using a Kontes Motor Cordless tissue homogenizer (CAT). A protease inhibitor cocktail (Sigma, P2714) (diluted 100-times) was added to all samples just prior to homogenization (1  $\mu$ L/mL homogenate). Homogenates were centrifuged for 15 min at 15,000 *g* (Beckman-Coulter Microfuge R) and 4 °C. The supernatant was collected and used immediately for enzyme assays.

*Assay conditions:*

For CS, assay conditions were (in mM) 0.15 DTNB (5,5'-dithiobis 2-nitrobenzoic acid), 0.15 acetyl-CoA, 0.5 oxaloacetate (substrate) and diluted homogenate (1:10) in 50 mM Tris-HCl buffer (pH 8.0). Absorbance was monitored for 10 min at 412 nm and at room temperature.

For CPT, assay conditions were (in mM) 0.15 DTNB, 0.035 palmitoyl-CoA, 5 carnitine (substrate) and diluted homogenate (1:2) in 50 Tris-HCl buffer (pH 8.0). Absorbance was monitored for 10 min at 412 nm and at room temperature.

For HOAD, assay conditions were (in mM) 1 EDTA, 0.2 NADH ( $\beta$ -nicotinamide adenine dinucleotide, reduced form), 0.1 acetoacetyl-CoA (substrate)

and diluted homogenate (1:2) in 50 imidazole buffer (pH 7.4). Absorbance was monitored for 10 min at 340 nm and at room temperature.

For CAT, assay conditions were a 170:1 mix of 50 mM potassium phosphate buffer and 30% H<sub>2</sub>O<sub>2</sub> and the appropriate amount of the sample homogenate. Samples were assayed for 2 min at 240 nm and at room temperature.

### 3.2.5 Gene expression

Hepatic gene expression of PPAR $\alpha$ / $\beta$ / $\gamma$ , CS, HOAD, CPT1b, acyl CoA oxidase (ACO) and elongation factor 1  $\alpha$  (EF1 $\alpha$ ) were measured using real-time RT-PCR (QPCR). Total RNA was extracted and DNase treated using the RNeasy® Micro Kit (Qiagen, Mississauga, ON). Total RNA quantity was measured using a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) and only RNA with a 260/280 ratio of >1.9 was used for cDNA synthesis. First strand cDNA synthesis was prepared from 2  $\mu$ g total RNA and 200 ng random hexamer primers (Invitrogen, Burlington, ON) using Superscript II RNase H–reverse transcriptase (SSII) as described by the manufacturer. A total of n=8 (EF1 $\alpha$  and PPAR $\alpha$ / $\beta$ / $\gamma$ ) and n=7 (CS, HOAD, total CPT and ACO) was used to calculate relative transcript levels. Except for PPAR $\gamma$ , each PCR reaction contained the following final concentrations: 20 ng first-strand cDNA template, 1X QPCR buffer, 5 mM MgCl<sub>2</sub>, 0.5X SYBR green (Invitrogen), 200  $\mu$ M dNTPs, 1.25U HotStarTaq (Invitrogen), and 20  $\mu$ M ROX reference dye, in a 25  $\mu$ L reaction volume. The thermal cycling parameters, primer sequences and primer concentrations in the PCR reactions are described in Kolditz et al. (2008) (PPAR $\alpha$ , CS, HOAD, CPT1b, ACO and EF1 $\alpha$ ) and Morash et al. (2008) (PPAR $\beta$ ). For PPAR $\gamma$  3.5 mM MgCl, 200 nM primers and an annealing temperature

of 59 °C was used. PPAR $\gamma$  primers were designed according to Mimeault et al. (unpublished). Briefly, fish sequences for PPAR $\gamma$  (goldfish: AY894893; plaice: AJ243956; salmon: AJ292962, AJ416951 and AJ416952; sea bass: AY590303; seabream: AY590304) were obtained from GenBank. Degenerate PCR primers (purchased from Invitrogen) were designed. [5'- GGR TTC ACH GAK CTG GAY MT-3' (F) and 5'- TTC TTS AGC AGS TGG ATG WG -3' (R)] using the Primer3 web site. PCR conditions were 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1 min; concentration of MgCl<sub>2</sub> was 2 mM. Non-degenerate primers for PPAR $\gamma$  [5'-ATG AAC AAG GAC GGC ACA CT-3' (F) and 5'-GCA GTA GCT TGG CGA ACA G-3' (R)] were designed based on sequencing results and PPAR $\gamma$  hepatic gene expression was measured as described above. Dilutions of cDNA pooled from all individual samples (both control and treated) were used to construct a relative standard curve for each primer set. After the reaction was completed, a dissociation curve was produced starting at 55 °C (+1 °C/30 s). QPCR used a MX4000® Multiplex Quantitative PCR system (Stratagene, La Jolla, CA, USA) and data were analyzed using the MX4000 Software Package. Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using the amplified PCR product from a pool of all individual samples as a template, and were used to calculate mRNA copy number in each sample. Genes were normalized to the expression of the control gene EF1 $\alpha$  since its expression did not change between experimental groups. In all cases, only standard curves with slopes between -3.2 and -3.5 with R<sup>2</sup> values of >0.98 were used. After each run, a random subset of PCR products (total n=4) from control and treatment groups were pooled and purified using a Minelute Reaction Cleanup kit (Qiagen, Mississauga, ON, Canada)

and sequenced with a CEQ™8000 Genetic Analysis System (Beckman Coulter, Mississauga, ON, Canada) to confirm specificity of the assays.

### 3.2.6 Protein assay

Protein content of tissue and mitochondria was determined using the Bicinchoninic Acid method (Sigma, TPRO-562) and bovine serum albumin as standard.

### 3.2.7 Statistical analyses

Experimental results are presented as means  $\pm$  standard error of the mean (s.e.m.). All statistical analyses were conducted using SigmaPlot™ 11.0 software (SPSS Corp., Chicago, IL). Statistical significance was tested with One-Way ANOVA on raw data, or in the case of percentages and ratios, on transformed data (arcsin\* $\sqrt{\text{data}}$ ), followed by a Bonferroni correction for multiple comparisons. A value of  $P < 0.05$  was considered significant.

## 3.3 Results

### 3.3.1 Body parameters and liver composition

The body parameters and liver composition of rainbow trout exposed to GEM are presented in Table 3.1. The final body weights of both the control and treatment groups decreased from the start of the experiment (-10%,  $P < 0.05$ ); there were no inter-group differences in initial or final body weights. A significant increase in HSI (+27%,  $P < 0.001$ ) was found, with the increase being associated with an increase in liver size ( $P < 0.001$ ) as body weights did not differ between treatment and control

groups. GEM-treatment significantly decreased the concentration of liver glycogen (-38%,  $P=0.038$ ), but had no effect on protein, moisture or lipid content, despite the increase in liver size.

### 3.3.2 Fatty acid composition

The FA composition of red muscle and liver are shown in Table 3.2 and 3.3, respectively. Changes in hepatic FA composition were detected after a t-test (MUFA +4.5%  $P=0.017$ ; PUFA -3.6%  $P=0.023$ ), however, following an ANOVA with a Bonferroni correction, no changes in the FA content of either the red muscle or liver remained.

### 3.3.3 Potential for fatty acid oxidation

The activities of enzymes related to fatty acid oxidation were measured in both the liver and red muscle and are presented in Table 3.4. No change to the activity of any of these enzymes occurred in the red muscle. In the liver, HOAD activity decreased per mg protein (-25%  $P=0.004$ ); CS, CPTI and CPTII activity did not differ from control.

