The effects of statin and fibrate drugs on cholesterol metabolism and steroid production in two fish species.

By

Aziz Al-Habsi

Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

University of Ottawa

In partial fulfillment of the requirements for the

PhD degree in the

Ottawa-Carleton Institute of Biology

Thèse soumise à la
Faculté des Études Supérieurs et Postdoctorales
Université d'Ottawa
En vue de la réalisation partielle du doctorat à
L'Institut de Biologie Ottawa-Carleton

©Aziz Al-Habsi, Ottawa, Canada, 2014

This thesis is dedicated to my wife and children who have always stood by me and dealt with all my absence from many family occasions with a smile.

Acknowledgments

Completing a PhD is truly a marathon event, and I would not have able to complete this journey without the aid and support of countless people over the past seven years. First and foremost I would like to gratefully and sincerely thank my supervisor Dr. Thomas W. Moon for his guidance, understanding, friendship, and most importantly, his patience during my graduate studies at University of Ottawa. His mentorship was paramount in providing a well-rounded experience consistent my long-term career goals. He encouraged me not only to be a biologist but also be an instructor and independent thinker. I am not sure many graduate students are given the opportunity to develop their own individuality and self-sufficiency by being allowed to work with such independence. For everything you've done for me, Dr. Moon, I thank you.

I would like to extend my thanks the Department of Biology at University of Ottawa, especially those members of my doctoral committee for their input, valuable discussions and accessibility. In particular, I would like to thank Drs. Vance L. Trudeau, William Willmore, Kathleen M. Gilmour, and Jean-Michel Weber.

Furthermore I would like to sincerely thank the members of Moon lab for some much needed humor and entertainment in what could have otherwise been a somewhat stressful laboratory environment. These include Dr. Andrey Massarsky, Dr. Paul M. Craig, Kim Mitchell, Pamela Stroud, and Rance Nault. Special thanks go to Bill Fletcher and Vishal Saxena for taking care of the fish facility and ensuring that fish were always available for my experiments in such a short notice.

Finally, and most importantly, I would like to thank my wife Mouza. Her support, encouragement, quite patience and unwavering love were undeniably the bedrock upon

which the past fifteen years of my life have been built. Her tolerance of my occasional vulgar mood is testament in itself of her unyielding devotion and love. I thank my mother for always wanting me to aim high and reach for the best. Furthermore, I would like to thank all my brothers, especially, Saif and Mubarak (Abu Hisham) for their constant encouragement and support.

Abstract

Statins and fibrates are the most widely used pharmaceuticals in developed countries for the treatment of hyperlipidemia. They reach the aquatic environment mainly via wastewater treatment plants and have been detected at concentrations of ng to µg/L. They are "pseudopersistent" due to their continuous and increasing input into the aquatic environment. Cholesterol is essential to all animals, so inhibition of its synthesis by these drugs could have negative consequences in non-target species. Currently little is known regarding the possible effects of statins and fibrates on aquatic organisms. Thus, this thesis investigates the effects of atorvastatin (ATV; statin) and gemfibrozil (GEM; fibrate) on rainbow trout and zebrafish *in vivo* and *in vitro*.

Intraperitoneal injection of ATV, GEM, or the combination of the two drugs (A+G) into rainbow trout resulted in a nearly 30% reduction of cellular cholesterol content. Additionally, gene expression related to lipid homeostasis (LDL-r, HMGCR-1, and SREBP-1) was elevated. Furthermore, plasma creatine kinase activity and skeletal muscle gene expression related to rhabdomyolysis (atrogin-1 and f-box 25) were elevated. Plasma cortisol concentration was reduced in injected trout, suggesting that either the reduction in cholesterol resulted in treated fish lacking a proper stress response or that the treated fish were simply not responsive to the stress protocol.

Feeding zebrafish ATV, GEM, or A+G daily over a 30 day period resulted in nearly a 30% reduction of whole-body cholesterol content and a concomitant change in gene expression related to lipid homeostasis (SREBP-1, SREBP-2, HMGCR-1, PPARα, and PPARγ). Moreover, sex steroids (testosterone and estradiol) were also reduced.

Finally, exposing rainbow trout hepatocytes to ATV at 4.5 or 45 μg/L for 3 or 6 h resulted in reduced ^{14}C incorporation into cholesterol and cholesteryl ester. Elevated gene expression related to lipid homeostasis (HMGCR-1, SREBP-1, and PPARγ) also occurred.

This thesis demonstrated that ATV and GEM affected fish cholesterol and steroid hormone contents, as well as molecular markers of rhabdomyolysis. Whether these changes impact fish fitness remains to be determined.

Résumé

Les statines et les fibrates sont les médicaments les plus consommés dans les pays développés dans le traitement de l'hyperlipidémie. Par conséquent, ils se répandent dans l'environnement aquatique en quantités de ng à µg le litre lorsqu'ils font leur passage via les usines de traitement d'eau. Grâce à leur introduction continuelle et croissante dans l'environnement aquatique, les statines et les fibrates sont 'pseudopersistent`. Le cholestérol joue un rôle essentiel chez les animaux, donc l'inhibition de sa synthèse peut avoir des effets néfastes chez les espèces non-ciblées. Présentement, il y a encore plusieurs inconnus en ce qui concerne les effets des statines et des fibrates chez les organismes aquatiques. Donc, cette thèse enquête les effets d'atorvastatine (ATV; une statine) et de gemfibrozil (GEM; un fibrate) chez la truite arc-en-ciel et le poisson zèbre *in vivo* et *in vitro*.

Les truites arc-en-ciel qui ont été administrées de l'ATV, du GEM ou la combinaison de ces deux médicaments (A+G) par injection intrapéritonéale ont démontré une réduction de près de 30 pour cent du contenu cellulaire de cholestérol. De plus, l'expression des gènes reliée à l'homéostasie des lipides (LDL-r, HMGCR-1, et SREBP-1) était élevée. En outre, l'activité de la créatine kinase de plasma ainsi que l'expression de gènes reliée au rhabdomyolyse (atrogin-1 et f-box 25) dans les muscles squelettiques étaient élevées. Le niveau de cortisol dans le plasma a diminué, ce qui suggère que soit les niveaux diminués de cholestérol ont réduit la capacité des poissons à répondre au stress, ou que soit les poissons n'étaient tout simplement pas sensibles au protocole de stress employé.

Par ailleurs, les poissons zèbre qui ont été administrés de l'ATV, du GEM, ou de l'A+G par alimentation quotidienne pendant 30 jours ont démontré une réduction de près de 30 pour cent du contenu de cholestérol du corps entier accompagné d'un changement

d'expression de gènes reliée à l'homéostasie des lipides (SREBP-1, SREBP-2, HMGCR-1, PPARα et PPARγ). De plus, les niveaux de stéroïdes sexuels (la testostérone et l'estrogène) ont été réduits.

Finalement, l'exposition des hépatocytes de truite arc-en-ciel à l'ATV (4.5 ou 45 µg le litre) pendant 3 ou 6 heures a diminué l'incorporation de ^{14}C dans le cholestérol et dans l'ester de cholestérol. De plus, l'expression de gènes reliée à l'homéostasie des lipides (HMGCR-1, SREBP-1 et PPARγ) étaient élevée.

Cette thèse a donc démontré que l'ATV et le GEM ont eu des effets sur les contenus de cholestérol et des stéroïdes sexuels ainsi que sur les marqueurs moléculaires de rhabdomyolyse. Il reste toujours à déterminer si ces changements pourront affecter la valeur sélective des poissons.

Table of Contents

Acknowledgments iii

Abstract v

Résumé vii

Table of Contents ix

List of Figures xiii

List of Tables xix

List of Abbreviations xx

Chapter 1: General Introduction 1

1.1. Thesis Rationale..........1

1.2. Cholesterol synthesis and metabolism.......... 2

1.3. Regulation of cholesterol biosynthesis 6

1.4. Cholesterol in fish 10

1.5. Pharmaceuticals in the environment 12

1.6. Statin drugs 15

1.7. Pharmacokinetics of Atorvastatin and Gemfibrozil 19

1.8. Stress response in fish..........23

1.9. Thesis outline and hypothesis 25

Chapter 2: The effects of intraperitoneal injection of atorvastatin and gemfibrozil on cholesterol metabolism and steriod production in rainbow trout *Oncorhynchus mykiss* 28

2.1. Introduction 28

2.2. Materials and methods 31
2.2.1. Fish used 31
2.2.2. Intraperitoneal injection method 31
2.2.3. Blood and tissue collection 32
2.2.4. Biochemical analyses 33
2.2.5. RNA isolation 34
2.2.6. Real-time quantitative RT-PCR 35
2.2.7. Statistical analysis 36
2.3. Results 39
2.4. Discussion 59
2.5. Conclusions 66
Chapter 3: Exposure to Atorvastatin (ATV) and Gemfibrozil (GEM) affects cholesterol metabolism and steroid production in the zebrafish *Danio rerio* 68
3.1. Introduction 68
3.2. Materials and methods 72
3.2.1. ATV and GEM water-borne exposure 72
3.2.2. ATV and GEM feeding experiment 73
3.2.3. Lipid and steroid extraction and estimates 73
3.2.4. RNA isolation 75
3.2.5. Real-time quantitative RT-PCR 76
3.2.6. Tissue distribution of hmgcr transcripts...79
3.2.7. Statistical analyses 79
3.3. Results 80

3.3.1. Tissue distribution of hmgcr1 and hmgcr2 80

3.3.2. Water-borne exposure 80

3.3.3. Drug feeding experiment 82

3.4. Discussion 101

3.5. Conclusions 106

Chapter 4: The effects of atorvastatin on lipid metabolism in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes 108

4.1. Introduction 108

4.2. Materials and methods 111

4.2.1. Chemicals and standards 111

4.2.2 Hepatocytes preparation and isolation 111

4.2.3. Exposure to ATV 112

4.2.4. Non-Radioactive exposure 113

4.2.5. $^{14}CO_2$ collection 113

4.2.6. Isolation of [^{14}C]-Glucose 114

4.2.7. Determination of [^{14}C]-Glycogen 114

4.2.8. Lipid isolation and fractionation 114

4.2.9. RNA isolation 115

4.2.10. Real-time qantative RT-PCR 116

4.2.11. Statistical analysis 118

4.3. Results 118

4.4. Discussion 134

4.5. Conclusions 139

Chapter 5: General conclusions and perspectives 140

5.1. Hypothesis tested 141

5.2. Effects of ATV and GEM on cholesterol synthesis and related genes 142

5.3. Effects of ATV and GEM on cortisol and sex steroid production 145

5.4. Effects of ATV and GEM on muscle and kidney 147

5.5. Summary 148

References 150

List of Figures

Chapter 1: General introduction 1

Figure 1.1. The cholesterol biosynthesis pathway…………………………..............5

Figure 1.2. Chemical structures of selected HMGCoAR inhibitors (statins) and the fibrate drug gemfibrozil…………………………………………….....................17

Chapter 2: The effects of intraperitoneal injection of atorvastatin and gemfibrozil on cholesterol metabolism and steriod production in rainbow trout *Oncorhynchus mykiss* 28

Figure 2.1. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV, 0.29 μg/g), Gemfibrozil (GEM, 6.35 μg/g), and the combination of the two drugs (A+G) on plasma cholesterol concentration of rainbow trout that were not fed and stressed (nFS), fed and stressed (FS), or not fed and not stressed (nFnS) …..........................45

Figure 2.2. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on liver LDLR, HMGCR-1, and HMGCR-2 transcript abundance from rainbow trout that were fasted and stressed (nFS) …………..……………………………………………………46

Figure 2.3. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma triglyceride concentrations of rainbow trout ………………………………………47

Figure 2.4. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on liver SREBP-1, PPARα, PPARβ and PPARγ transcript levels ……………………………………..48

Figure 2.5. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma creatinine concentration of rainbow trout ……………………………………….49

Figure 2.6. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma lactate concentration of rainbow trout …………………………………......................50

Figure 2.7. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma cortisol concentration of rainbow trout …………………………………………………..51

Figure 2.8. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on hematocrit of rainbow trout ………………………………………………………………………….52

Figure 2.9. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma protein concentration of rainbow trout …………………………………………………..53

Figure 2.10. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma creatine kinase concentration of rainbow trout …………………………………………...54

Figure 2.11. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on white muscle f-box 25, atrogin-1, and MURF-1 transcript abundance ……………………………55

Figure 2.12. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on red muscle f-box 25, and atrogin-1 transcript abundance ……………………………………….56

Figure 2.13. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma estradiol concentration of rainbow trout …………………………………………..57

Figure 2.14. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma testosterone concentration of rainbow trout …………………..............................58

Chapter 3: Exposure to Atorvastatin (ATV) and Gemfibrozil (GEM) affects cholesterol metabolism and steroid production in the zebrafish *Danio rerio* ……………………...68

Figure 3.1. Tissue distribution of (A) hmgcr-1 and (B) hmgcr-2 mRNA abundance in various tissues of adult female zebrafish using qPCR …………………………......87

Figure 3.2. Effects of Atorvastatin (ATV, 45 μg/L), Gemfibrozil (GEM, 1.5 mg/L), and the combination of the two drugs (A+G) on whole-body cholesterol in male and female adult zebrafish following a 30 day water-borne exposure to the drugs……88

Figure 3.3. Effects of water-borne Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body triglyceride content of male and female adult zebrafish exposed for 30 days to the drugs ………….………………..89

Figure 3.4. Effects of water-borne Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body cortisol content of male and female adult zebrafish exposed for 30 days to the drugs …………………………..90

Figure 3.5. Effects of water-borne Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body testosterone and estradiol contents of male and female adult zebrafish exposed for 30 days to the drugs …..91

Figure 3.6. Effects of feeding Atorvastatin (ATV, 0.53 μg/g), Gemfibrozil (GEM, 16 μg/g), and the combination of the two drugs (A+G) on whole-body cholesterol contents in male and female adult zebrafish ……………………………...…92

Figure 3.7. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on liver SREBP-2, LDLR and hmgcr-1 and -2 transcript abundance in male and female adult zebrafish …………………………93

Figure 3.8. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on brain hmgcr-1 and -2 transcript abundance in male and female adult zebrafish …………………………………………………...94

Figure 3.9. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body triglyceride content of male and female adult zebrafish ………………………………………………………………...95

Figure 3.10. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on liver SREBP-1, PPARα, PPARβ and PPARγ transcript abundance in adult male and female zebrafish …………………………96

Figure 3.11. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on skeletal muscle atrogin-1 transcript abundance in adult male and female zebrafish ………………………………….....97

Figure 3.12. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on liver cyp3a65 transcript abundance in adult male and female zebrafish ……………………………………………………………98

Figure 3.13**.** Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body cortisol content in adult male and female zebrafish ………………………………………………………………99

Figure 3.14. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body testosterone and estradiol contents in adult male and female zebrafish …………………………………….....100

Chapter 4: The effects of atorvastatin on lipid metabolism in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes …………………………………………………..…108

Figure 4.1. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-cholesterol. Hepatocytes were exposed for 3 and 6 h; control hepatocytes were exposed to DMSO (final concentration <0.01%) ……………………………………………………………………………..…………123

Figure 4.2. The effects of exposure to Atorvastatin (ATV, 45 µg/L), Gemfibrozil (GEM, 150 µg/L), and the combination of the two drugs (A+G) on trout hepatocyte LDLR, HMGCR-1 and HMGCR-2 transcript abundance at 3 and 6 h. Control hepatocytes were exposed to DMSO (final concentration <0.01% ....…………...124

Figure 4.3. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-cholesterol ester at 3 and 6 h ……………125

Figure 4.4. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-triglycerides at 3 and at 6 h ………………..126

Figure 4.5. The effect of exposure of Atorvastatin (ATV, 45 μg/L), Gemfibrozil (GEM, 150 μg/L), and the combination of the two drugs (A+G) on hepatocytes SREBP-1, PPARα, PPARβ and PPARγ transcript abundance ………..………….127

Figure 4.6. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-phospholipids at 3 and at 6 h ……………...128

Figure 4.7. The effect of exposure of Atorvastatin (ATV, 45 μg/L), Gemfibrozil (GEM, 150 μg/L), and the combination of the two drugs (A+G) on hepatocytes CYP3A27 transcript abundance …………………………………………………...129

Figure 4.8. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into $^{14}CO_2$ at 3 and at 6 h …………………………..130

Figure 4.9. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-glucose at 3 and at 6 h …………………..131

Figure 4.10. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-glycogen at 3 and at 6 h …………………...132

Figure 4.11. Total [^{14}C]-acetate incorporation into different lipid fractions by hepatocytes isolated from rainbow trout. Hepatocytes were incubated for 6 h in the presence of 0.31 μCi/mL [^{14}C]-acetate ……………………………..…………..133

List of Tables

Chapter 2: The effects of intraperitoneal injection of atorvastatin and gemfibrozil on cholesterol metabolism and steriod production in rainbow trout *Oncorhynchus mykiss* 28

Table 2.1. Primers used to quantify mRNA levels in liver, red muscle, and white muscle of rainbow trout, their sequences, amplicon size and GenBank accession number ……….38

Chapter 3: Exposure to Atorvastatin (ATV) and Gemfibrozil (GEM) affects cholesterol metabolism and steroid production in the zebrafish *Danio rerio* ……….68

Table 3.1. Primers used to assess mRNA abundance in liver, brain, and muscle of zebrafish, their sequences, amplicon size and GenBank accession number ……….78

Chapter 4: The effects of atorvastatin on lipid metabolism in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes ……….108

Table 4.1. Primers used to assess mRNA transcript abundance in hepatocytes from rainbow trout, their sequences, amplicon size and GenBank accession number..…117

List of abbreviations

17, 20-DP	17,20-dihydroxy-4-pregnen-3-one
A+G	Atorvastatin and Gemfibrozil
ACTH	Adrenocorticotropic hormone
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATV	Atorvastatin
BCA	Bicinchoninic acid
bHLH-Zip	Basic helix-loop-helix-leucine zipper
BSA	Bovine serum albumin
CK	Plasma creatine kinase
COPII	Coat protein complex II
CRF	Corticotrophin-releasing factor
CVT	Cerivastatin
CYP450	Cytochrome P450
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EE2	17 alpha-ethinylestradiol
EIA	Enzyme immunoassay
ER	Endoplasmic reticulum
FS	Fed and stressed
GEM	Gemfibrozil
GSI	Gonadosomatic index

HDL	High density lipoproteins
HMGCoA	3-hydroxy-3-methylglutaryl-CoA
HMGCoAR	3-hydroxy-3-methylglutaryl-CoA reductase
HPI	Hypothalamus-pituitary-interrenal
HSD3B	3β-hydroxysteroid dehydrogenase
HSPs	Heat shock proteins
IP	Intraperitoneal injection
K_{ow}	Octanol-water coefficient
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
LPs	Lipoproteins
MC2R	Melanocortin receptor
mRNA	Messenger RNA
nFnS	Not fed and not stressed
nFS	Not fed and stressed
P450scc	Cytochrome P450 side chain cleavage enzyme
P45c17	17 α-hydroxylation
PBS	Phosphate buffered saline
PGC	Primordial germ cell
PPAR	Peroxisome proliferator activated receptor
PPCPs	Pharmaceutical and personal care products
RIA	Radioimmunoassay
RNA	Ribonucleic acid

SCAP	SREBP cleavage-activating protein
SREBPs	Sterol regulatory element-binding proteins
SSD	Sterol-sensing domain
StAR	Steroidogenic Acute Regulatory protein
VLDL	Very low density lipoproteins
WWTP	Wastewater treatment plant

CHAPTER 1

General Introduction

1.1. Thesis rationale

Pharmaceutical and personal care products (PPCPs) in the aquatic environment generally occur at low concentrations (ng to µg/L), and there is little evidence that they exert acute toxicity to aquatic organisms (fish) based on these low concentrations (Daughton and Ternes, 1999; Fent et al., 2006; Halling-Sörensen et al., 1998). On the other hand, aquatic organisms may be chronically exposed to PPCPs because of their continuous introduction into the aquatic environment. Although, PPCPs are designed to target specific metabolic and molecular pathways in the target organism (humans or farm animals), they could have many effects on non-target organisms since basic mechanisms such as cell division, signal transduction, and key metabolizing enzymes (different cytochrome P450 isoforms) are conserved in a wide variety of organisms (Buhler and Wang-Buhler, 1998). Therefore, PPCPs may have similar effects or side effects on different organisms that share these basic mechanisms. This is shown at least for the fibrate drugs in both goldfish and rainbow trout (Mimeault et al., 2006; Mimeault et al., 2005).

Statins are human pharmaceuticals that block the mevalonic acid pathway and consequently cholesterol biosynthesis. There are several rare side effects associated with statin treatment, the most prominent of which is rhabdomyolysis or the breakdown of skeletal muscle, with degradation products from these muscle cells causing further damage in the body. Rhabdomyolysis is a rare but potentially fatal complication of statin treatment when combined with the fibrate drug Gemfibrozil (GEM), a lipid lowering drug (Christopher-Stine, 2006; Jennie et al., 2004; SoRelle, 2001).

Given that pharmaceuticals are now found and continuously introduced into the aquatic environment, and that fish are continuously exposed to them throughout their life and as vertebrates have the same systems that these drugs target in humans, clearly fish may be affected by these drugs as the humans for which they were originally designed. I will study statins and fibrates that are known to impact cholesterol and lipids in humans and given the importance of cholesterol and lipids in fish, do these drugs pose a problem to fish? The following will introduce the critical components of this subject that form the basis of my thesis.

1.2. Cholesterol synthesis and metabolism

Cholesterol is an essential biological molecule. It is an amphipathic molecule that is an essential structural component of eukaryote cell membranes where it provides stability and modulates fluidity by hindering the packing of phospholipids molecules (Burger et al., 2000; Norum et al., 1983; Robertson and Hazel, 1995; Simons and Ikonen, 2000). Cholesterol increases the order within membranes by aligning the long fatty acid acyl chains of phospholipids more perpendicular to the plane of the membrane resulting in a thicker and more rigid membrane (Burger et al., 2000; Maxfield and Tabas, 2005). Cholesterol is an essential structural component of the outer layer of blood lipoproteins (Faust et al., 1988; Siperstein, M. D., 1984) and a precursor of steroids including corticosteroids (cortisol), sex hormones (estrogen, testosterone), bile acids, and vitamin D (Espenshade and Hughes, 2007; Norum et al., 1983). Cholesterol plays a major regulatory role in several cellular processes including lipoprotein synthesis, cell growth, receptor function, gene expression and DNA replication (Morris et al., 1995; Russell, 1992). Cholesterol arises either from the diet (exogenous) or by biosynthetic (endogenous) pathways.

Cholesterol contains 27 carbon atoms, all of which arise from a two carbon acetate group acetyl-CoA. The biosynthetic pathway which takes place within the cytosolic compartment of cells involves at least 27 enzymes and is referred to as the mevalonate pathway (Russell, 1992). Cytosolic acetyl-CoA is the primary building block for cholesterol and fatty acid biosynthesis (Espenshade and Hughes, 2007; Faust et al., 1988; Goldstein and Brown, 1990; Russell, 1992). The pathway for this process is shown in Figure 1.1. In a broad sense, the biosynthetic pathway can be divided into the squalene portion and the post-squalene portion. The squalene portion starts with the production of acetyl-CoA in the mitochondria that comes either from the conversion of pyruvate by pyruvate dehydrogenase or from β-oxidation of fatty acids. Within the mitochondria, acetyl-CoA combines with oxaloacetate and is converted to citrate by citrate synthase; citrate can then be transported to the cytosol through a carrier-mediated dicarboxylate transporter. This citrate is re-converted into acetyl-CoA and oxaloacetate by citrate lyase. In the cytosol, acetyl-CoA acts as a precursor for cholesterol and fatty acid biosynthesis, while oxaloacetate provides NADPH after conversion to malate for lipid biosynthesis. Acetoacetyl-CoA is generated via the condensation of two molecules of cytosolic acetyl-CoA by the thiolase reaction, and then another molecule of acetyl-CoA is incorporated with acetoacetyl-CoA to produce 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) in a reaction that is catalyzed by 3-hydroxy-3-methylglutaryl-CoA synthase (Fig. 1.1) (Espenshade and Hughes, 2007; Faust et al., 1988; Goldstein and Brown, 1990; Russell, 1992). In the rate-limiting step in cholesterol biosynthesis, HMGCoA is converted to mevalonate by the action of HMGCoA Reductase (HMGCoAR; E.C. 1.1.1.88) (Espenshade and Hughes, 2007; Faust et al., 1988; Goldstein and Brown, 1990; Russell, 1992). HMGCoAR is bound to the membranes of the

endoplasmic reticulum and to perform the reductive deacylation of HMGCoAR requires reducing equivalents as NADPH (Faust et al., 1988). Squalene is formed from mevalonate through a series of six additional reactions (Fig. 1.1) (Espenshade and Hughes, 2007; Faust et al., 1988). In the post-squalene portion of cholesterol pathway, lanosterol is formed via the cyclization of squalene in a two-step enzymatic reaction involving squalene expoxidase and lanosterol cyclase (Espenshade and Hughes, 2007; Faust et al., 1988). Lanosterol is then converted to cholesterol by a series of oxidation, reduction, and demethylation steps. The required enzyme reactions have been defined and metabolic intermediates identified; however, the precise sequence of reactions between lanosterol and cholesterol remains to be established (Waterham and Wanders, 2000) (Fig. 1.1).

The fate of newly synthesized cholesterol is variable; cholesterol may be catabolized to end products (e.g., bile acids, steroid hormones, vitamin D, and lipoproteins); it may be excreted directly in the bile; it may be stored as cholesteryl esters; or, used in the biosynthesis of new membranes in dividing cells (Goldstein and Brown, 1990).

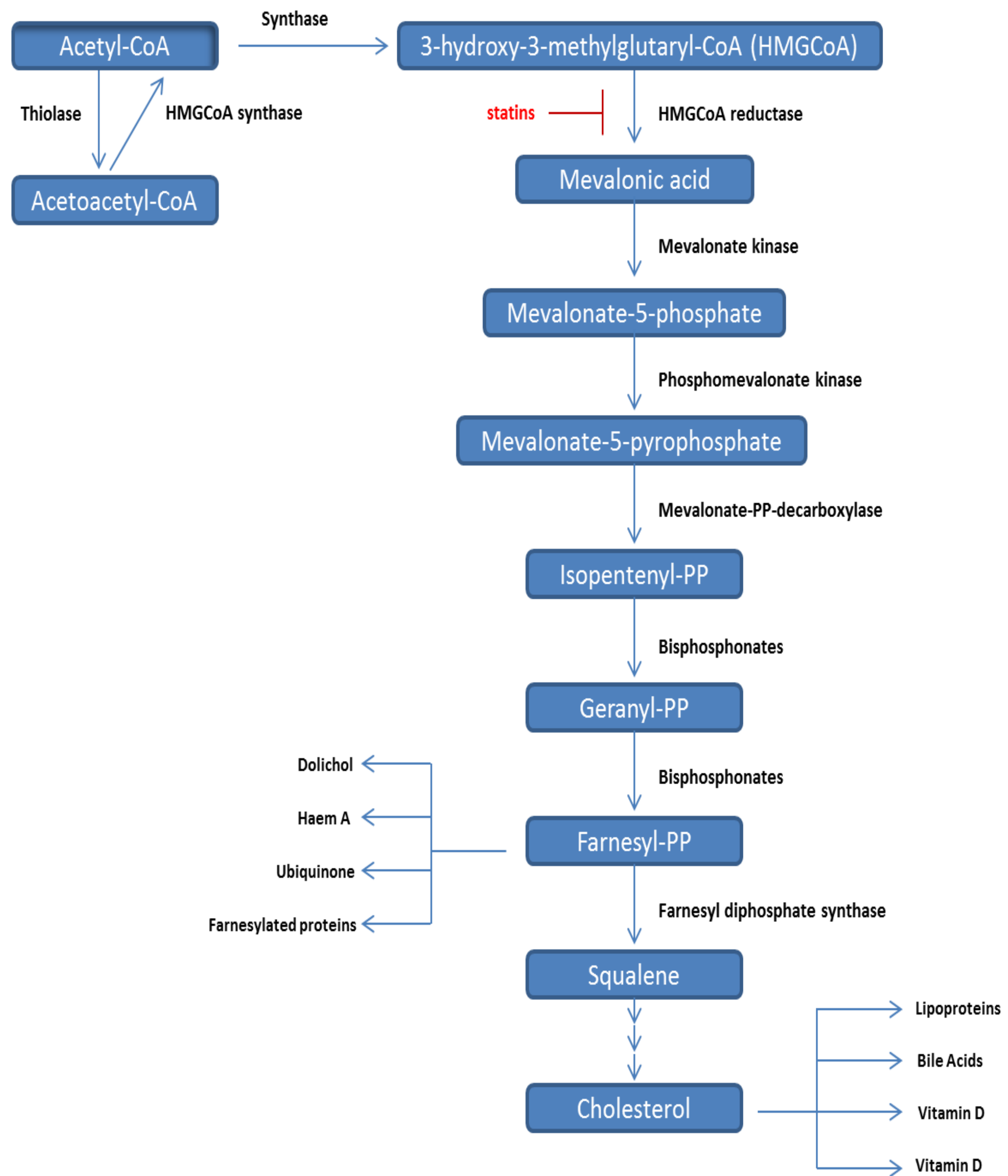


Figure 1.1. The cholesterol biosynthesis pathway

1.3. Regulation of cholesterol biosynthesis

Cholesterol and other fatty acids are transported in the blood in sphere-shaped particles called lipoproteins. In addition to free and esterified forms of cholesterol, lipoproteins (LPs) are composed of phospholipids, triglycerides, and proteins (Lee et al., 2003). Lipoproteins are classified according to their size and densities into four main classes each with a different function (Lewis and Rader, 2005). The largest and the least dense are called chylomicrons; these transport dietary cholesterol and triglycerides from the intestinal mucosa to the rest of the body. Very low density lipoproteins (VLDL) are rich in triglycerides and deliver fatty acids for storage to adipocytes and to skeletal and cardiac muscles for metabolism (Lee et al., 2003). Low density lipoproteins (LDL), also known as "bad cholesterol", are LPs rich in cholesterol ester and deliver cholesterol to the peripheral tissues for steroidogenesis and the maintenance of cell membrane integrity (Lee et al., 2003). High density lipoproteins (HDL) or "good cholesterol" transport excess cholesterol from extrahepatic cells to the liver for recycling or bile acids catabolism (Lee et al., 2003; Lewis and Rader, 2005).

Two-thirds of the total cholesterol found in the body is of endogenous origin, with the major site of cholesterol biosynthesis being the liver (Brown and Goldstein, 1980). Liver-derived cholesterol is the main cause of the development of hypercholesterolemia, whereas cholesterol in non-hepatic cells is needed for normal cell functions (Russell, 1992). Therefore, the synthesis and utilization of cholesterol must be tightly regulated to prevent over-accumulation and abnormal deposition within the body. Of particular importance clinically is the abnormal high deposition of cholesterol and cholesterol-rich LPs in the coronary arteries. Such deposition, eventually leading to atherosclerosis, is the leading

contributory factor in diseases of coronary arteries and in North America is considered one of the leading causes of cardiovascular mortality and morbidity; it is estimated that each year 50% of mortality in the United States is caused by atherosclerosis or atherosclerosis-related diseases (Choy et al., 2000; Choy et al., 2004). Moreover, high cholesterol is associated with the formation of amyloid-β peptides, the principal constituents of senile plaques found in the brains of patients with Alzheimer's disease (Corrdy et al., 2006; Marx, 2001; Shobab et al., 2005). Low plasma cholesterol levels are associated with mood disorders and an increased suicidal behavior in humans (Boston et al., 1996; Lűtjohann, 2007).