Activities of enzymes related to antioxidant potential in both liver and red muscle are shown in Figure 3.1. The activity of CAT, measured as the rate of breakdown of 1  $\mu$ mole of  $H_2O_2$  per minute, did not differ between control and treatment in the liver (Control: 36.2 and GEM: 43.5;  $P=0.264$ ) or red muscle (Control: 4.2 and GEM: 4.9;  $P=0.494$ ) per mg of protein. The activity of SOD, measured as its ability to inhibit the activity of the superoxide-generating enzyme xanthine oxidase, was lower in the liver of the GEM-treated group (67% inhibition)

compared to the Control group (61% inhibition) (t-test,  $P=0.003$ ). There was no difference between SOD activity in the red muscle of the GEM-treated and Control groups (53% inhibition for both groups,  $P=0.875$ ).

#### 3.3.4 Gene expression

To explore the possibility that GEM acts as a transcriptional regulator in fish, hepatic gene expression of three PPAR subtypes (Fig 3.2) and enzymes involved in mitochondrial and peroxisomal  $\beta$ -oxidation (Fig. 3.4) were estimated. There were no detectable changes in the transcript levels of any of the genes measured.

**Table 3.1.** Morphometric parameters and carbohydrate levels in Control and GEM-treated rainbow trout. Data are presented as means  $\pm$  s.e.m. and an asterisk indicates a significant difference from Control values ( $P < 0.05$ ),  $n = 8$

Condition	Treatment	
	Control	GEM
Initial Body mass (g)	206.2 $\pm$ 5.3	215.1 $\pm$ 4.4
Final Body Mass (g)	188.6 $\pm$ 4.6	194.6 $\pm$ 5.5
Liver Mass (g)	2.0 $\pm$ 0.1	2.8 $\pm$ 0.1*
HSI <sup>1</sup>	1.1 $\pm$ 0.05	1.4 $\pm$ 0.05*
Liver TG + PL <sup>2</sup>	41.6 $\pm$ 1.3	45.6 $\pm$ 2.0
Liver Moisture (%)	75.7 $\pm$ 3.1	81.1 $\pm$ 2.3
Liver Protein (mg/g)	133.6 $\pm$ 18.1	133.5 $\pm$ 19.2
Liver Glycogen <sup>3</sup>	80.9 $\pm$ 7.3	58.5 $\pm$ 6.1*

1- Hepatosomatic index (liver mass/body mass)\*100%

2- Units are  $\mu$ mol fatty acids per gram liver wet weight

3- Units are  $\mu$ mol glycosyl units per gram tissue wet weight (see 3.2.2)

**Table 3.2.** Fatty acid (FA) composition of triglycerides and phospholipids in the red muscle of rainbow trout. Data are presented as mean  $\pm$  s.e.m (n=8). Values are percentages of total FA by concentration (nmol/g tissue), except the last row where the absolute concentration is indicated. There was no difference between control and GEM-treated groups ( $P>0.05$ ). SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA.

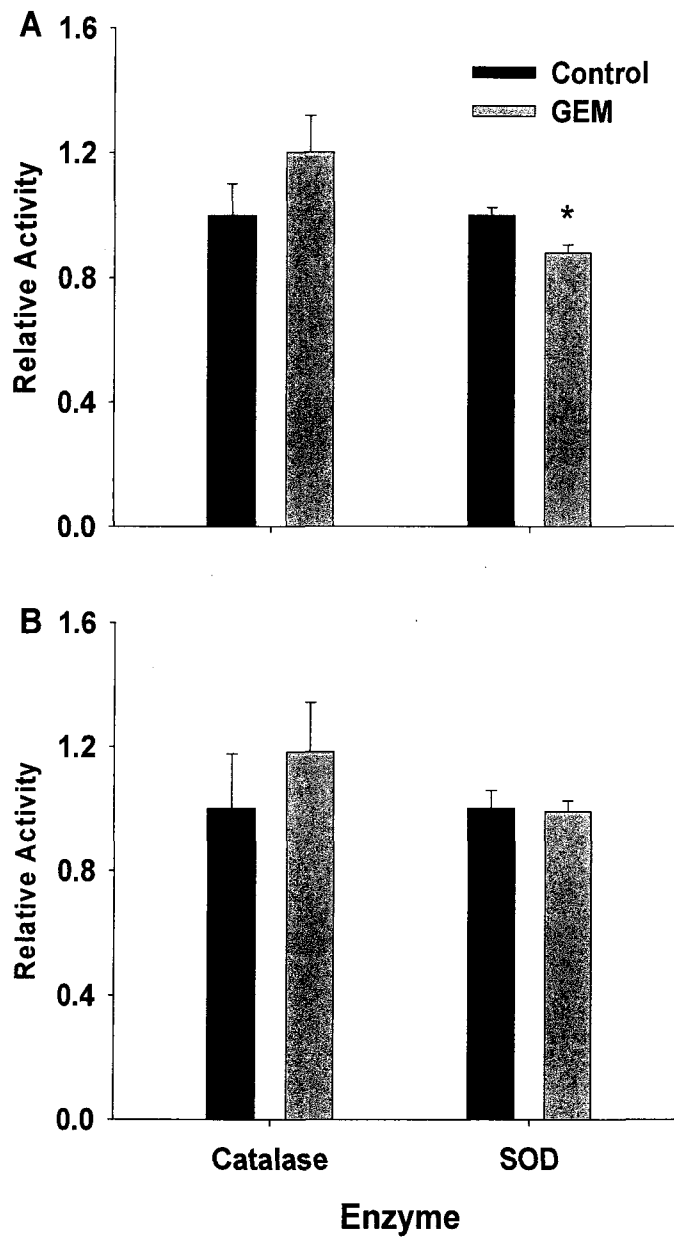
FA (% of total)	TG Composition		PL Composition	
	Control	Treatment	Control	Treatment
14:0	3.3 $\pm$ 0.1	3.5 $\pm$ 0.1	0.6 $\pm$ 0.1	0.6 $\pm$ 0.05
16:0	11.6 $\pm$ 0.4	11.7 $\pm$ 0.2	17.6 $\pm$ 0.4	16.6 $\pm$ 0.4
16:1	6.1 $\pm$ 0.2	6.3 $\pm$ 0.2	1.2 $\pm$ 0.03	1.2 $\pm$ 0.03
18:0	2.6 $\pm$ 0.3	2.5 $\pm$ 0.03	0.9 $\pm$ 0.1	0.9 $\pm$ 0.06
18:1(n-9)	2.8 $\pm$ 0.09	2.6 $\pm$ 0.1	8.9 $\pm$ 0.7	8.8 $\pm$ 0.4
18:1(n-7)	21.6 $\pm$ 0.3	21.5 $\pm$ 0.2	1.1 $\pm$ 0.02	1.1 $\pm$ 0.02
18:2(n-6)	2.3 $\pm$ 0.02	2.2 $\pm$ 0.04	3.6 $\pm$ 0.1	3.6 $\pm$ 0.09
18:2(n-3)	12.3 $\pm$ 0.2	12.4 $\pm$ 0.2	-----	-----
18:3(n-3)	1.0 $\pm$ 0.02	1.0 $\pm$ 0.01	-----	-----
18:4	1.2 $\pm$ 0.04	1.2 $\pm$ 0.01	-----	-----
20:1(n-9)	12.5 $\pm$ 0.2	12.2 $\pm$ 0.1	1.7 $\pm$ 0.1	1.8 $\pm$ 0.07
20:4(n-6)	-----	-----	1.9 $\pm$ 0.06	2.1 $\pm$ 0.08
20:5(n-3) EPA	2.3 $\pm$ 0.1	2.5 $\pm$ 0.05	10.1 $\pm$ 0.4	10.4 $\pm$ 0.4
22:1 (n-9)	13.4 $\pm$ 0.4	13.2 $\pm$ 0.3	0.9 $\pm$ 0.1	1.2 $\pm$ 0.1
22:5 (n-3)	1.2 $\pm$ 0.04	1.1 $\pm$ 0.08	3.1 $\pm$ 0.1	3.1 $\pm$ 0.2
22:6(n-3) DHA	5.9 $\pm$ 0.2	5.9 $\pm$ 0.2	48.5 $\pm$ 0.7	48.5 $\pm$ 0.7
$\Sigma$ n-3	20.5 $\pm$ 0.2	20.4 $\pm$ 0.3	51.6 $\pm$ 0.6	51.6 $\pm$ 0.7
$\Sigma$ n-6	2.3 $\pm$ 0.02	2.3 $\pm$ 0.05	5.5 $\pm$ 0.2	5.6 $\pm$ 0.1
SFA	17.0 $\pm$ 0.6	17.8 $\pm$ 0.4	19.1 $\pm$ 0.5	18.1 $\pm$ 0.5
MUFA	56.4 $\pm$ 0.7	55.8 $\pm$ 0.2	13.8 $\pm$ 0.8	14.2 $\pm$ 0.5
PUFA	26.3 $\pm$ 0.2	26.4 $\pm$ 0.4	67.1 $\pm$ 0.6	67.7 $\pm$ 0.6
Concentration ( $\mu$ mol/g wet weight)	68.7 $\pm$ 9.3	98.4 $\pm$ 15.03	6.7 $\pm$ 0.4	7.2 $\pm$ 0.3