Cholesterol homeostasis is achieved by a feedback mechanism that monitors membrane cholesterol levels and controls transcription of genes mediating cholesterol biosynthesis and uptake (Sakakura et al., 2001). This homeostatic process is regulated by a family of membrane-bound transcription factors called sterol regulatory element-binding proteins (SREBPs). SREBPs directly activate the expression of a suite of genes that are dedicated to the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids, and they are considered as global regulators of lipid synthesis (Sakakura et al., 2001; Shimano, 2009).

SREBPs belong to the basic helix-loop-helix-leucine zipper (bHLH-Zip) family of proteins that are synthesized as inactive precursors residing in the endoplasmic reticulum (ER) (Brown and Goldstein, 1997). Each SREBP is about 1150 amino acids in length, organized into three domains: an NH_2-terminal domain, that is about 480 amino acids which contains the bHLH-ZIP region for DNA binding; two hydrophobic transmembrane-spanning segments that are interrupted by a short loop of about 30 amino acids that project into the

lumen of the ER; and, a COOH-terminal regulatory domain of about 590 amino acids (Brown and Goldstein, 1997; Goldstein et al., 2002).

There are two SREBP genes which encode three isoforms which have been described in several mammalian species: SREBP-1a and SREBP-1c produced from a single gene (*SREBF1*) located on human chromosome 17p11.2, and SREBP-2 from a separate gene (*SREBF2*) located on human chromosome 22q13 (Hua et al., 1995). SREBP-1 and SREBP-2 share 47% amino acids sequence identity and have similar domain structures (Miserez et al., 1997). Moreover, these three SREBP isoforms play different roles in lipid synthesis. SREBP-2 controls the expression of genes that are primarily involved in cholesterol synthesis (e.g. HMGCoAR) and the uptake of LDL (e.g. LDL receptor); SREBP-1c controls the expression of genes primarily involved in the synthesis of fatty acids, phospholipids, and triglycerides; SREBP-1a tends to have specificities that overlap with those of SREBP-1c and SREBP-2, such as the activation of fatty acids and cholesterol synthesis (Brown and Goldstein, 1997; Goldstein et al., 2006; Shao and Espenshade, 2012).

SREBPs are produced in the ER as a membrane bound precursor, and their activation depends on a polytopic membrane domain called the SREBP cleavage-activating protein (SCAP) (Horton et al., 2002; Shimano, 2009). SCAP plays a dual role in cellular cholesterol homeostasis, both as an escort for SREBPs and as a cholesterol sensor (Horton et al., 2002; Shimano, 2009). When cellular cholesterol is depleted, SCAP facilitates the assembling of SREBP into coat protein complex II (COPII)-coated vesicles which carry the SCAP-SREBP complex to the Golgi apparatus where the inactive SREBP is cleaved sequentially by two proteases to release the transcriptionally active SREBP into the cytosol (Horton et al., 2002).

The released active fragment travels to the nucleus where it activates a suite of genes involved in cholesterol synthesis and uptake (Horton et al., 2002; Shimano, 2009).

Conversely, when cellular cholesterol achieves a high level or when cells become satiated with cholesterol, SCAP interacts with cholesterol and adopts a conformation that would allow the SCAP-SREBP complex to bind to a closely related polytopic membrane protein called insulin-induced gene-1 or -2 (Insig-1 or Insig-2) (Yabe et al., 2002; Yang et al., 2002). When the SCAP-SREBP complex binds to Insig, the complex no longer can be incorporated into COPII-coated vesicles, resulting in the ER retaining SREBP and the transcription of the target genes to decline, decreasing cholesterol synthesis and uptake (Yabe et al., 2002).

Levels of cholesterol and other sterols are monitored in mammalian cells by two membrane-embedded proteins that are also found in the ER, namely SCAP and HMGCoAR (Goldstein et al., 2006). These two proteins share a polytopic intramembrane sequence named the sterol-sensing domain (SSD), and through this domain, cholesterol ensures SCAP and HMGCoAR bind Insigs in the ER (Goldstein et al., 2006). Upon binding SCAP and HMGCoAR, Insigs exert actions that limit cholesterol synthesis; therefore by controlling SCAP and HMGCoAR, Insigs play a pivotal role in the transcriptional and post-transcriptional regulatory mechanisms that maintain cholesterol homeostasis (Sun et al., 2007).

HMGCoAR, the rate limiting enzyme in the cholesterol biosynthetic or more specifically the mevalonate pathway, is a membrane protein that resides in the ER membrane (Goldstein and Brown, 1990). Mammalian HMGCoAR consists of 887 or 888 amino acids that can be separated into two distinct domains (Liscum et al., 1985). The hydrophobic N-

terminal domain that consists of 339 amino acids and it is integrated into the membrane by virtue of eight membrane-spanning segments that are separated by short loops (Liscum et al., 1985; Roitelman et al., 1992). The hydrophilic C-terminal domain that projects into the cytosol which is catalytically active and comprises 548 amino acids (Roitelman et al., 1992). The amino acid sequence of the membrane domain of the HMGCoAR is well conserved among mammalian species (Gertler et al., 1988; Luskey and Stevens, 1985). The control of HMGCoAR activity is mediated through feedback mechanisms which includes sterol (cholesterol) and non-sterol isoprenoid (farnesol and farnesyl pyrophosphate) factors (Goldstein and Brown, 1990; Hampton, 2002; Mo and Elson, 2004; Xu and Simoni, 2003). Cholesterol appears as a key component of this feedback mechanism by inducing a rapid degradation of HMGCoAR protein (Goldstein and Brown, 1990). In mammalian cells, the SSD of HMGCoAR is required for the enzyme to bind Insig-1 and Insig-2 (Sever et al., 2003a). Such binding leads to sterol-mediated, accelerated degradation of HMGCoAR (Sever et al., 2003b). Moreover, HMGCoAR activity may also be regulated through covalent modification which results in the conversion of the enzyme between active and inactive forms (Russell, 1992). HMGCoAR is most active in its dephosphorylated form, and becomes inactive when phosphorylated primarily by AMP-activated protein kinase (AMPK) (Clarke and Hardie, 1990; Russell, 1992; Sun et al., 2006).

1.4. Cholesterol in fish

Fish possess cholesterol synthesizing abilities that are similar to other vertebrates and as discussed in section 1.3. Furthermore, fish, as with other vertebrate species, obtain cholesterol by dietary intake or by *de novo* synthesis (Pederson, 1988). Cholesterol biosynthesis is believed to proceed by the same pathway in fish as in higher vertebrates

(Henderson and Tocher, 1987). However, when using the standards applied to higher vertebrates or humans in particular, fish are classified as hyperlipidemic and hypercholesterolemic (Babin and Vernier, 1989). Hyperlipidemia and hypercholesterolemia are most common among teleost fishes, while elasmobranchs often show lipid contents in the same range reported for more phylogenetically advanced vertebrates (Babin and Vernier, 1989). For example, total plasma lipid concentrations in rainbow trout (*Oncorhynchus mykiss*) range between 960 and 4700 mg/100 mL (Hille, 1982). Moreover, when comparing the concentration of plasma lipids in fed rainbow trout and fed rats, the rat has three times less lipids (685 mg/100 mL vs 1940 mg/100 mL) and cholesterol (106 mg/100 mL vs 303 mg/100 mL) than the trout (Babin and Vernier, 1989).

Hypercholesterolemia seems to be a normal condition in teleost fishes in contrast to the condition in mammals where this condition is associated with coronary heart disease and atherosclerosis (Larsson and Fange, 1977). Fish, and salmonids in particular, are susceptible to atherosclerotic lesion formation in their coronary arteries which is attributed not to hypercholesterolemia *per se* but to growth rate and sexual maturity (Saunders et al., 1992). Moreover, the lesions in the salmonids lack intra- or extracellular lipid or calcium accumulations which is contrary to mammalian plaques; however, these lesions are similar to those found in the early stages of mammalian arteriosclerosis (Saunders et al., 1992).

Generally plasma cholesterol concentrations in most fish species are between 2- and 6-times higher than that of mammals (Babin and Vernier, 1989; Larsson and Fange, 1977). In contrast to mammals, the lipoprotein profile of teleosts fish is dominated by HDL, the concentrations of which may reach values between 1500-2000 mg/100 mL (Babin, 1987; Babin and Vernier, 1989; Chapman et al., 1978), a condition which may be related to or is

speculated to be responsible for the lack of lipid-containing lesions in fish (Farrell and Munt, 1983).

There is, however a lack of knowledge regarding cholesterol dynamics in fish. Few attempts to measure the synthesis of cholesterol in fish *in vivo* are reported. In northern pike (*Esox lucius* L.) injected with ^{14}C-acetate, cholesterol had the lowest specific activity of all the lipid classes examined (Kluytmans and Zandee, 1974). However, the researchers attributed the low cholesterol radioactivity to the fact that ^{14}C-acetate was injected into the dorsal white muscles, which generally has a low blood circulation/flow and a low oxidative metabolism. Thus these studies may be an artifact of the injection protocol.

The difficulty of measuring cholesterol *in vivo* led researchers to use isolated hepatocytes to make estimates of the rate of cholesterol synthesis in relation to that of fatty acid synthesis. Hepatocytes isolated from rainbow trout acclimated to different temperatures had rates of fatty acid synthesis from acetate exceeding that of sterol synthesis by 2-4 fold (Hazel and Prosser, 1979). Moreover, the rate of cholesterol synthesis in trout hepatocytes was related to the glycogen content of these cells. The rate of acetate incorporated into cholesterol at 20 ºC in hepatocytes containing low glycogen content was 31 nmol/g/h compared with 20.7 nmol/g/h in hepatocytes of high glycogen content (Voss et al., 1986).

1.5. Pharmaceuticals in the environment

PPCPs are an extraordinarily diverse group of chemicals used in veterinary medicine, agriculture practices, human health and cosmetic care (Daughton and Ternes, 1999; Fent et al., 2006; Halling-Sörensen et al., 1998). Specially, pharmaceuticals are created with the intent of causing a biological effect; they often have similar types of physiochemical behaviors that are characteristics of harmful xenobiotics including their ability to pass across

membranes, and their relative persistence in order to avoid inactivation before achieving their therapeutic intended effect (Daughton and Ternes, 1999). Many PPCPs are highly bioactive, many are polar but equally many are non-polar, and when present in the environment usually their concentrations are in the ng/L to µg/L range (Daughton and Ternes, 1999; Fent et al., 2006; Metcalfe et al., 2003; Trudeau et al., 2005). Moreover, PPCPs are complex molecules with different functionalities, physiochemical and biological properties, and their molecular weight typically ranges from 300 to 1000 (Daughton and Ternes, 1999).

Many PPCPs are biotransformed in the body, and undergo biodegradation that modifies their active chemical structure, which in turn results in a change in their physiochemical and pharmacological properties (Corcoran et al., 2010; Daughton and Ternes, 1999). Metabolism may lower activity or enhances water solubility; however, metabolism most often is incomplete, with excretion rates range from 10 to 100% but averaging around 60% of the parent compound (Daughton and Ternes, 1999). In human there are two important pathways of drug metabolism. Phase I metabolites results from modification of the active compound by hydrolysis, oxidation and reduction (Baillie et al., 2002; Daughton and Ternes, 1999). Phase II metabolites are Phase I metabolites which have been modified by glucuronidation or sulfation or other addition steps to further enhance their water solubility and thus excretion (Baillie et al., 2002; Daughton and Ternes, 1999). Depending on the type of pharmaceutical, up to 90% of certain drugs can become conjugated (Daughton and Ternes, 1999; Ternes, 1998). Moreover, conjugates can essentially act as a storage reservoir from which the free drugs can later be released into the environment since

some degradation products can even be more bioactive than the parent compound (Daughton and Ternes, 1999).

Pharmaceuticals are excreted through feces and urine as a mixture of metabolites and the unchanged parent substance. Thus, these compounds predominately enter the environment through wastewater effluents, aggravated by the fact that many people flush unused drugs down the drain or dispose of them within the household garbage (Jones et al., 2001). Other sources of pharmaceuticals in the Canadian environment are from the effluents of sewage treatment facilities, sludge from these plants spread on agricultural lands, terrestrial wet weather runoff and septic systems (Daughton and Ternes, 1999). Drugs are designed to have specific biological effects in the target organism or tissue. Despite having relatively short environmental half-lives, they may assume the quality of persistent pollutants as they are continuously introduced into the environment (Daughton and Ternes, 1999; Oaks and Gilbert, 2004). One extreme catastrophic toxic effect of a pharmaceutical was the decline in the population of three species of vultures in India and Pakistan due to the use of the anti-inflammatory drug diclofenac. Diclofenac exposure caused renal failure and visceral gout in vultures when they fed on the carcasses of domestic cattle that were treated with a normal dose of the drug shortly before death (Oaks and Gilbert, 2004). Additionally, some pharmaceuticals are found to bioconcentrate (e.g., GEM) (Mimeault et al., 2005; Trudeau et al., 2005) and to bioaccumulate (e.g., 17 alpha-ethinylestradiol (EE2)) (Al-Ansari et al., 2010) in non-target aquatic organisms, which means there is the enhanced likelihood of adverse biological effects.

1.6. Statin drugs

Pharmaceuticals are a class of new, so-called emerging contaminants that have raised concern in recent years because of their pseudopersistence in the environment, their increased usage patterns and the fact they are designed specifically to be bioactive (Daughton and Ternes, 1999). For example, in 2001 the annual sales of the cholesterol lowering statin drug Atorvastatin (Lipitor®) in Canada was over 5000 kg (Metcalfe et al., 2004). Canadians spent more than $18 billion and $20.7 billion on pharmaceuticals in 2005 and 2007, respectively (Cavallucci, 2007; Robinson et al., 2007). According to IMS Health (Cavallucci, 2007) cholesterol reducers (statins) were the fastest growing drug among Canada's most prescribed drugs, and for four straight years (2004-2007), Atorvastatin (Lipitor®) was Canada's most prescribed drug. Statins or HMGCoAR inhibitors are drugs used to treat hypercholesterolemia (Stancu and Sima, 2001). Statins are also recommended for the treatment and prevention of other conditions including stroke, osteoporosis, progression of multiple sclerosis and Alzheimer's disease to mention only a few (Amarenco et al., 2004).

There are several different statins available on the world market, and all share an HMG-like moiety (Istvan and Deisenhofer, 2001). Chemical structures of selected statins are presented in Figure 1.2. Mevastatin, a fungal product extracted from *Penicillium citrinum* was the first statin developed (Endo et al., 1976). Lovastatin, simvastatin and pravastatin are also derivatives of fungal products, but lovastatin is the only truly natural product and it is derived from *Aspergillus terreus* whereas simvastatin and pravastatin are semi-synthetic products that are derived from lovastatin and compactin, respectively (Endo, 1992; Morris et al., 1995). Lovastatin and simvastatin possess a lactone ring in their structure and are

transformed into the active open acid form in the body while pravastatin is administered as the biologically active open ring acid form (Corsini et al., 1999). Atorvastatin (ATV), fluvastatin, cerivastatin (CVT) and rosuvastatin are completely synthetic compounds with very different structures from the statins derived from fungal products (Metcalfe et al., 2004). All the complete synthetic statins are administered as an open acid form and have higher affinity for HMGCoAR and exert more potent inhibitory effects than their fungal counterparts (Holdgate et al., 2003; Istvan and Deisenhofer, 2001). Atorvastatin (ATV) is the most prevalent drug in the aquatic environment of the statin class. The reported concentrations of ATV in surface waters were 15 ng/L and 44 ng/L in wastewater treatment plant (WWTP) effluents (Metcalfe et al., 2004).