**Table 3.3.** Fatty acid (FA) composition of triglycerides and phospholipids in the liver of rainbow trout. Data are presented as mean  $\pm$  s.e.m (n=8). Values are percentages of total FA by concentration (nmol/g tissue), except the last row where the absolute concentration is indicated. There was no difference between control and GEM-treated groups ( $P>0.05$ ). SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA.

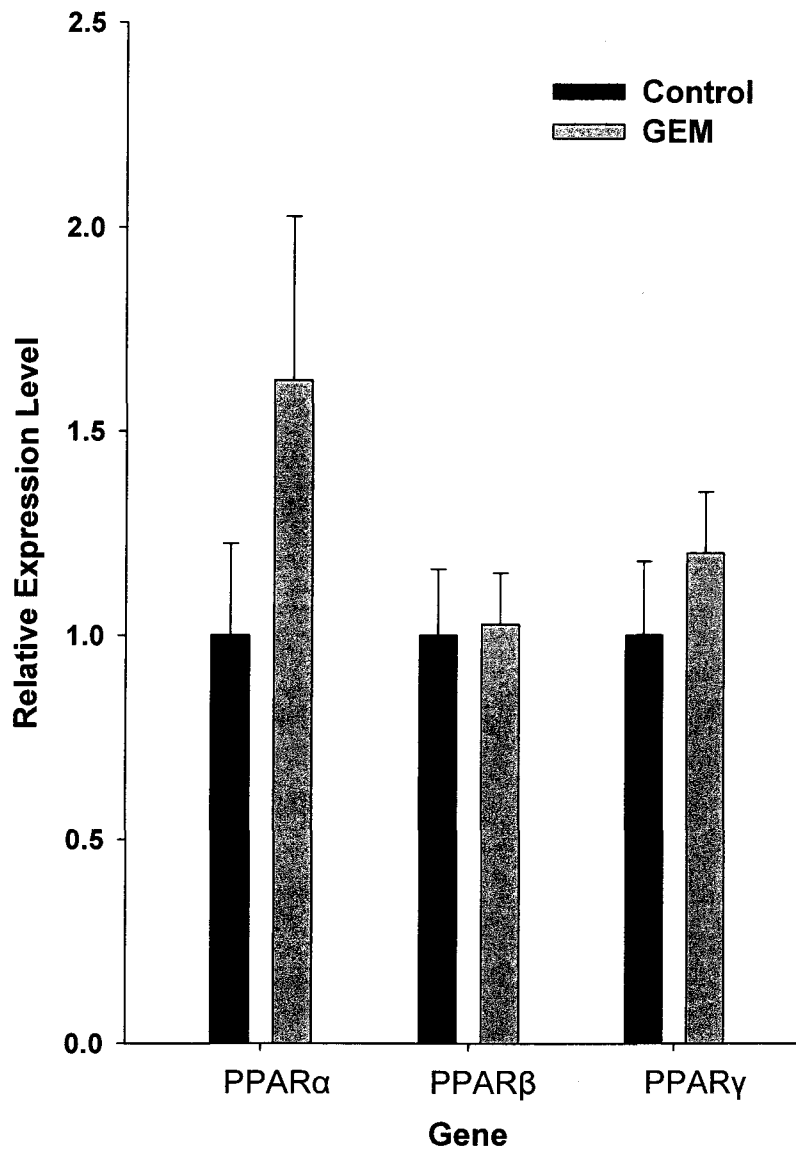
FA (Molar %)	TG Composition		PL Composition	
	Control	Treatment	Control	Treatment
14:0	1.8 $\pm$ 0.1	1.6 $\pm$ 0.1	0.9 $\pm$ 0.03	1.0 $\pm$ 0.05
16:0	11.7 $\pm$ 0.3	11.0 $\pm$ 0.2	21.2 $\pm$ 0.2	22.4 $\pm$ 0.2
16:1	6.1 $\pm$ 0.2	5.8 $\pm$ 0.2	1.5 $\pm$ 0.06	1.6 $\pm$ 0.05
18:0	4.0 $\pm$ 0.5	4.0 $\pm$ 0.3	2.9 $\pm$ 0.2	3.4 $\pm$ 0.1
18:1(n-9)	3.0 $\pm$ 0.1	3.2 $\pm$ 0.1	1.6 $\pm$ 0.05	1.6 $\pm$ 0.07
18:1(n-7)	22.2 $\pm$ 0.6	24.0 $\pm$ 0.6	9.5 $\pm$ 0.1	9.9 $\pm$ 0.1
18:2(n-6)	2.6 $\pm$ 0.03	2.8 $\pm$ 0.05	1.5 $\pm$ 0.02	1.7 $\pm$ 0.05
18:2(n-3)	10.4 $\pm$ 0.2	9.7 $\pm$ 0.3	4.8 $\pm$ 0.1	4.9 $\pm$ 0.1
18:3(n-6)	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1	-----	-----
18:3(n-3)	1.1 $\pm$ 0.08	0.7 $\pm$ 0.1	-----	-----
18:4	1.8 $\pm$ 0.1	1.5 $\pm$ 0.07	-----	-----
20:1(n-9)	7.2 $\pm$ 0.2	8.4 $\pm$ 0.4	1.6 $\pm$ 0.05	1.7 $\pm$ 0.3
20:4(n-6)	1.9 $\pm$ 0.7	1.6 $\pm$ 0.4	1.1 $\pm$ 0.03	1.2 $\pm$ 0.07
20:5(n-3) EPA	4.9 $\pm$ 0.3	3.6 $\pm$ 0.2	7.8 $\pm$ 0.1	7.4 $\pm$ 0.2
22:1 (n-9)	5.5 $\pm$ 0.3	7.3 $\pm$ 0.4	-----	-----
22:5 (n-3)	1.6 $\pm$ 0.06	1.6 $\pm$ 0.2	2.1 $\pm$ 0.1	2.3 $\pm$ 0.1
22:6 (n-3) DHA	12.8 $\pm$ 0.9	11.7 $\pm$ 0.8	42.3 $\pm$ 0.4	40.8 $\pm$ 0.2
$\Sigma$ n-3	30.8 $\pm$ 1.0	27.3 $\pm$ 0.9	49.2 $\pm$ 0.3	48.1 $\pm$ 0.2
$\Sigma$ n-6	11.1 $\pm$ 1.5	14.0 $\pm$ 0.5	2.6 $\pm$ 0.05	2.9 $\pm$ 0.08
SFA	17.5 $\pm$ 0.4	16.7 $\pm$ 0.3	26.2 $\pm$ 0.3	26.9 $\pm$ 0.3
MUFA	44.2 $\pm$ 1.3	48.9 $\pm$ 1.1	14.2 $\pm$ 0.2	14.8 $\pm$ 0.2
PUFA	38.2 $\pm$ 1.0	34.5 $\pm$ 1.0	59.6 $\pm$ 0.2	58.4 $\pm$ 0.2
Concentration ( $\mu$ mol/g wet weight)	6.6 $\pm$ 0.5	7.8 $\pm$ 1.2	35.0 $\pm$ 1.0	37.7 $\pm$ 1.3