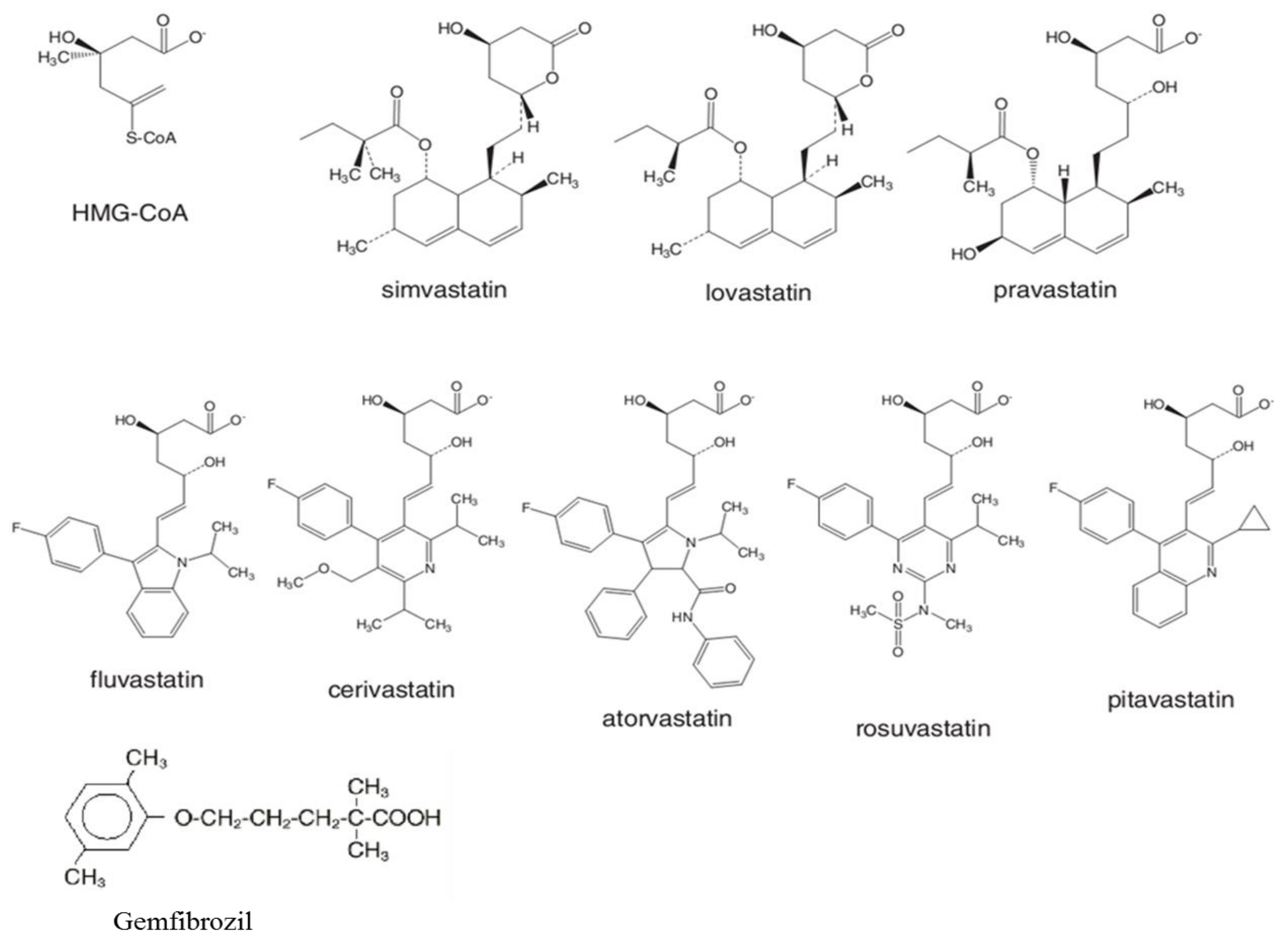


Figure 1.2. Chemical structures of selected HMGCoAR inhibitors (statins) and the fibrate drug gemfibrozil. The figure is adopted from (Pfizer, 2010; Shitara and Sugiyama, 2006).

Simvastatin and lovastatin are lipophilic statins with lactone forms, which easily cross the plasma membrane; therefore, they are easily absorbed from the intestine. They are eliminated from the liver by CYP3A4-mediated metabolism (Corsini et al., 1999). Pravastatin, rosuvastatin and pitavastatin are not metabolized by P450. These statins are mainly eliminated from the body by a transporter-mediated excretion mechanism in the liver, primarily by the organic anionic transporting polypeptide 1B1 (Olsson et al., 2002; Wensel et al., 2010). Fluvastatin is metabolized mainly by CYP2C9, and to a lesser extent by CYP3A4 (Corsini et al., 1999). Cerivastatin is no longer available on the market, and it is metabolized by 2 different enzymes, CYP2C8 and CYP3A4 (Mück, 2000). This dual metabolic pathway is unique among statins, and their estimated contributions are 61% and 37%, respectively (Shitara et al., 2004). Atorvastatin is a hydrophilic statin and undergoes CYP3A4-mediated metabolism (Lau et al., 2006). Moreover, ATV binds to the organic anionic transporting polypeptide and is actively taken up into the liver prior to its metabolism (Lau et al., 2006).

All statin drugs target the liver and inhibit HMGCoAR, the enzyme that converts HMGCoA into mevalonate, a cholesterol precursor (see Fig. 1.2). X-ray crystallography studies have determined the structures of the catalytic portions of HMGCoAR complexed with statins (Istvan and Deisenhofer, 2001). These studies showed that the HMG-like moiety of the statin occupies the HMG binding site of HMGCoAR, thus competing with the normal substrate for the active site of the enzyme and also altering the conformation of the enzyme when it binds to the active site preventing HMGCoAR from attaining a functional structure (Istvan and Deisenhofer, 2001). Recent studies have shown that the zebrafish (*Danio rerio*) HMGCoAR enzyme has a conserved binding site for statins within its primary structure

(Eisa-Beygi et al., 2012). By inhibiting HMGCoAR, statins reduce the hepatocyte cholesterol content, stimulate expression of LDL receptors, and ultimately enhance removal of cholesterol from the circulation (Blumenthal, 2000). This results in an overall decrease in the amount of cholesterol produced by the liver (Blumenthal, 2000). Several other products synthesized within the mevalonate pathway (see Fig. 1.1) are also inhibited in the presence of statins which might account for the effects of statins on processes other than simply cholesterol reduction (Liao and Laufs, 2005). For example proteins which need intermediates within the mevalonate pathway for their function include Ras, which require the addition of a fernesyl moiety for activity in cell signaling, and Rho GTP-binding proteins which require geranylgeranylation for activity in cell signaling (Liao and Laufs, 2005).

The passive membrane permeability of statins increases along with their lipophilicity (Hamelin and Turgeon, 1998). Therefore, statins with lipophilic structures are more readily distributed into the peripheral tissues than hydrophilic statins, such as pravastatin (Sirtori, 1993). Statins concentrate in the human liver while little drug circulates in the plasma including only 14% ATV, 17% pravastatin, 20-30 % fluvastatin, 5% simvastatin and lovastatin, while cerivastatin has a circulatory distribution of more than 60% (Blumenthal, 2000). All statins are metabolized in the liver. Pravastatin is hydrophilic, whereas the remaining members of the class are lipophilic (Ishingami et al., 2001).

1.7. Pharmacokinetics of Atorvastatin and Gemfibrozil

My thesis deals primarily with two pharmaceuticals, the statin ATV and the fibrate gemfibrozil (GEM) so I will discuss some of the properties of these two drugs specifically.

Atorvastatin, which belongs to the second generation or synthetic group of statins, is a reversible inhibitor of the microsomal enzyme HMGCoAR. ATV (molecular weight

1209.42 g/mol) is administered orally as the calcium salt of the active hydroxy acid form (Cilla et al., 1996b; Pfizer, 1996). The dosage range used clinically is 10-80 mg/day (Pfizer, 1996). The acid form is a surface active molecule, since it consists of a lipophilic part and a more hydrophilic part (Lennernas, 2003). This surface activity of ATV is an important physiochemical property, since it will affect its partition into biological membranes and diffusion across the membranes (Lennernas, 2003). ATV is lipophilic with a K_{ow} (octanol-water coefficient) of 4.2 for its lactone form, although the acid form has a K_{ow} 1.53 (Ishingami et al., 2001). This difference in physiochemical properties between the two forms, i.e. the lactone for being more lipophilic than the acid form, has an impact on their pharmacokinetics. ATV-calcium is insoluble in water of pH 4 and below, but very slightly soluble in distilled water, pH 7.4 phosphate buffer, and acetonitrile, lightly soluble in ethanol, and freely soluble in methanol (Pfizer, 1996)

After oral administration, ATV is rapidly absorbed achieving maximum plasma concentrations within 1 to 2 h. The oral bioavailability of ATV is low (around 14%) and the systemic availability of HMGCoAR inhibitory activity is approximately 30%, largely as a result of its CYP3A4-mediated first-pass metabolism in the intestinal wall and liver (Scheen, 2012). Liver metabolism results in the production of two active hydroxy metabolites, ortho-hydroxy-atorvastatin (o-OH-ATV) and para-hydroxy-atorvastatin (p-OH-ATV), and three corresponding inactive lactone metabolites (Jacobsen et al., 2000). *In vitro*, inhibition of HMGCoAR by ortho- and para-hydroxylated metabolites is equivalent to that of the parent compound and depending on the dose administered the extent of absorption increases in proportion to the dose with plasma protein binding exceeds 98% (Pfizer, 1996). In humans the total plasma clearance of ATV is 625 mL/min and the half-life in humans is about 14 h,

however the half-life of inhibition of HMGCoAR is 20 to 30 h due to its active metabolites contributing to this inhibition (Cilla et al., 1996a). The renal route is of minor importance (< 2%) for the elimination of ATV (Pfizer, 1996; Schachter, 2005). Furthermore, ATV is extensively metabolized in both the gut and liver by oxidation, lactonization and glucuronidation, and metabolites are eliminated by biliary excretion into the gut and direct secretion from the blood to the intestine (Lennernas, 2003).

Gemfibrozil (GEM) is a lipid regulating agent that belongs to the fibrate class of pharmaceuticals, which is prescribed primarily for the treatment of hypertriglyceridemia and the reduction of very low density lipoprotein (VLDL) cholesterol, and increases in high density lipoprotein (HDL) cholesterol (Miller and Spence, 1998). GEM (molecular weight of 250.35) is a white waxy crystalline solid that is practically insoluble in water but soluble in alcohol, chloroform and methyl alcohol (Pfizer, 2010).

The mode of action of GEM has been well studied in mammals. GEM crosses the cell membrane and binds to and activates the peroxisomal-proliferating activating receptor alpha (PPARα) subtype, a transcription factor that specifically affects the expression of more than 50 genes after its heterodimerization with the retinoid X receptor (RXR) and binding to the PPRE (peroxisome proliferator hormone response element) of the promoter region of these genes (Spencer and Barradell, 1996; Staels et al., 1998).

The typical oral dosage of GEM in human ranges from 1200 to 1500 mg/day (Spencer and Barradell, 1996). Oral absorption of GEM is found to be 100%, reaching peak plasma concentrations 1 to 2 h after dosing, with a plasma half-life of 1.5 h following multiple doses (Miller and Spence, 1998). Approximately 70% of the administered dose is excreted in the urine, mostly as the glucuronide conjugate, with less than 2% excreted as

unchanged GEM, and 6% of the dose is accounted for in the feces (Prueksaritanont et al., 2002).

Statins have a few known side effects, although cerivastatin (CVT) was voluntarily removed from the US market because of its association with higher than expected numbers of fatal cases of rhabdomyolysis (breakdown of skeletal muscles, with degradation products from these muscle cells causing further damage in the body). Rhabdomyolysis is a rare but potentially fatal complication of the combined use of fibrate and statin drugs (SoRelle, 2001).

There is little research reported on statin drugs in non-human organisms. Brain et al. (2006) demonstrated that ATV is phytotoxic in the aquatic plant *Lemna gibba* and they found biochemical alterations in the products downstream of HMGCoAR within the mevalonate pathway. A single study found that significant disruptions occurred during zebrafish development when ATV was used at pharmacological doses (10 μM) (Thorpe et al., 2004). This experiment clearly demonstrated that primordial germ cell (PGC) migration was blocked when HMGCoAR was inhibited. However, this effect may be related not to reduced cholesterol but to the reduction in prenylated protein production (isoprenoid addition to proteins, catalyzed by geranylgeranyl transferase 1). A similar requirement for protein prenylation is thought to be responsible for changes in early heart morphogenesis (D'Amico et al., 2007) and for cerebro-vascular stability (Eisa-Beygi et al., 2012) in zebrafish. In addition, Estey et al. (2008) demonstrated that HMGCoAR activities and mRNA transcript levels and cholesterol levels are affected in rainbow trout both *in vitro* and *in vivo* when treated with statin drugs.

1.8. Stress response in fish

The main corticosteroid that is released by teleost fish in response to stress is cortisol which plays a key role in enabling the fish to overcome an exposure to a stressor and regain homeostasis (Barton, 2002; Geslin and Auperin, 2004; Mommsen et al., 1999; Wendelaar Bonga, 1997). In teleost fish, cortisol is considered both a corticosteroid and mineralocorticoid as no specific mineralocorticoid is reported in fish (Jeffrey et al., 2012; Mommsen et al., 1999). Cortisol promotes gluconeogenesis, suppresses inflammation and plays a role in electrolyte and water balance in fish by increasing the surface epithelia of the gills to water and ions (Gilmour et al., 2012; Mommsen et al., 1999; Wendelaar Bonga, 1997). Circulation of cortisol is regulated by the activation of the hypothalamus-pituitary-interrenal axis (HPI) (Mommsen et al., 1999; Wendelaar Bonga, 1997). The release of adrenocorticotropic hormone (ACTH), the main "secretagogue" for cortisol biosynthesis is released by the anterior pituitary which itself is stimulated by the release of corticotrophin-releasing factor (CRF) from the hypothalamus in the brain (Barton, 2002). ACTH is released into the blood, travels to and binds to the melanocortin receptor (MC2R) located on membranes of interrenal cells. These interrenal cells are located within the post-cardinal vein of the anterior part of the head kidney in teleost fish and ACTH binding activates the steroidogenic pathway leading to the synthesis and release of cortisol (Geslin and Auperin, 2004; Hontela, 2005; Mommsen et al., 1999; Wendelaar Bonga, 1997).

Cortisol and sex steroids are synthesized from the common precursor cholesterol. Cytochrome P450 side chain cleavage enzyme (P450scc) is the first and rate-limiting enzyme that converts cholesterol into pregnenolone (Miller, 1988; Stocco, 2001). The activity of P450scc is regulated by the availability of its substrate, cholesterol. Cholesterol is

usually stored in cytosolic lipid droplet away from the mitochondria, where P450scc resides. Cholesterol has to be transported from the lipid droplet to the inner mitochondrial membrane in order to be accessed by P450scc. This step is relatively slow and requires the facilitation of Steroidogenic Acute Regulatory protein (StAR) (Stocco and Clark, 1996).

Pregnenolone is then transferred from the mitochondria to the endoplasmic reticulum to be converted to 17-OH-pregnenolone by 17 α-hydroxylation (P45c17) and 3β-hydroxysteroid dehydrogenase (HSD3B) ensures the formation of 17-OH-progesterone (Mommsen et al., 1999). Following synthesis of pregnenolone, the steroidogenic pathway diverges in three different directions for the synthesis of the final products: glucocorticoids, mineralocorticoids, and sex steroids (Miller, 1988). These steroids control sugar and salt balance, sexual characteristics, as well as metabolic homeostasis.

Teleost fish steroidogenesis is thought to be very similar to mammalian steroidogenesis (Mommsen et al., 1999). The steroid intermediates in the mammalian steroidogenic pathways are present in fish, although the fish ovary contains a few more steroids, such as 17,20-dihydroxy-4-pregnen-3-one (17,20-DP), which is responsible for oocyte maturation (Nakamura et al., 2005).

Fish continually face environmental stressors including temperature change and hypoxia. According to Barton (2002), physiological responses to such environmental stressors are grouped broadly as primary and secondary responses. The primary response involves the release of catecholamines from the chromaffin tissue and the release of cortisol into the circulation from the interrenal tissue upon the stimulation of the HPI axis. The secondary response involves changes in hematological parameters, plasma and tissue ions and metabolites (glucose), and the stimulation of heat shock or stress proteins (HSPs).

1.9. Thesis outline and hypothesis

Surveys and reports on the occurrence of PPCPs in the aquatic environment show that the pharmaceuticals are ubiquitously distributed (Daughton and Ternes, 1999; Fent et al., 2006). PPCPs are created with the intent of causing a biological effect, many pass through cell membranes, therefore they have similar physiochemical characteristic behavior of harmful xenobiotics, and furthermore many PPCPs are relatively persistent in the environment (Daughton and Ternes, 1999; Fent et al., 2006).

There remains a data scarcity and unknown risks associated with pharmaceuticals in the environment. Fish are perhaps the most likely vertebrate organism to be affected by PPCPs in the aquatic environment because of their ecological niche and the similarities in many of their physiological processes compared with mammals (Erickson et al., 2008). Therefore, this thesis investigates the effects of two human lipid/cholesterol lowering drugs on fish (non-target species). ATV is mainly prescribed for cholesterol reduction and GEM is prescribed to reduce triglyceride (see above). Moreover, the two drugs are detected in the Canadian environment and in a few instances, at comparable levels (Metcalfe et al., 2004). Given the presence of these drugs and their known effects on cholesterol and triglycerides in humans, do these drugs demonstrate these intended effects in fish and are there any unintended or unexpected effects noted by these drugs in fish?

Two fish species will be used in my studies: the rainbow trout *Oncorhynchus mykiss* and the zebrafish *Danio rerio*. The rainbow trout is one of the most intensively studied fishes in a wide range of research areas. They are the most-widely cultivated cold freshwater fish in the world and are considered by many to be “aquatic lab-rat” (Craig, 2013). They are a member of the Salmonidae, native to the Pacific coast of North America and Russia, and

have been widely introduced across the world especially in regions with cool water temperatures (MacCrimmon, 1971). Interests in utilization of rainbow trout as a model species for a wide range of research activities such as toxicology, comparative immunology, physiology, and nutrition have been well documented (Craig, 2013; Thorgaard et al., 2002).

The zebrafish is a small tropical fish species that has been used extensively in the laboratory and toxicity tests (Froehlicher et al., 2009; Hill et al., 2005; Stegman et al., 2010). They are freshwater fish belong to the cyprinid family in the class of Actinopterygii (ray-finned fishes), along with minnows and carps. They are omnivorous fish that primarily feed on zoo- and phyto-plankton, as well as insects, in the wild. In the aquarium, zebrafish are usually fed various types of dry food (typically with a fat content of 8-15%) along with live food, most commonly *Artemia* (brine shrimp) (Stegman et al., 2010; Westerfield, 2000). Zebrafish have a short generation time and spawn regularly under laboratory conditions. While endpoints like spawning activity, egg production and gonadosomatic index (GSI) are readily determined for the zebrafish, their small size makes this species difficult to collect more than a few microliters of blood for the determination of plasma sex steroids and other lipid profiles.

This research will be guided by the following general hypothesis and predictions:

Hypothesis: Cholesterol metabolism and production are modified by statin and fibrate drugs which will impact the physiological role of steroids in fish.

Prediction 1: Rainbow trout and zebrafish exposed to a statin (ATV) and a fibrate (GEM) drug will decrease their plasma cholesterol and lipid contents compromising the ability of the fish to mount a proper stress response as indicated by a reduction in circulating cortisol content upon exposure to a stressor. Moreover, these fish will have reduced reproductive

capacity as assessed by a reduction in the content of sex steroids the secretion of which also depends upon the availability of cholesterol as a precursor.

Prediction 2: Trout and zebrafish exposed to the combination of ATV and GEM will demonstrate biomarkers of muscle damage (rhabdomyolysis) as reported in mammals when exposed to the combination of the two drugs.

Prediction 3: HMGCR-1 and -2 mRNA abundance will increase with exposure to ATV. This hypothesis and these predictions will be examined in the following 3 chapters using injection and in-tank drug exposures, biochemical assessments of metabolites, and molecular identification of gene transcript levels. This study does demonstrate that these drugs have similar effects in fish as they do in humans/rodents, but that they also show unintended effects that could challenge the fitness of these fish in their aquatic environment.

CHAPTER 2

The effects of intraperitoneal injection of Atorvastatin and Gemfibrozil on cholesterol metabolism and steroid production in the rainbow trout *Oncorhynchus mykiss*

2.1. Introduction

Pharmaceutical and personal care products have received increasing attention as emerging aquatic pollutants over the last decade (Fent et al., 2006). In the aquatic environment they generally occur at low concentrations (ng to µg/L), and there is little evidence that they exert acute toxicity to non-target species (fish) based on these low concentrations (Daughton and Ternes, 1999; Fent et al., 2006). Although pharmaceuticals are designed to act in humans, it is likely that non-target organisms that possess similar biochemical pathways will also be affected since basic mechanisms such as cell division, signal transduction, and key metabolizing enzymes (different cytochrome P450 isoforms) are conserved in a wide variety of organisms and especially across the vertebrates (Buhler and Wang-Buhler, 1998; Fent et al., 2006).

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) inhibitors or statins are a group of pharmaceuticals used for reducing blood cholesterol levels (Fent et al., 2006). Statins suppress cholesterol biosynthesis by competitively inhibiting HMGCoAR, the enzyme that catalyzes the conversion of HMGCoA to mevalonate, a precursor of sterols including cholesterol (Blumenthal, 2000). Inhibition of cholesterol biosynthesis leads to up-regulation of LDL receptors (LDLr) in the liver and enhanced clearance of LDL from the plasma (Bellosta et al., 2000).

Statins are among the most prescribed human pharmaceuticals in North America (Drug Topics, 2011), and reach the aquatic environment in increasing concentrations (Walley et al., 2005). Atorvastatin (ATV), manufactured by Pfizer in 1997, is one of the most prescribed drugs within the statin family. According to Drug Topics (2011) the total sales of ATV in the United States in 2010 was $5.3 billion. ATV is administered as the calcium salt of its active acid form (Pfizer®, 1996). Moreover, pharmacokinetics studies demonstrate that after oral administration, ATV is rapidly metabolized by CYP3A4 (Kantola et al., 1998) into two active hydroxylated metabolites (*ortho*-hydroxy-ATV and *para*-hydroxy-ATV) and three unstable-inactive lactone metabolites that are readily hydrolyzed to their original acid form (Bullen et al., 1999; Hermann et al., 2005).

Gemfibrozil (GEM), a lipid-regulating agent, belongs to the fibrate class of drugs and is specifically prescribed to reduce elevated circulating triglyceride levels (Spencer and Barradell, 1996). GEM binds to and activates the transcription factor peroxisome proliferator activated receptor (PPAR), primarily PPARα, which affects the expression of a suite of genes after its heterodimerisation with the retinoid X receptor (Bishop-Bailey, 2000). Upon activation as a consequence of GEM administration, marked changes result in the expression of key enzymes involved in the biosynthesis of several apoliproteins and lipoprotein metabolism (Gervois et al., 2000; Staels et al., 1998) ultimately resulting in the reduction of plasma triglyceride concentration and the elevation of high density lipoprotein cholesterol (HDL) levels (Staels et al., 1998).

Until 2001, fibrate and statin drugs were frequently prescribed together to treat patients with mixed hyperlipidemia (Shek and Ferrill, 2001). However, there have been reports of increased threats of developing myopathy, including rhabdomyolysis resulting

from this co-administration (Murdock et al., 1999). In spite of being usually accepted as a class effect for all fibrate-statin combinations, this increased risk was observed at different incidences with different fibrate and stain drugs; most of the cases of rhabdomyolysis are reported with GEM-statin combined therapy than with other fibrate-statin combinations (Chang et al., 2004; Shek and Ferrill, 2001). In late 2001, cerivastatin (another statin drug) was removed from the market worldwide as a result of its association with higher numbers of fetal rhabdomyolysis cases (compared with other statins), many of which occurred in patients receiving GEM (Chang et al., 2004; Farmer, 2001; SoRelle, 2001).

It was reported by Metcalfe and colleagues (2004) that in Canadian wastewater treatment plant (WWTP) effluents where statin drugs were detected, fibrate drugs were also present at comparable or higher concentrations. ATV is the most prevalent drug in the aquatic environment of the statin class. The concentration of ATV in surface waters was 15 ng/L and 44 ng/L in the WWTP effluent (Metcalfe et al., 2004). The concentration of GEM in the surface water was 1500 ng/L and 2100 ng/L in WWTP (Metcalfe et al., 2004).

The main objective of this study was to evaluate the effects of ATV and GEM on cholesterol metabolism and steroid production in rainbow trout. To my knowledge there is no report regarding the combining effect of ATV and GEM in any fish species (non-target species). Given that a supply of cholesterol is required as substrate for steroid hormone synthesis (including cortisol) and these pharmaceuticals affect cholesterol content, I hypothesize that exposing rainbow trout to ATV and GEM will decrease steroid hormone levels and impact those processes that are affected by these hormones. Therefore I predict that rainbow trout exposed to ATV and GEM will have decreased plasma cholesterol, compromising the ability of the fish to produce cortisol and thus mount an appropriate stress

response. Moreover, these fish will have reduced levels of sex steroids including testosterone and estradiol that could affect reproductive capabilities.

2.2. Materials and Methods

2.2. 1. Fish used

Rainbow trout were purchased locally (Linwood Acres Trout Farm, Campbellcroft, ON, Canada). Fish were acclimated to 13 °C for at least 1 week in 1200 L tanks supplied with oxygenated and dechloraminated water from the City of Ottawa within the University of Ottawa Aquatic Care Facility. In experiment 1, trout weighed between 75 and 130 g and were fed 1% of body weight (maintenance ration) with commercial trout feed (3 PT Classic Floating Trout Grower Feed; Martin Mills, Elmira, ON Canada). In experiment 2, trout weighed between 155 and 212 g and were not fed for the entire period of the experiment. Trout in experiment 3 weighed between 120 and 244 g and again were not fed for the entire period of the experiment. All experiments were conducted under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhered to the guidelines set forth by the Canadian Council for Animal Care for the use of animal in teaching and research.

2.2.2. Intraperitoneal injection method

Rainbow trout were intraperitoneally (IP) injected with a human equivalent dose of ATV (0.29 µg/g) for a total of 8 injections over 28 days (every 4th day). The combined effects of the two drugs (A+G) on *in vivo* cholesterol metabolism was assessed by IP injection of ATV (0.29 µg/g) and GEM (6.35 µg/g) into trout. This GEM concentration represents 75% of a human equivalent dose. The doses of ATV (based upon an 80 mg dose

per 70 kg human per day) and GEM (based upon a 1500 mg dose per 70 kg human per day) were calculated by scaling down the human dose to fish estimating that fish total blood volume was approximately 5 mL /100 g fish (Estey et al., 2008; Olson, 1992). Three different experimental groups were used: experiment 1, the fish were not fed during the entire 28 days and on day 28 were stressed (nFS); experiment 2, the fish were fed daily to 1% of body mass (maintenance ration) and at day 28 the fish were stressed (FS); and experiment 3, the fish were not fed as in experiment 1, but they were not stressed at the end of the experiment (nFnS). The stress involves netting the fish in the air for 30s. Control groups were assessed for each group and consisted of fish that were injected with DMSO alone; each experiment was repeated three times (n = 3) and each experiment consisted in 10 fish per tank. All drugs were dissolved in DMSO at a total injected volume that corresponded to 1 μL/g fish body mass.

2.2.3. Blood and tissue collection

Forty five minutes poststress, fish were rapidly netted from each tank and anaesthetized in a 20-L bucket containing a lethal (200 mg/L) dose of benzocaine. Blood samples were taken from the caudal vessel using a 23-gauge needle attached into an EDTA-coated syringe. Blood was allowed to flow freely from the vein by applying gentle suction to avoid hemolysis. The blood samples were centrifuged (3 min at 1200 x g) to collect plasma. The plasma samples were stored at -80 °C until analyzed. Livers were rapidly removed, weighed, and a small piece removed (~200 mg) and snap frozen on dry ice in a 2.0 mL plastic centrifuge tube for analysis of molecular endpoints. The rest of the liver was snap frozen in liquid nitrogen. Samples of both white and red muscles were removed and a small

piece (~200 mg) treated as above for molecular analysis. All tissues were stored at -80 °C until analyzed.

2.2.4. Biochemical analyses

Protein was assessed using the bicinchoninic acid method (BCA; Sigma-Aldrich, St. Louis, MO, USA) according to Smith et al. (1985) using a SpectraMAX Plus 384 microplate reader and SOFTmax software 3.1 (Molecular Devices, Sunnydale, CA, USA). Content was compared to a standard curve using bovine serum albumen at a wavelength of 550 nm.

Total plasma cholesterol (catalog # C509) and triglycerides (catalog # T531) were assessed using a commercially available colorimetric assay using a quinoneimine dye (TECO Diagnostic, Anaheim, CA). Absorbance was read at 520 nm using the microplate spectrophotometer as above and the concentrations of unknown samples were calculated in comparison with a standard curve.

Plasma cortisol was assessed using a ^{125}I Radioimmunoassay (RIA) kit as per the manufacturer's protocol (MP Biomedicals, Orangeburg, NY, USA).

Plasma estradiol and plasma testosterone were determined using Enzyme immunoassay (EIA) test kits for estradiol (catalogue # ESTRA-96) and testosterone (catalogue # TEST-96) as per manufacturer's protocol (TECO Diagnostic). This assay is based on competitive binding between estradiol and testosterone in the plasma and estradiol- or testosterone-horseradish peroxidase conjugate for a constant amount of rabbit anti-estradiol or -testosterone. The estimated sensitivity of the kits as reported by the manufacturer is 10 and 50 pg/mL, for estradiol and testosterone, respectively.

Plasma creatine phosphokinase (EC 2.7.3.2) was assayed with a 50 mM imidazole-acetate buffer containing 1 mM NAD^+, 1 mM ADP, 5 mM $Mg(CH_3COO)_2$, 4 mM glucose,

10 mM AMP, 1 unit/ml glucose-6-phosphate dehydrogenase, and 2.5 units/ml hexokinase, pH 7.4. Activities were initiated with 100 mM creatine phosphate. Activities were assessed kinetically in 96-well plates using the microplate spectrophotometer at 340 nm. All chemicals were purchased from Sigma-Aldrich.

Plasma creatinine concentration was assessed using a commercially available spectrophotometric assay kit (catalogue # C513-480) as per the manufacturer's protocol (TECO Diagnostic) using alkaline picric acid. Absorbance was read at 510 nm using the microplate spectrophotometer and creatinine concentrations were determined against a standard curve.