**Table 3.4.** Effect of GEM on the activity of enzymes related to mitochondrial  $\beta$ -oxidation in the liver and red muscle of rainbow trout. Data are presented as means  $\pm$  s.e.m. (n=8). Statistically significant differences between groups is indicated by an asterisk ( $P < 0.05$ ).

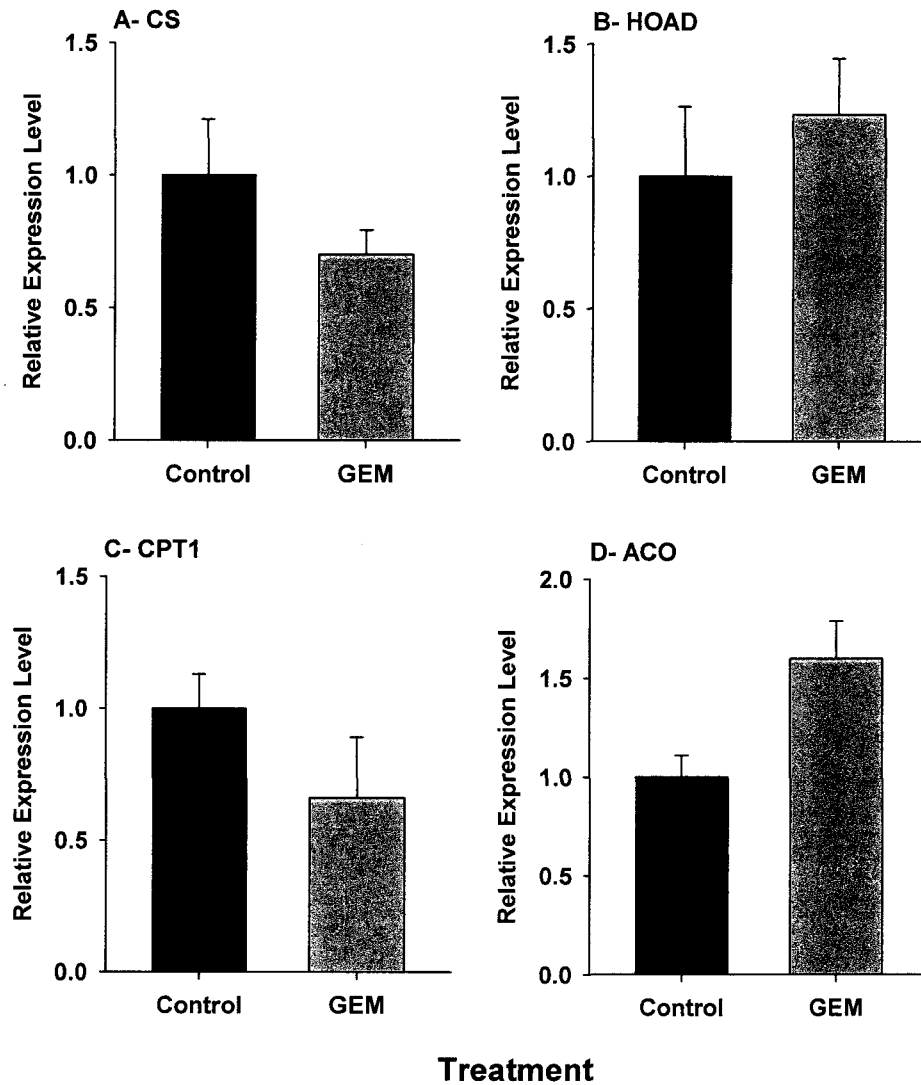
Enzyme	Liver			Red Muscle		
	Control	GEM	P	Control	GEM	P
CS <sup>1</sup>						
$\mu\text{mol min}^{-1} \text{mg}^{-1}$ total protein	8.7 $\pm$ 0.5	8.9 $\pm$ 0.4	0.76	147.4 $\pm$ 7.4	165.0 $\pm$ 17.8	0.37
HOAD <sup>2</sup>						
$\mu\text{mol min}^{-1} \text{mg}^{-1}$ total protein	16.7 $\pm$ 0.9	12.6 $\pm$ 0.8*	0.004	160.4 $\pm$ 21.0	185.0 $\pm$ 26.3	0.47
CPT <sup>3</sup>						
$\mu\text{mol min}^{-1} \text{mg}^{-1}$ total protein	3.0 $\pm$ 0.2	2.0 $\pm$ 0.2	0.1	5.0 $\pm$ 0.7	2.2 $\pm$ 0.5	0.51
CPT <sup>4</sup>						
$\mu\text{mol min}^{-1} \text{mg}^{-1}$ mitochondrial protein	0.09 $\pm$ 0.01	0.09 $\pm$ 0.01	0.79	0.21 $\pm$ 0.03	0.20 $\pm$ 0.01	0.37
1- Citrate Synthase						
2- 3-hydroxyacyl-CoA dehydrogenase						
3- Carnitine palmitoyl transferase 2						
4- Carnitine palmitoyl transferase 1						



**Figure 3.1.** The activity of catalase and superoxide dismutase (SOD) in the liver (A) and red muscle (B) of rainbow trout. Fish were injected with either 100% DMSO (Control) or 100 mg/kg of Gemfibrozil (GEM). Data are means + s.e.m. (n=8) and activities are presented as fold-change from Control values. A significant difference between treatment groups is indicated by an asterisk ( $P < 0.05$ ).



**Figure 3.2.** The relative transcript levels of PPAR isoforms in the liver of Control and GEM-treated rainbow trout. Data represent means relative to control values + s.e.m. Genes are normalized to the internal control gene EF1 $\alpha$  because its expression did not differ between treatment and control groups. n= 7 for PPAR $\alpha$ ; n=8 for PPAR $\beta$  and PPAR $\gamma$ .



**Figure 3.3.** The relative expression of genes involved in mitochondrial and peroxisomal  $\beta$ -oxidation in the liver of Control and GEM-treated rainbow trout. A) Citrate Synthase (CS), B) 3-hydroxyacyl-CoA dehydrogenase (HOAD), C) Carnitine Palmitoyl Transferase 1b (CPT1b), D) Acyl CoA Oxidase (ACO). Data are means relative to control values + s.e.m. Genes are normalized to the internal control gene EF1 $\alpha$ , because its expression did not differ between treatment and control groups. n= 8 for CPT1b; n=7 for CS, HOAD and ACO.

### **3.4 Discussion**

Fibric acid derivatives are known to cause hepatomegaly and peroxisomal proliferation in rodents. The results of this study demonstrate that GEM induced hepatomegaly, but did not disrupt tissue lipid metabolism in rainbow trout.

#### **3.4.1 Liver composition**

The enlargement of the liver seen here is consistent with a previous study by Scarano et al. (1994) using GEM-treated rainbow trout. However, another study of rainbow trout, on this occasion fed fenofibrate (100 mg fenofibrate/g fish), reports no change in liver size (Du et al., 2004). More studies are needed to determine whether the difference in hepatic response to the different fibrates is related to drug potency or whether it is more related to the different exposure regimes (i.e. oral vs i.p.)

The change in liver size that follows exposure to some xenobiotic agents (Klaunig et al., 2003) can be from an increase in cell size, an increase in cell number, or both. In this study there was no change in liver protein, fat (triglyceride and phospholipid) or moisture content, suggesting that the increase in liver size here is a result of hyperplasia, rather than cellular hypertrophy. Despite that the composition of the liver was largely static, there was an unexpected decrease in concentration of liver glycogen that appeared following GEM-treatment. It seems unlikely that the decrease in hepatic glycogen concentration is due to hepatic glucose oxidation since PPAR $\alpha$  agonists consistently increase hepatic lipid oxidation in animal models (Minnich et al., 2001). It is possible that the decrease in hepatic

glycogen is because GEM stimulated extra-hepatic glucose oxidation, but more studies are needed to investigate this hypothesis.

#### 3.4.2 Tissue fatty acid content

No changes in the FA content of red muscle or liver were detected after GEM-treatment, and therefore their FA content does not reflect physiological changes occurring in the plasma lipoproteins (see Chapter 2). Like changes in hepatic morphology, the stable hepatic FA content after GEM-treatment lays in stark contrast to other studies that report changes to this endpoint in fish fed with fenofibrate (Du et al., 2004; Du et al., 2008) and rodents fed GEM (Sanchez et al., 1993; Vazquez et al., 1995b). Most studies that do show changes in tissue lipid content expose the organisms orally (i.e. gavage or feeding), rather than i.p. as in this study. It is well known that the route of exposure to a chemical (e.g. gavage, waterborne etc.) has profound effects on its physiological outcomes. More studies are needed to discern whether the absence of change in tissue FA described in this study is simply characteristic of GEM in fish, or whether the route of exposure limited the capacity of GEM to perturb tissue lipid metabolism.

#### 3.4.3 Potential for lipid oxidation

The fibrate drug fenofibrate increases both peroxisomal and mitochondrial  $\beta$ -oxidation in rainbow trout *in vivo* (Du et al., 2004); however, the effects of different fibrates on lipid metabolism can be strikingly different, even within individuals of the same species (Vazquez et al., 1995a; Vazquez et al., 1995b; Weston et al., 2009). In this study, increased peroxisomal activity was not measured directly but rather

investigated for by assaying the anti-oxidant enzymes catalase and SOD. Mitochondrial density and capacity for  $\beta$ -oxidation was estimated by assaying for CS and CPT1, and CPT2 and HOAD, respectively. Other than minor decreases in HOAD and SOD, no other changes in activities of enzymes involved in  $\beta$ -oxidation or antioxidant defence were observed after exposure to GEM, when measured per gram of liver. Once again, these results are in contrast to other studies of rainbow trout where peroxisomal/mitochondrial markers were significantly increased after injections with ciprofibrate, feeding with fenofibrate, or exposing hepatocytes to GEM (Donohue et al., 1993; Du et al., 2004; Yang et al., 1990). It is possible that the discrepancies between the results of this study and those of others are a result of differences in the potency of the different fibrates, or the different dosing and exposure regimes. The transient and variable nature of the response of antioxidant enzymes to xenobiotics (Livingstone, 2001) may also be limiting the detection of any changes in the activities of these enzymes.

#### 3.4.4 Gene expression

Piscine PPARs share similarities with their mammalian homologues, including transcriptional activation of 'target' genes and similar structural requirements of promoter sequences, but their roles in FA metabolism are not well characterized and may not match those of mammals (Leaver et al., 2005). In mammals, fibrates bind to PPAR $\alpha$  that, in turn, regulates the transcription of multiple target genes downstream of a peroxisome proliferator response element (PPRE) (Desvergne et al., 2006). While there has been some attempt to link the bioactivity of fibrates in non-target organisms to PPAR gene regulation, results have been conflicting and studies show

that the expression of all three PPAR subtypes of teleost fish are affected by exposure to fibrates (Ibabe et al., 2005; Mimeault et al., 2006; Ruyter et al., 1997; Weston et al., 2009) and even that changes in PPAR expression are not needed to achieve a pharmacological response (Raldúa et al., 2008). In this study, the relative transcript levels of trout hepatic PPAR $\alpha$ , PPAR $\beta$  and PPAR $\gamma$  were estimated to investigate whether PPARs were associated with possible changes in hepatic FA metabolism. Although assessing transcript levels of PPARs does not necessarily confirm activation of the PPAR protein via ligand binding, there is evidence from mammals that PPAR genes, particularly  $\alpha$  and  $\gamma$ , are up-regulated after exposure to fibrates (Soria et al., 2005); an example of *in vivo* auto-regulation of the PPAR protein. In this study, the fact that GEM did not stimulate the expression of PPAR genes is insufficient evidence to eliminate the possibility that PPARs are involved in the reported physiological responses. This is because the activation and use of PPAR pathways can occur without increased expression of the PPAR genes, and any increase in PPAR expression that may occur is likely to be transient and appear only at an earlier stage of exposure. It is also possible that since this study only considered one isoform of each PPAR $\beta$  and PPAR $\gamma$  when two are identified in another salmonid species (Atlantic salmon, *Salmo salar*) (Andersen et al., 2000; Leaver et al., 2006), changes in PPAR gene expression may have been missed. It is interesting however, that after two weeks of exposure, no changes in the gene expression of any mitochondrial or peroxisomal marker enzymes were detected, especially given the increase in HSI and that other studies show ligands of mammalian PPARs up-regulate PPAR $\alpha$ , PPAR $\gamma$  transcripts and also the CPT1 activity of rainbow trout (Morash et al., 2008). It is possible that the absence of

change is due to the lack of PPRE in gene promoter sequences of ACO or CPT1 since the genes regulated by PPARs in fish do not necessarily correspond to those in humans and rodents (Mandard et al., 2004; Mukherjee et al., 1994). However, any up-regulation of expression is likely transient and disappears as *in vivo* drug concentrations decrease post injection, making the detection of gene expression changes 24 h after the final dose of GEM -as done in this study- difficult.

#### 3.4.5 Conclusion

The first objective of this study was to establish whether the PPAR $\alpha$  agonist GEM promotes selective lipid and FA metabolism in the oxidative tissues of rainbow trout. GEM-treated fish were found to have an increased HSI, but not in the fatty acid composition of tissues. However, the second objective, to find an enzyme-related mechanism for changes in hepatic morphology, remains elusive.