Plasma lactate was estimated using an endpoint assay by its complete oxidation to pyruvate using lactate dehydrogenase (LDH). Hydrazine traps the pyruvate, allowing the reaction to run to completion in the samples. The concentration of lactate in the sample is proportional to the increased in absorbance of NADH assayed spectrophotometrically at 340 nm. Samples (10 µl) were assayed using a glycine/NAD^+ buffer containing, 320 nM glycine, 250 nM hydrazine, and 2.5 nM NAD^+. The reaction was initiated by the addition of 2 units/mL LDH and the reaction was run for 45 min. The plates were read using a microplate spectrophotometer at 340 nm. Lactate concentrations were estimated from a standard curve using L-(+)-Lactic acid. All chemicals were purchased from Sigma-Aldrich.

2.2.5. RNA isolation

RNA was isolated using Trizol reagent, following manufacturer's instructions (Gibco BRL, Burlington, ON). Briefly, 10-30 mg of tissues or 80-100 mg (muscle), were suspended in 1 ml Trizol in 2 ml safe lock Eppendorf tubes and homogenized using a Polytron homogenizer (Kinematica, Luzern, Switzerland). The homogenate was incubated at room

temperature for 5 min, followed by the addition of 0.3 mL chloroform and then shaken vigorously by hand for about 15 sec. The homogenate was then incubated at room temperature for 2-3 min, and then centrifuged at 12000 xg for 15 min at 4 °C. After centrifugation, the upper aqueous phase was removed carefully without disturbing the lower (Trizol phase) and placed in a new autoclaved 1.5 mL tube. RNA precipitation was performed by the addition of 0.5 mL isopropanol alcohol and 4 μL linear acrylamide (2 mg/mL) to the aqueous phase (Gaillard and Strauss, 1990) and mixed by flicking. The aqueous phase was incubated on dry ice for 30 min, and then centrifuged for at 12000 xg for 15 min at 4 °C. Following centrifugation, the supernatant was removed and the RNA pellet was rinsed with cold 75% ethanol. RNA pellets were vortexed and centrifuged at 7500 xg for 5 min at 4 °C. The rinsing of the RNA pellets was repeated 3-times to ensure the removal of any contaminants. After the rinsing, the supernatant was removed and the RNA pellet was allowed to air dry for 10-15 min, and then dissolved in an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water depending on the size of the pellet. The dissolved pellet was incubated at 65 °C for 10 min while vortexing every 2-3 min until dissolved. The quantity and quality of RNA was measured using a NanoDrop 2000 (ThermoScientific, USA). Only RNA with a 260/280 ratio of greater than 1.9, and with 260/230 of greater than 2 was used for cDNA synthesis. Qualified RNA samples were stored at -80 °C.

2.2.6. Real-time quantitative RT-PCR

Transcript abundance was assessed in duplicate using quantitative real-time RT-PCR and a MX3000P Multiplex Quantitative PCR system (Stratagene, La Jolla, CA, USA). Data were analyzed using the MX3000P Software Package. Total RNA from selected tissues were used to synthesize cDNA. Briefly, 1 μg total RNA was DNase treated using DNase I

amplification grade (Invitrogen, Burlington, ON, Canada). Following DNase treatment, first strand cDNA synthesis was prepared by the addition of 300 ng random hexamer primers (Invitrogen) and 200 units of Superscript II Reverse transcriptase (Invitrogen). A relative standard curve for each primer set used was constructed by pooling dilutions of cDNA of all samples (treated and control). In each run, a standard curve was generated that was used to calculate relative changes in mRNA abundance between control and treated samples. Genes were normalized to the expression of the house-keeping gene β-actin which did not change between treatments. Each PCR reaction contained the following final concentrations: 10 µL Brilliant III Ultra-fast SYBR Green QPCR Master Mix (Agilent Technologies, Stratagene, La Jolla, CA, USA), 15 nM ROX reference dye, 500 nM gene-specific primers (forward and reverse), and 10 ng first-strand cDNA template, in a 20 µL reaction volume. The thermal cycling parameters were 3 min initial denaturation step at 95 °C, followed by 40 cycles of 95 °C for 20 s, and 60 °C for 20 s. Unless otherwise indicated, all primers were purchased from Invitrogen and designed with OligoPerfect Designer (Life Technologies). Other primers came from the literature [SREBP-1 (Skiba-Cassy et al., 2009); HMGCR-1 and HMGCR-2 (Estey et al., 2008); Atrogin-1 and f-Box 25 (Cleveland and Evenhuis, 2010b); MURF-1 (Cleveland and Burr, 2011); PPAR-α (Kolditz et al., 2008); PPAR-β (Morash et al., 2008); and, PPAR-γ (Prindiville et al., 2011)].

2.2.7. Statistical analyses

Experimental results are presented as means ± standard error of the mean (SEM). Statistical analyses were conducted using SigmaPlot 11.0 (SPSS Corporation, Chicago, IL, USA). Log-transformed data were used wherever necessary to satisfy homogeneity of variance while data that failed the normality test after log transformation were assessed using

a Kruskal-Wallis One Way ANOVA on ranks. All data presented in figures and tables of this chapter are non-transformed. Whenever appropriate, statistical significance was tested with One way ANOVAs followed by Tukey tests (for biochemical end-point) or Student-Newman-Keuls (for gene expression). A value of $p < 0.05$ was accepted as significant.

Table 2.1. Primers used to quantify mRNA levels in liver, red muscle, and white muscle of rainbow trout, their sequences, amplicon size and GenBank accession number.

Gene	Primer	Sequence (5'→3')	Amplicon size (base pairs)	Accession number
β-actin	Forward	GGCATCCTGACCCTGAAGTA	203	AJ438158
	Reverse	GGGGTGTTGAAGGTCTCAAA		
HMGCR-1	Forward	CTCTGGCCAGGTTACAGGAG	180-200	Not Available [a]
	Reverse	TCTACCAGGGCTTCAGTGCT		
HMGCR-2	Forward	TCCCTGAGCTCCAGGTTCTA	180-200	Not Available [a]
	Reverse	CATGGCTGAGCCCACTAGAT		
SREBP-1	Forward	GACAAGGTGGTCCAGTTGCT	59	Not Available [b]
	Reverse	CACACGTTAGTCCGCATCAC		
LDLR	Forward	CAGCGAAGGACTGGAGAAAC	108	AF542091
	Reverse	TTCAGCCCACTCTTCTCGAT		
PPAR-α	Forward	CTGGAGCTGGATGACAGTGA	192	AY494835
	Reverse	GGCAAGTTTTTGCAGCAGAT		
PPAR-β	Forward	CTGGAGCTGGATGACAGTGA	195	NM_001197207
	Reverse	GTCAGCCATCTTGTTGAGCA		
PPAR-γ	Forward	ATGAACAAGGACGGCACACT	310	NM_001197212
	Reverse	GCAGTAGCTTGGCGAACAG		
Atrogin-1	Forward	TGCGATCAAATGGATTCAAA	114	HM189693
	Reverse	GATTGCATCATTTCCCCACT		
F-box 25	Forward	CCAGCTCATAGCCAGGTCTC	119	HM189692
	Reverse	TAAGGCGAGGGTTATGATGG		
MURF-1	Forward	AGAGACTCCACTGAAGCCCA	Not Available [c]	TC134460
	Reverse	ACACTTTGCACATGGAGCAG		

[a]See Estey et al. (2008)
[b]See Skiba-Cassy et al. (2009)
[c]See Cleveland and Burr (2011)

2.3. Results

Since the analysis of plasma endpoints were very similar across the three experiments, all molecular work was performed on tissues that were collected only from the nFS (non-fed, stressed) experiment.

Plasma cholesterol concentrations were significantly reduced in all treated fish in the three experiments compared with the control (no drug treatment). In the nFS experiment (non-fed, stressed), all treated fish had significantly reduced plasma cholesterol compared with the control ($p < 0.001$ for all treatments, One-Way ANOVA) (Fig. 2.1). In the FS experiment (fed, stressed) ANOVA on Ranks followed by Dunn's method detected significant differences in treated fish compared with the control (Fig. 2.1). In the nFnS experiment (non-fed, not stressed), all treated fish had significantly reduced plasma cholesterol compared with the control ($p < 0.001$, < 0.001, and 0.004, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.1).

Moreover, the reduction of plasma cholesterol concentration in the nFS experiment coincided with up-regulation of liver LDL-r mRNA transcript abundance in treated fish compared with the control (p = 0.003, 0.077, and <0.001, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.2). Furthermore, hmgcr-1 mRNA transcript abundance were up-regulated in ATV and A+G-treated fish compared with the control (p = 0.009, 0.791, and <0.001, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.2). Hmgcr-2 mRNA abundance in treated fish was not statistically significant when compared with hmgcr-2 of the control fish (Fig. 2.2).

Plasma triglycerides concentrations in the nFS experiment were significantly reduced in GEM and A+G treated fish only compared with the control (ANOVA on Ranks, followed

by Dunn's method) (Fig. 2.3). In the FS experiment, plasma triglycerides concentrations were not statistical significant compared with the control (Fig. 2.3). Plasma triglycerides concentration in the nFnS experiment were significantly reduced in all treated fish compared with the control (p = 0.011, 0.002, and 0.023, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.3).

In the nFS experiment, the reduction in plasma triglycerides concentration in GEM and A+G groups coincided with significant up-regulation of liver SREBP-1 transcript levels compared with control (p = 0.786, <0.001, and 0.011, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.4). Moreover, One-way ANOVA detected statistical significance when comparing GEM-treated fish to ATV and A+G treated fish (p <0.001 and 0.016, for ATV and A+G, respectively), and also, when comparing ATV with A+G (p <0.016).

Furthermore, PPAR-α transcript abundance was up-regulated in all treated fish in the nFS experiment compared with the control (p <0.001, <0.001, and <0.001, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.4). Moreover, One-way ANOVA detected statistical significance when comparing ATV-treated fish with A+G-treated fish (p = 0.005). PPAR-β mRNA abundance was not affected by the drugs (Fig. 2.4), while PPAR-γ transcript abundance was elevated in all treated fish, but only statistically significant in A+G-treated fish compared with the control (Fig. 2.4).

Plasma creatinine concentrations in the nFS experiment were significantly elevated in all treated fish compared with the control (p = 0.004, <0.001, and <0.001, for ATV, GEM and A+G, respectively; One-way ANOVA). Moreover, in the FS experiment, plasma creatinine concentrations were significantly elevated in all treated fish compared with the

control (*p* = 0.002, <0.001, and <0.001, for ATV, GEM and A+G, respectively; One-way ANOVA). Furthermore, in the nFnS experiment, plasma creatinine concentrations were significantly elevated in the ATV and A+G treatments but not in GEM-treated fish compared with the control (*p* <0.001, 0.291, and <0.001, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.5). One-way ANOVA detected statistical significance when comparing GEM-treated fish to ATV and A+G-treated fish (*p* = 0.038, and 0.035, for ATV and ATV + GEM, respectively.

Plasma lactate concentrations were reduced in all treated fish in the nFS experiment, but were only statistically significant in ATV-treated fish compared with the control (ANOVA on Ranks followed by Dunn's method) (Fig. 2.6). Furthermore, plasma lactate concentrations in the FS experiment were significantly reduced compared with the control (*p* <0.001, 0.006, and <0.001, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.6). In the nFnS experiment, plasma lactate concentrations were not affected by the drugs but were well below concentrations noted in the other two treatments (Fig. 2.6).

Plasma cortisol concentrations were significantly reduced in all treated-fish in the nFS experiment compared with the control (*p* = 0.002, <0.001, and 0.001, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.7). In the FS experiment, plasma cortisol concentrations were reduced in all treated fish but only statistically significant in A+G-treated fish compared with the control (ANOVA on Ranks followed by Dunn's method) (Fig. 2.7). In the nFnS experiment, plasma cortisol was elevated in all treated fish but only statistically significant in ATV and GEM treated fish compared with control (ANOVA on Ranks followed by Dunn's method) (Fig. 2.7). Again, levels of cortisol in the nFnS group were low compared with the other two experiments.

Hematocrit values were significantly reduced in all treated fish in the nFS experiment compared with the control (ANOVA on Ranks followed by Dunn's method) (Fig. 2.8). Moreover, hematocrit values were statistically significantly reduced in all treated-fish in the FS experiment compared with the control (p <0.001, <0.001, and <0.001, for ATV, GEM and A+G, respectively; One-way ANOVA). However, hematocrit values were not significant different in the nFnS experiment-treated fish compared with the control (Fig. 2.8).

Plasma protein concentrations were not significantly different in either the FS or nFnS experiments compared with their controls (Fig. 2.9). In the nFS experiment plasma protein concentrations were statistically significant in ATV and A+G-treated fish but not in GEM-treated fish compared with the control (ANOVA on Ranks followed by Dunn's method) (Fig. 2.9). ANOVA on Ranks detected significance when comparing GEM-treated fish to ATV and A+G-treated fish.

Plasma creatine kinase (CK) concentrations were elevated in all treated fish in the nFS experiment but only statistically significant in ATV-treated fish compared with the control (p = 0.003, 0.327, and 0.486, for ATV, GEM and A+G, respectively; One-way ANOVA). Furthermore, plasma CK concentrations were elevated in all treated fish in the FS experiment but were only statistically significant in GEM and A+G-treated fish compared with the control (p = 0.102, 0.022, and 0.001, for ATV, GEM and A+G, respectively; One-way ANOVA). Finally, in the nFnS experiment plasma CK concentrations were significantly elevated in all treated-fish compared with the control (p = 0.003, 0.050, and <0.001, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.10).

The white muscle F-box 25 transcript abundance was statistically elevated in all treated fish compared with the control (p <0.001, <0.001, and <0.001, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.11). One-way ANOVA detected significance when comparing GEM-treated fish to ATV and ATV + GEM-treated fish (p <0.001, and <0.001, for ATV and A+G, respectively). Moreover, atrogin-1 transcripts levels were statistically elevated in the GEM and A+G groups but not in ATV-treated fish compared to the control (p = 0.263, 0.002, and 0.002, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.11). One-way ANOVA detected significance when comparing GEM-treated fish to ATV-treated fish (p = 0.012), and when comparing ATV with A+G- treated fish (p = 0.009). Furthermore, MURF-1 transcript abundance was elevated in all treated fish compared with the control but these differences were not significant (Fig. 2.11).

Red muscle F-box 25 transcript abundance was significantly elevated in all treated fish compared with the control (p = 0.041, 0.002, and 0.002, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.12). Moreover, atrogin-1 mRNA levels were statistically elevated in all treated-fish compared with the control (p = 0.012, 0.002, and 0.01, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.12).

Plasma estradiol concentrations in the nFS experiment were statistically reduced in GEM-treated fish compared with the control and comparing GEM-treated fish to ATV and A+G-treated fish (ANOVA on Ranks followed by Dunn's method) (Fig. 2.13). In the FS experiment, plasma estradiol content was significantly elevated in ATV-treated fish but not in GEM or A+G-treated fish compared with the control (p <0.001, 0.994, and 0.977, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.13). One-way ANOVA detected a statistical significance when comparing ATV-treated fish to GEM and A+G-

treated fish (*p* <0.001 and 0.004, respectively). In the nFnS experiment, plasma estradiol concentrations were reduced in all treated-fish but not significantly compared with the control (Fig. 2.13).