This study contrasts with others that demonstrate fibrates stimulate the activities of mitochondrial and peroxisomal enzymes and the transcripts of PPARs in teleost fish (Du et al., 2008; Du et al., 2004; Mimeault et al., 2006). However, these other studies used different routes of exposure and different doses of different fibrates. Future work should investigate whether there are mechanistic differences that explain the different effects of different fibrates in fish, or whether the different drug strengths and routes of exposure account for the discrepancies.

This study serves as an important reminder that the effects of PhACs are unpredictable in non-target species. Since fibrates are ubiquitous aquatic contaminants, fully understanding their toxicodynamics in fish may be useful for determining biomarkers that identify fish species unintentionally exposed to

hypolipidemic drugs present in their environment and for identifying organisms that are sensitive to drugs affecting their performance.

## **Chapter 4: General Conclusion**

The main objective of this thesis was to characterize how exposure to a mammalian PPAR $\alpha$  agonist affects lipid transport and use in the rainbow trout. To achieve this objective, experiments presented in this thesis were designed to test the hypothesis that the pharmacological effects of fibrates on lipid metabolism are conserved across vertebrates. Results of these experiments were presented in two chapters, where changes in plasma lipoprotein content were examined in Chapter 2 and changes to tissue lipid metabolism were examined in Chapter 3.

### **4.1 Changes to plasma lipoproteins**

Results presented in Chapter 2 provide evidence that *in vivo* exposure to pharmacological concentrations of GEM dramatically changed both plasma lipid and lipoprotein metabolism. This study is the first to characterize the effects of a human pharmaceutical on the complete lipoprotein profile of a non-mammalian vertebrate. Furthermore, this study is one of only a few that demonstrates changes to the FA content of both lipid and lipoprotein classes after exposure to a PPAR $\alpha$  agonist. The results show that despite large decreases in the concentration of circulating lipoproteins, the plasma-lipoprotein profile remained remarkably stable, indicating that circulating lipoprotein classes are well regulated. Despite similarities between mammalian and fish lipoprotein metabolism (Gjoen and Berg, 1992a; Gjoen and

Berg, 1992b; Gjoen and Berg, 1993a), differences do exist. For instance, lipoproteins in rainbow trout are considered as a lipid reservoir used to shuttle lipids to tissues, sustaining processes like low-intensity exercise (Magnoni and Weber, 2007; Magnoni et al., 2006), thermal adaptation (Wallaert and Babin, 1993; Wallaert and Babin, 1994b), and yolk protein provision to oocytes (Wiegand, 1996). The elevated plasma lipoprotein concentration in rainbow trout reflects their pivotal role in these physiological processes [2119 mg/100 ml (depending on age and seasonality) for rainbow trout compared to 736 mg/100 ml for humans] (Babin and Vernier, 1989). The divergent roles of lipoproteins may affect how lipoprotein metabolism responds to xenobiotic queues and therefore may be responsible for some of the disparities in the effects of GEM between mammals and fish seen in my study.

#### **4.2 Changes to tissue lipid metabolism**

The experiment presented in Chapter 3 was designed to detect whether GEM affected the lipid metabolism of oxidative tissues. The results demonstrated a large change in hepatic morphology (e.g. HSI), suggesting that the liver is involved in the *in vivo* response to fibrate exposure. However, hepatic lipid and FA content was unchanged after GEM-treatment. This study contrasted others that showed changes in tissue fatty acids after exposure to fibrates (Du et al., 2008; Du et al., 2004). Likewise, attempts to link changes in hepatic morphology and lipoprotein metabolism to mitochondrial and peroxisomal marker enzymes and PPAR gene transcripts were without success. Piscine PPARs share similarities with their

mammalian homologues, including transcriptional activation of 'target' genes and similar structural requirements of gene promoter sequences (Leaver et al., 2005). However, differences exist in that *Salmoniformes* have additional PPAR paralogs with unique tissue distributions and transactivation properties, at least for PPAR $\beta$  (Leaver et al., 2007), and that fish show critical amino acid differences in the ligand binding domains of PPAR $\alpha$  and PPAR $\gamma$  (Leaver et al., 2005). Given the similarities between mammalian and fish PPARs, it seems likely that PPARs were involved in the disruption of lipoprotein metabolism seen in this thesis, but perhaps species differences in PPAR subtypes, their ligand-binding domains and their tissue distributions were at least partly responsible for the observed disparities in the effects of GEM on tissue lipid metabolism.

The changes in hepatic morphology and circulating lipoproteins shown in this chapter were independent of changes in enzyme activity and gene transcript levels. Hence, these endpoints may not be reliable indicators of fibrate exposure, at least in rainbow trout. Furthermore, the results of this chapter highlight that when searching for physiological effects of xenobiotics *in vivo*, careful consideration needs to be given to how the test organisms are exposed.

### **4.3 Conclusions and future studies**

The results of this thesis support the hypothesis that the pharmacological effects of fibrates are conserved across vertebrates. Moreover, the observed hypolipidemic effects support a similar regulatory scheme for lipoprotein metabolism in trout and mammals and the evolutionary conservation of the pharmacological

target of fibrates, which are the PPARs. Fibrates are mammalian PPAR $\alpha$  agonists, but despite efforts to link their bioactivity in fish to PPARs (Mimeault et al., 2006; Weston et al., 2009), no study to date has provided unequivocal evidence for this link in any fish species. A study by Liu et al. (2005) developed a reporter gene assay based upon transfecting a luciferase expression vector containing a mammalian peroxisome proliferator response element (PPRE) into the rainbow trout (rt)-gill-W1 cell line and assaying luminescence with various PPAR agonists. This reporter gene assay was sensitive to PPAR $\alpha$  and PPAR $\beta$  agonists but not PPAR $\gamma$  agonists. These studies provide evidence for agonist binding to PPARs, but do not provide adequate evidence for specific bioactivity of fibrates in fish species. Future work, therefore should concentrate on verifying that the bioactivity of fibrates in fish is mediated by binding a particular PPAR subtype. PPAR-knockout fish models and specific ligand-PPAR binding studies would provide more direct evidence if this is also linked to downstream activation of known PPRE-sensitive genes. In addition, measuring PPAR protein levels, rather than mRNA transcript levels as in this study, may prove useful for this research direction. To this point, using fibrates as a tool to pharmacologically modify the lipoproteins of fish may be valuable in uncovering features of lipoprotein metabolism that are uniquely present in these organisms.

One of the original intents of this thesis was to compare the high dose effects of fibrates to those appearing from exposure to waterborne, environmentally-relevant concentrations. Unfortunately, difficulties inherent with the experiments presented here precluded this possibility. I showed that pharmacological doses of fibrates have major effects on the lipoproteins of rainbow trout; not all of which are analogous to the effects of fibrates in humans. As fibrates are ubiquitous

contaminants of aquatic environments, their ability to affect the lipid and lipoprotein metabolism at environmental concentrations should be determined since maintaining lipid transport serves vital physiological roles in teleosts. In this regard, disruption of lipid homeostasis may prove to be a sensitive indicator of chronic exposure to fibrates or other lipid-regulating pharmaceuticals present in the environment.

## **Appendix A: Bioactivity of pharmacological doses of gemfibrozil**

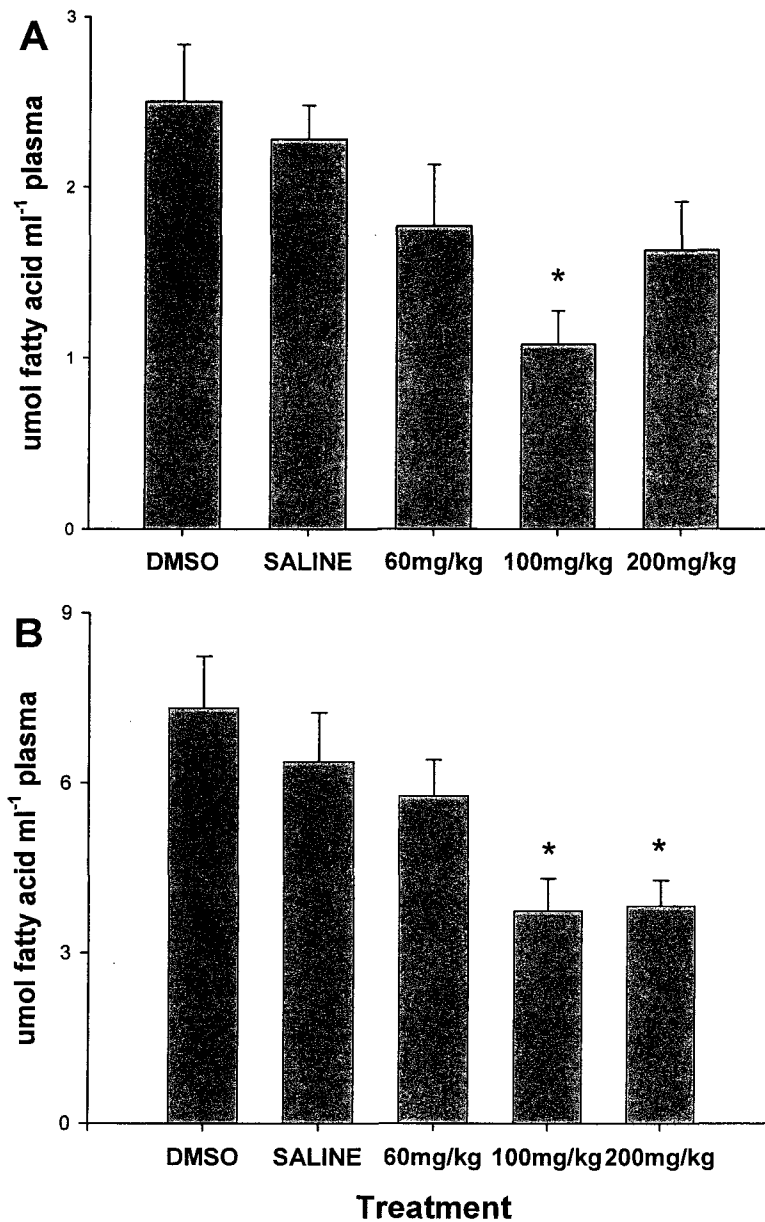
### **(GEM) in rainbow trout**

**Objective:** This experiment was a preliminary, proof-of-principle analysis designed to show that GEM acts as a plasma-lipid regulator in rainbow trout.

**Methods:** Female rainbow trout ( $133.20 \pm 9.53\text{g}$ ) were exposed to GEM (Sigma) by i.p. injections. The control groups received 0.9% Saline or 100% dimethylsulfoxide (DMSO; Sigma) while the experimental groups received GEM dissolved in DMSO. Injections were given at  $0.5 \mu\text{l/g}$  of fish every third day. The final dose of GEM was either 200 mg GEM/kg, 100 mg GEM/kg or 60 mg GEM/kg. After 16 days, fish were anesthetized with Benzocaine (100 mg/l; Sigma) prior to blood sampling from the caudal vessel. Fish were then sacrificed by a swift blow to the head followed by cervical dislocation. Plasma was separated from blood by a 2 min centrifugation at 12,000 g and aliquots were stored at  $-80^{\circ}\text{C}$  until analyses of plasma lipids. Plasma was analyzed for triglyceride and phospholipid concentration by gas chromatography according to the protocol outlined in Chapter 2. Data were analyzed with one-way ANOVA and a value of  $P < 0.05$  was considered significant.

**Results:** At the concentration of 100 mg/kg, GEM maximally reduced plasma triglyceride (-60%;  $P=0.002$ ) and phospholipid (-49.3%;  $P=0.003$ ) (Fig A1) compared to the DMSO control.

**Interpretation:** The effects on triglycerides and phospholipids shown in this experiment suggest that both the concentration and profile of lipoproteins in the plasma of rainbow trout are affected by exposure to fibrates. The dose of 100 mg/kg is the most likely to clearly reveal such effects.



**Figure A1.** The concentrations of plasma triglycerides (A) and phospholipids (B) in rainbow trout exposed to GEM. Data represents means + s.e.m. (n=8). A significant difference between treatment groups and the DMSO control is indicated with an asterisk (ANOVA,  $P < 0.05$ ).

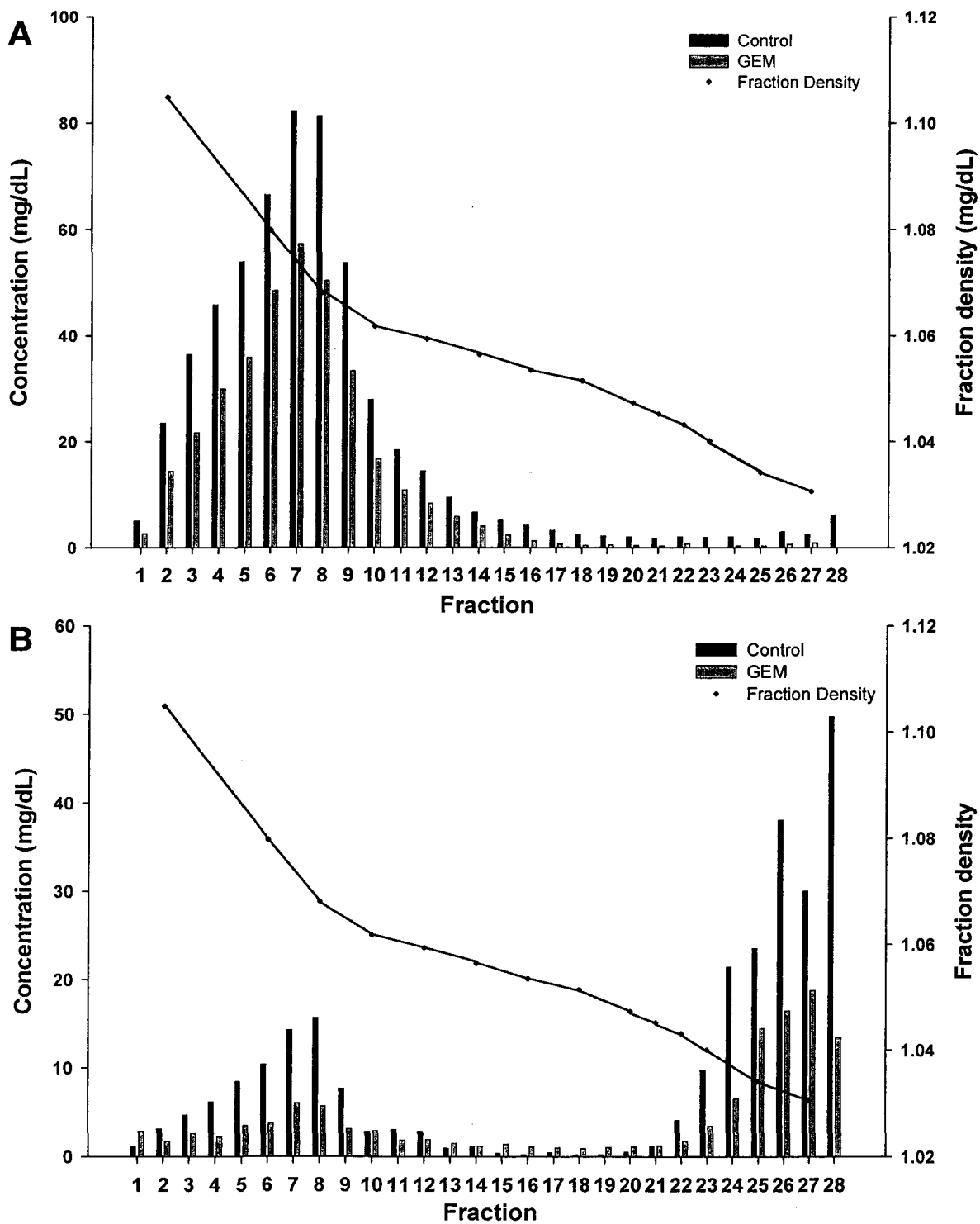
## **Appendix B: Lipid and density profiles created by ultracentrifugation**