Plasma testosterone concentrations in the nFS experiment were significantly elevated in the ATV and A+G-treated fish but not in GEM-treated fish compared with the control (ANOVA on Ranks followed by Dunn's method) (Fig. 2.14). In the FS experiment, plasma testosterone concentrations were statistically elevated in ATV-treated fish but not in GEM or A+G-treated fish compared with the control (ANOVA on Ranks followed by Dunn's method) (Fig. 2.14). Moreover, ANOVA on Ranks detected significance when comparing ATV-treated fish with GEM-treated fish. In the nFnS experiment, plasma testosterone concentrations were significantly reduced in all treated fish compared with the control (*p* <0.011, 0.006, and 0.013, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 2.14).

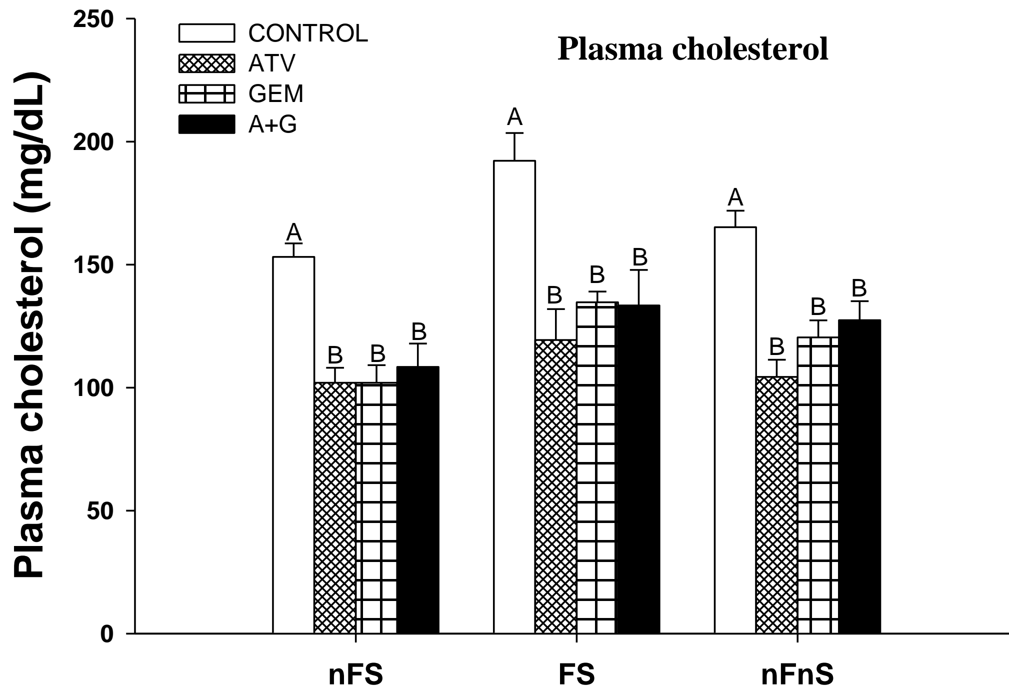


Figure 2.1. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV, 0.29 μg/g), Gemfibrozil (GEM, 6.35 μg/g), and the combination of the two drugs (A+G) on plasma cholesterol concentration of rainbow trout that were not fed and stressed (nFS), fed and stressed (FS), or not fed and not stressed (nFnS). Control fish were injected with DMSO. Data represent mean (± SEM, n = 8-10 for each group). Different letters above the bars denote statistical significance from the control ($p<0.05$; One-Way ANOVA).

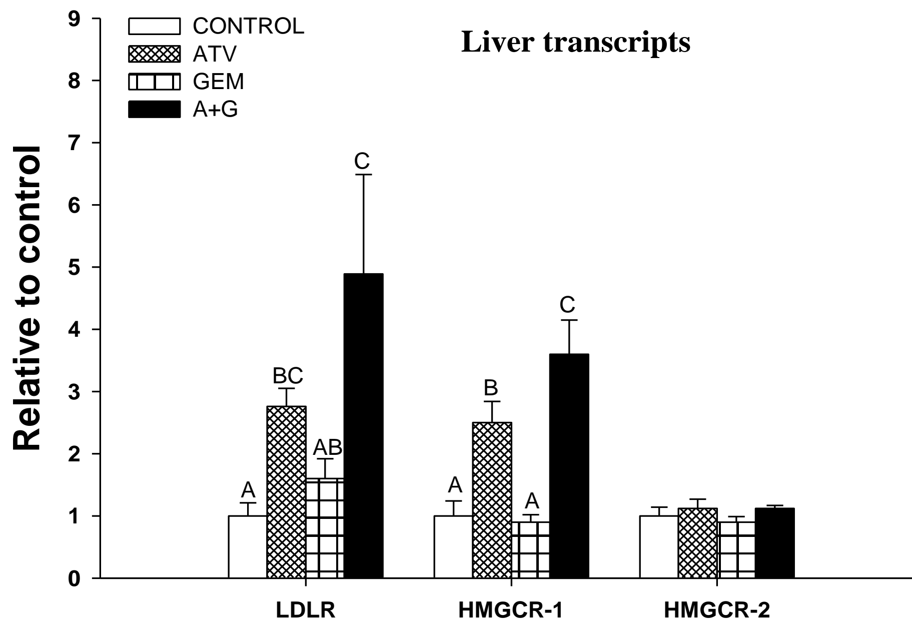


Figure 2.2. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on liver LDLR, HMGCR-1, and HMGCR-2 transcript abundance from rainbow trout that were fasted and stressed (nFS). Control fish were injected with DMSO. Data represent mean (± SEM, n= 5-6 for each group). Different letters on the bars denote statistical significance from the control ($p<0.05$; One-Way ANOVA).

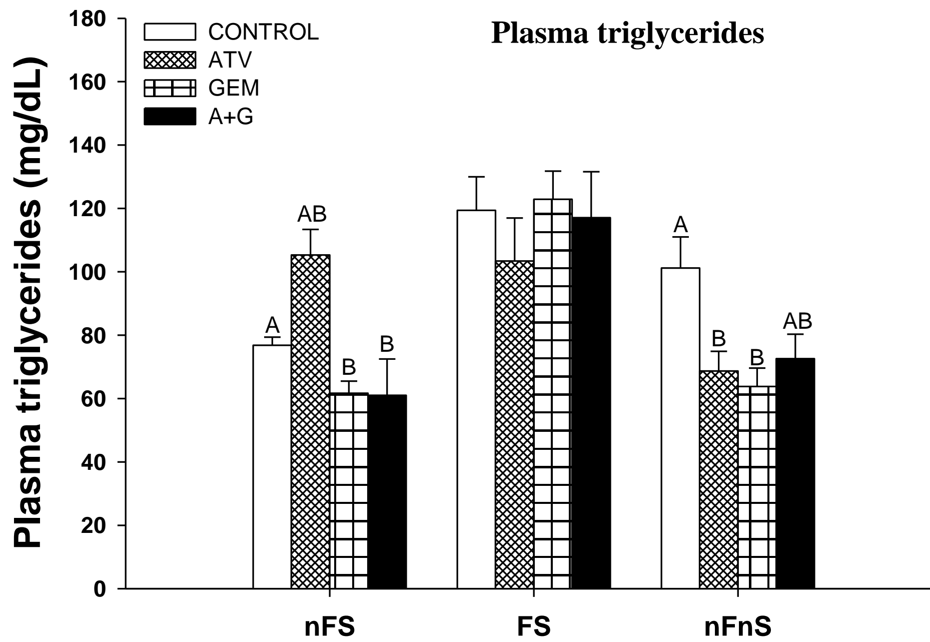


Figure 2.3. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma triglyceride concentrations of rainbow trout. See legend in Fig. 2.1 for details.

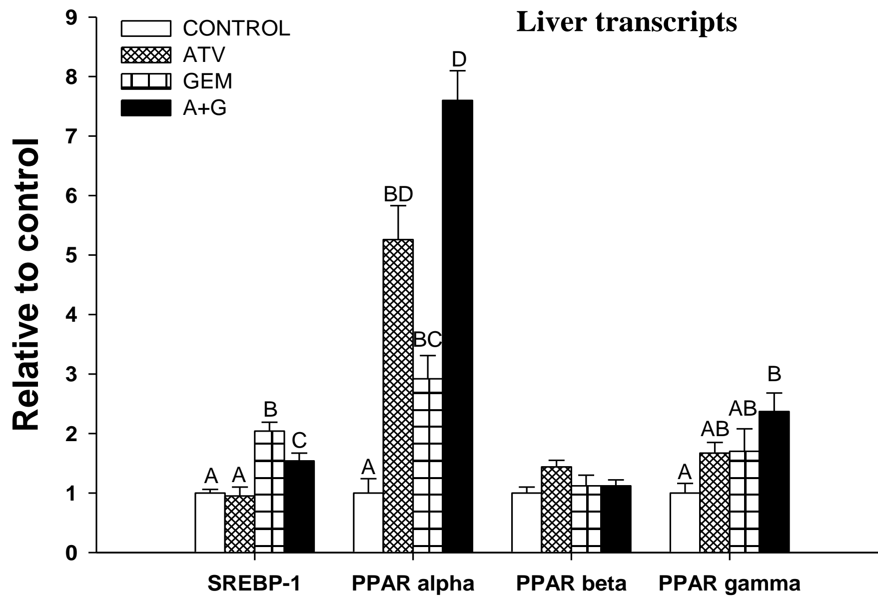


Figure 2.4. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on liver SREBP-1, PPARα, PPARβ and PPARγ transcript levels. See legend in Fig. 2.2 for details.

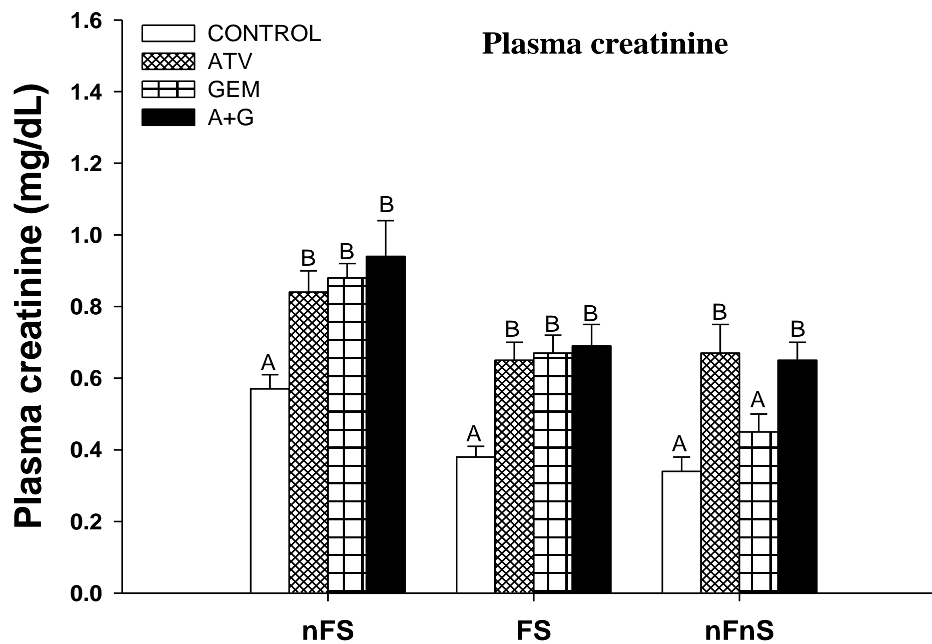


Figure 2.5. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma creatinine concentration of rainbow trout. See legend in Fig. 2.1 for details.

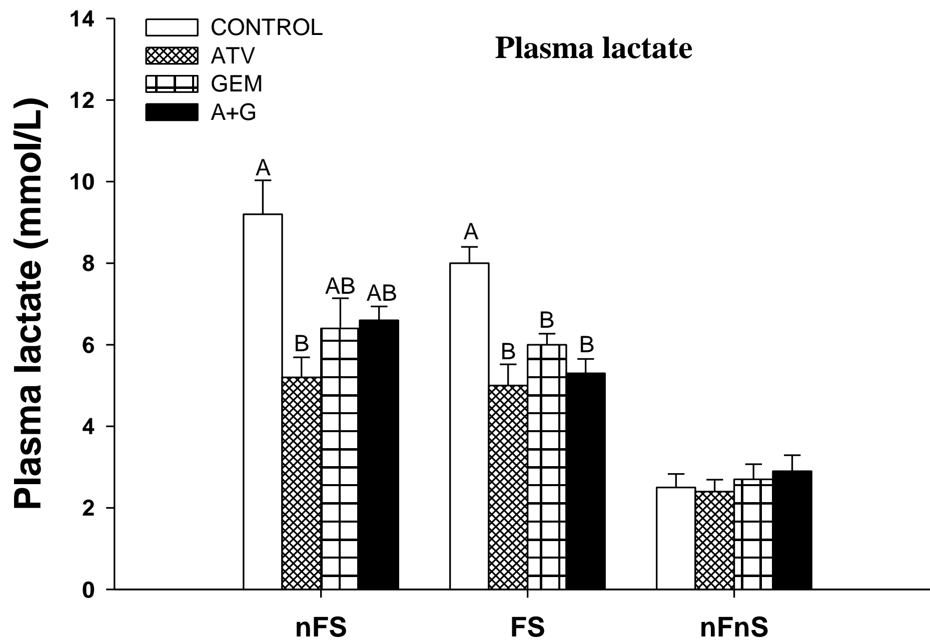


Figure 2.6. The effect of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma lactate concentration of rainbow trout. See legend in Fig. 2.1 for details.

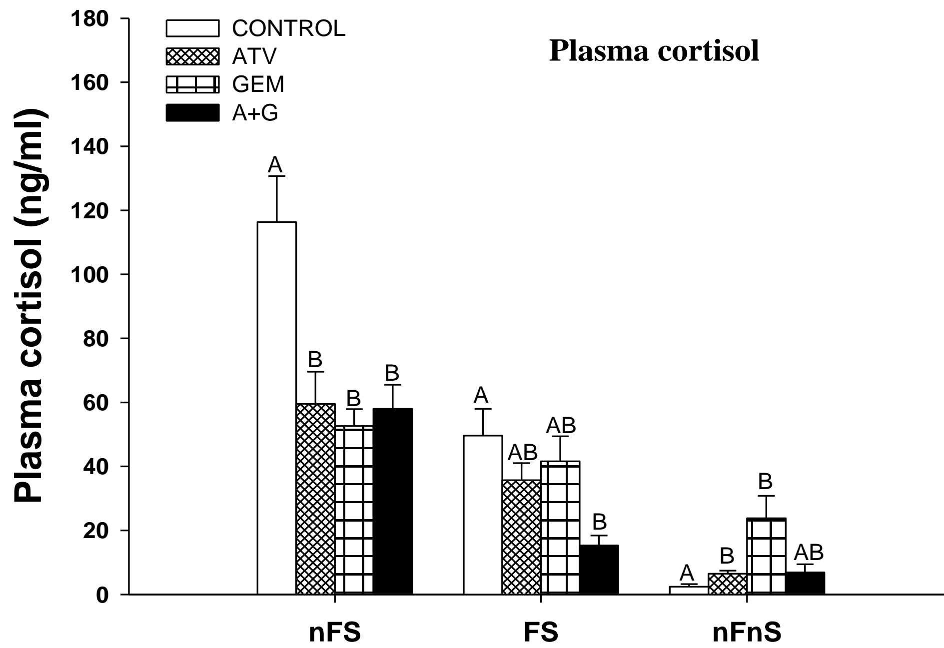


Figure 2.7. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma cortisol concentration of rainbow trout. See legend in Fig. 2.1 for details.