**Objective:** The objective of this experiment was to separate rainbow trout plasma in a continuous density gradient. Lipoproteins can be isolated based on the density of the gradient (e.g. HDL = fractions 1-8; LDL = fractions 9-23; VLDL = fractions 24-28).

**Methods:** Exposure duration and dose, the set-up of the density gradient, the isolation of lipoproteins and the quantification of triglycerides and cholesterol in separated plasma are as described in Chapter 2

**Results:** Results are shown in Figure B1-A and B1-B. The density gradient is sufficient to segregate separated plasma into density intervals that correspond to the densities of the HDL, LDL and VLDL of rainbow trout. The concentration profiles of triglycerides and cholesterol along the density gradient are different. The method of eluting plasma from the ultracentrifugation tube was insufficient to allow a statistical comparison of fractions between exposed and control experimental groups (e.g. fraction 1 control vs fraction 1 exposed).

**Interpretation:** Future studies using similar methods should focus on generating a more optimal density gradient and develop a highly reproducible way to elute the plasma from the ultracentrifugation tube.



**Figure B1.** The concentration of cholesterol (A) and triglyceride (B) in rainbow trout plasma isolated by density-gradient ultracentrifugation. Concentration data for the first 24 fractions represent means of 7 fish whereas fractions 25-28 represent means from 2-6 fish. The density gradient represents means from 7 fish.

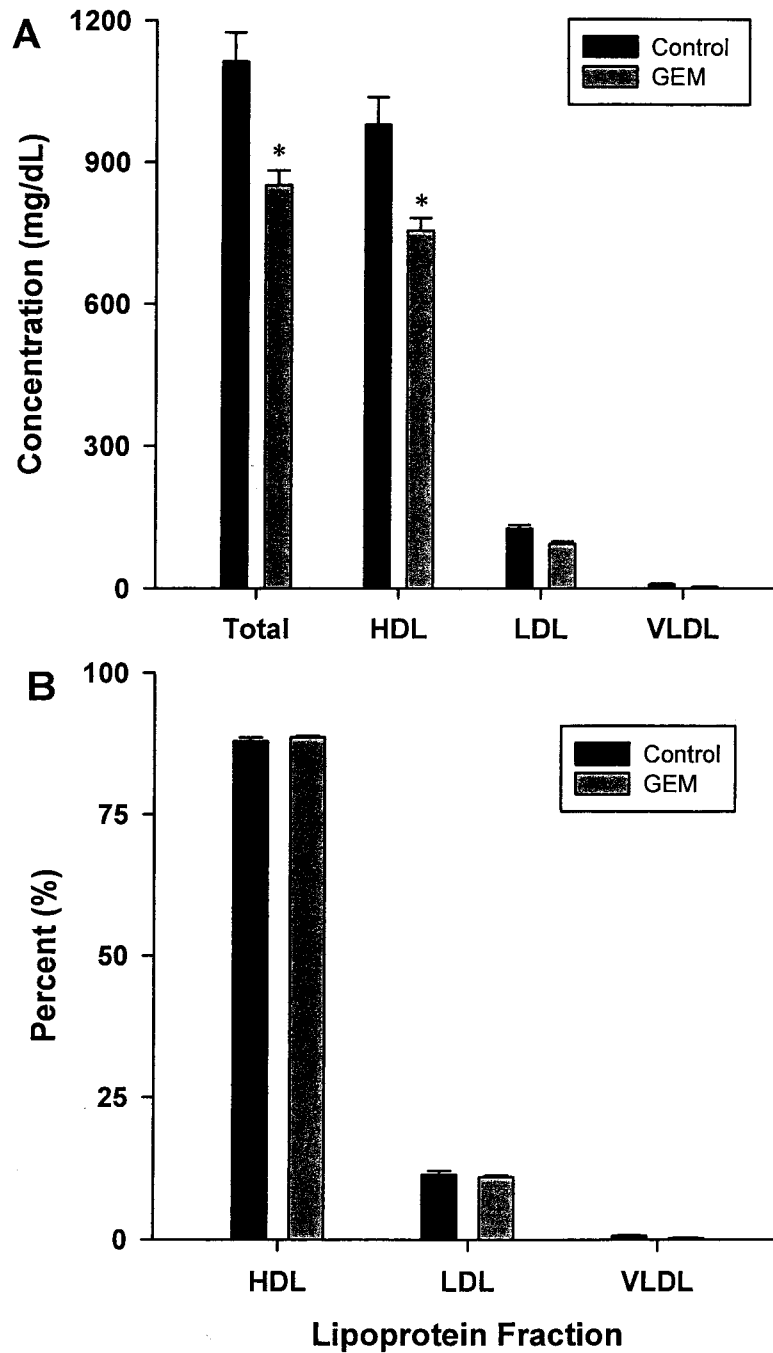
## **Appendix C: Protein content of lipoproteins**

**Objective:** The objective of this experiment was to analyze the protein content of lipoproteins isolated from rainbow trout plasma by density-gradient ultracentrifugation.

**Methods:** Exposure duration, dose, and estimates of protein content are as described in Chapter 2. Data were analyzed with a t-test and a value of  $P < 0.05$  was considered significant.

**Results:** Results are shown in Fig.C1A and Fig.C1B. GEM reduced both total plasma protein and HDL protein concentration by 23% ( $P = 0.002$  and  $P < 0.001$ , respectively compared to the DMSO control).

**Interpretation:** It is likely that the high protein concentration in HDL is influenced by the presence of albumin. In this study, the density gradient was insufficient to separate albumin from the HDL. To eliminate albumin from HDL, the density of the first fractions (see Appendix B) must be higher than the upper limit of HDL density outlined in Babin and Vernier (1989).



**Figure C1.** The protein content of rainbow trout lipoproteins. Panel A shows concentration of protein in the plasma and the plasma lipoproteins. Panel B shows the percent of total protein found in the lipoproteins. Therefore HDL+ LDL+ VLDL=100%. Data represents means + s.e.m. (n=7). A significant difference between treatment groups and the DMSO control is indicated with an asterisk (t-test,  $P < 0.05$ ).

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