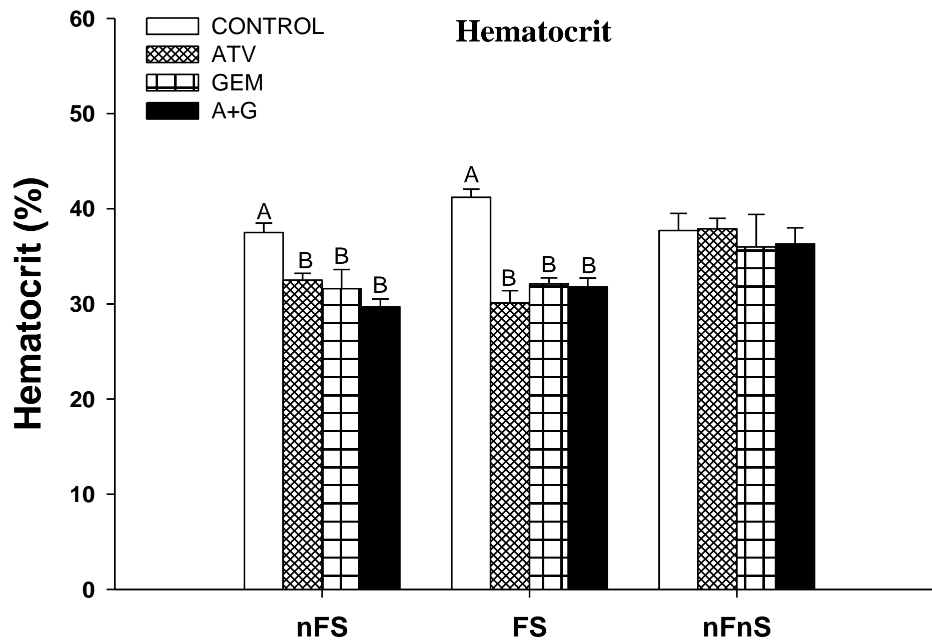


Figure 2.8. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on hematocrit of rainbow trout. See legend in Fig. 2.1 for details.

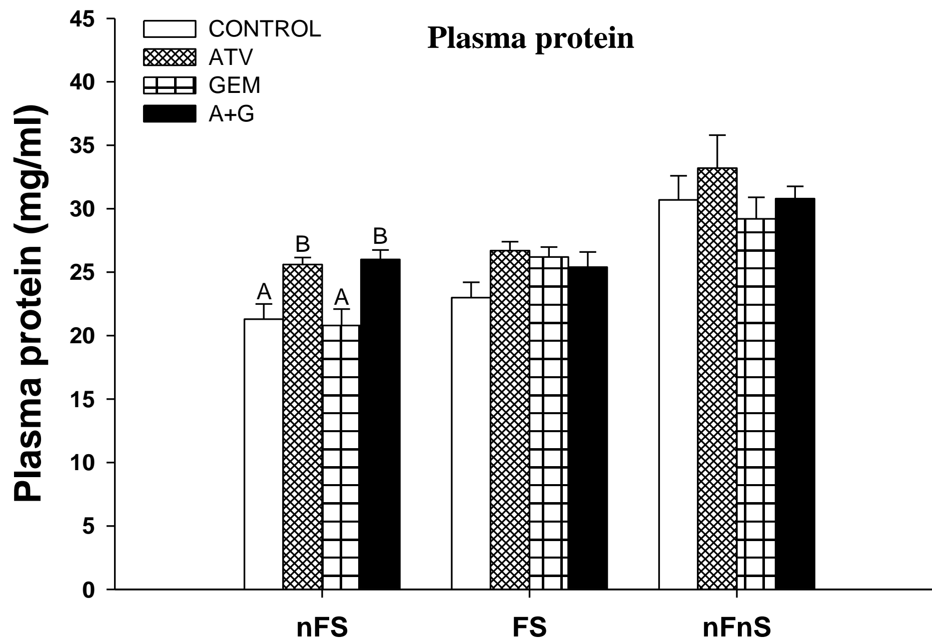


Figure 2.9. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma protein concentration of rainbow trout. See legend in Fig. 2.1 for details.

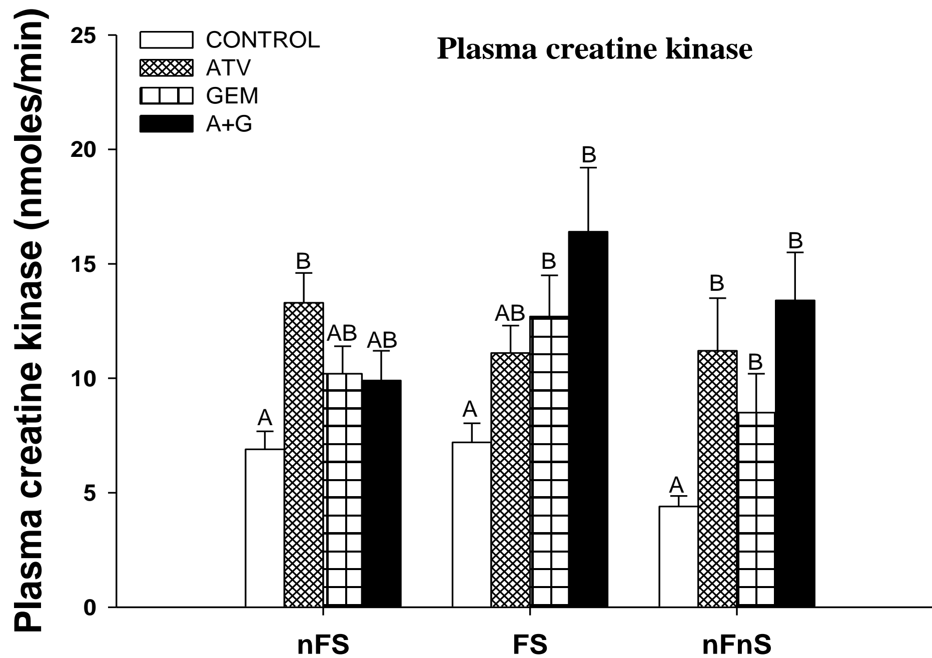


Figure 2.10. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma creatine kinase concentration of rainbow trout. See legend in Fig. 2.1 for details.

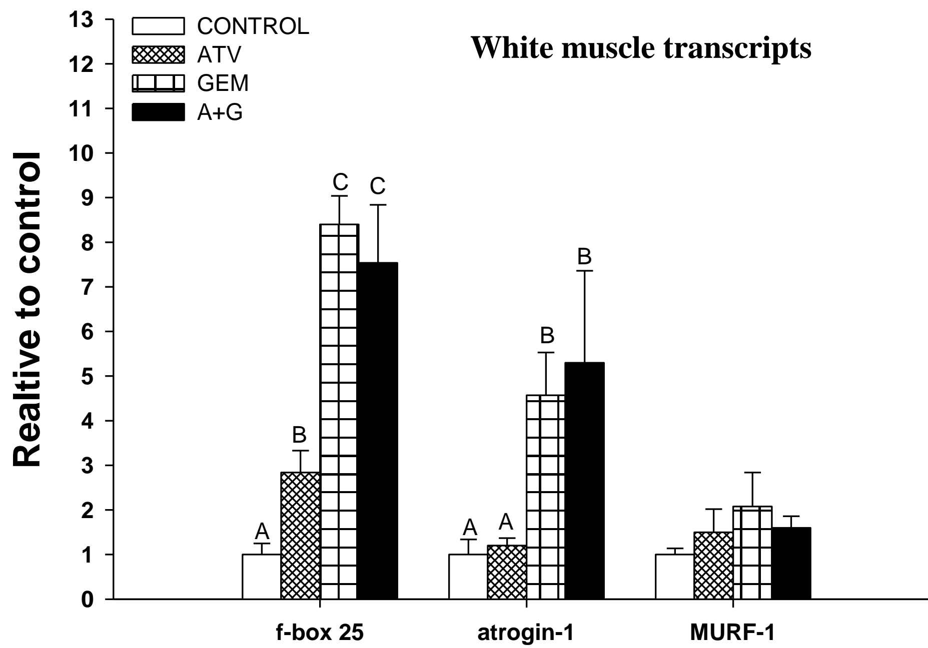


Figure 2.11. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on white muscle f-box 25, atrogin-1, and MURF-1 transcript abundance. See legend in Fig. 2.2 for details.

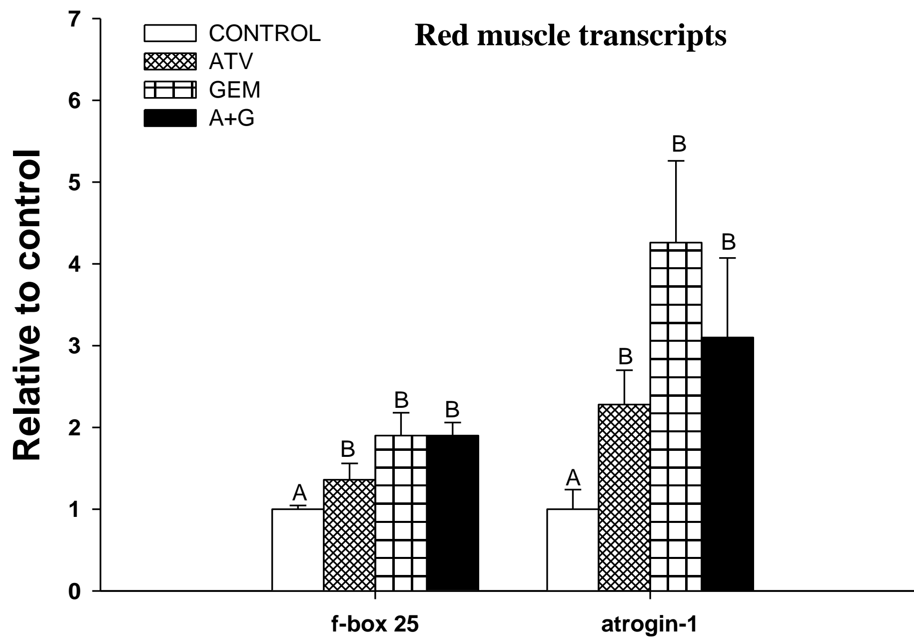


Figure 2.12. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on red muscle f-box 25, and atrogin-1 transcript abundance. See legend in Fig. 2.2 for details.

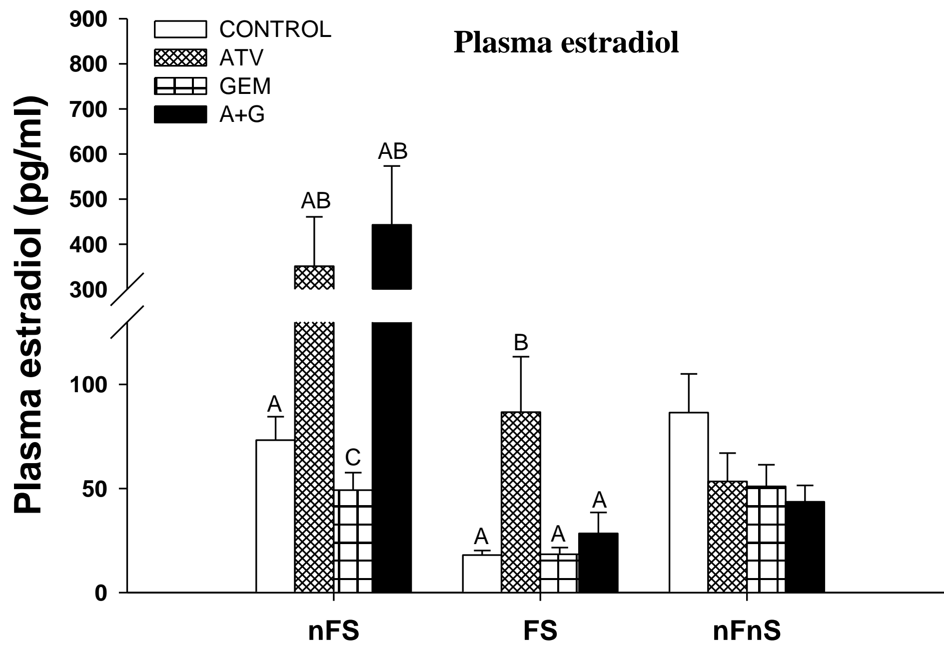


Figure 2.13. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma estradiol concentration of rainbow trout. See legend in Fig. 2.1 for details.

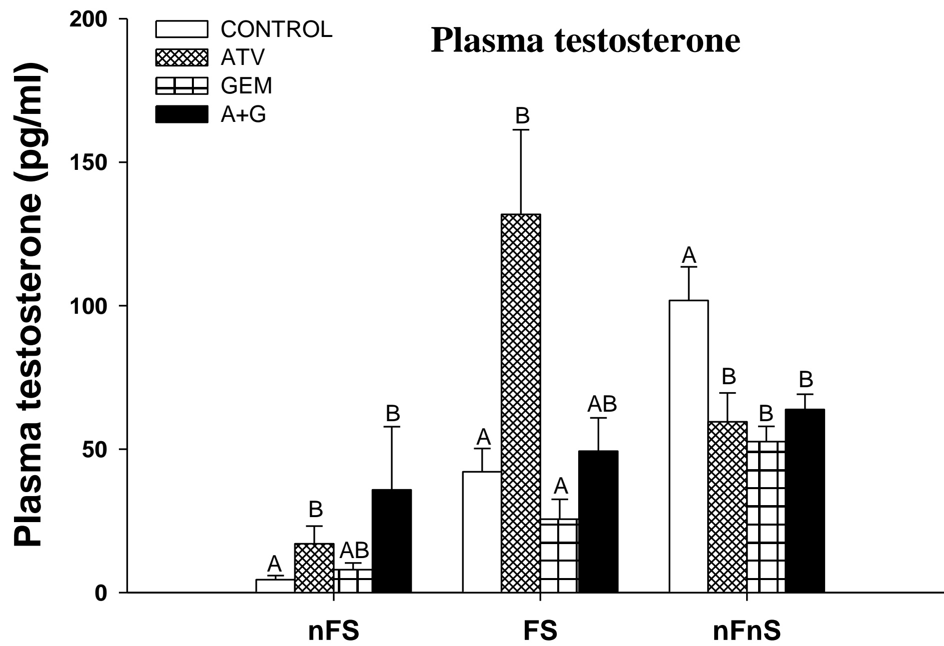


Figure 2.14. The effects of intraperitoneal injection (IP) of Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on plasma testosterone concentration of rainbow trout. See legend in Fig. 2.1 for details.

2.4. Discussion

The main objective of this study was to evaluate the effects of ATV and GEM on cholesterol metabolism and steroid production in rainbow trout. As predicted, cholesterol concentrations were significantly reduced in all experimental treatments compared with the control fish (Fig. 2.1); this decrease was approximately 30% regardless of feeding status of the fish. This reduction of cholesterol coincided with the elevation of hepatic hmgcr-1 mRNA abundance in the ATV and A+G groups, but not in the GEM group (Fig. 2.2). In contrast, neither drug alone or in combination affected hmgcr-2 transcript abundance (Fig. 2.2). The lack of a significant effect of ATV on hepatic hmgcr-2 is consistent with a previous study (Estey et al., (2008) where rainbow trout were injected with a pharmacological dose of cerivastatin, a more potent HMGCoAR inhibitor. ATV appears to specifically target liver hmgcr-1 and not hmgcr-2; the hmgc-1 transcript abundance is approximately 3-orders of magnitude greater than that of hmgcr-2 (Estey et al., 2008). This result is also consistent with the finding that hmgcr-2 expression in zebrafish is not liver-specific but is most abundant in the heart (D'Amico et al., 2007).

ATV alone and in combination with GEM, induced hepatic low-density lipoprotein receptor (LDLr) mRNA abundance in treated trout compared with the controls (Fig. 2.2). The greatest effects on LDLr mRNA occurred in the A+G group (approximately 5-orders of magnitude greater than the levels in control group). The significant elevation of LDLr mRNA is consistent with the reduction of plasma cholesterol and the significant elevation of hmgcr1 mRNA levels. Hepatic LDLr mediates the uptake of LDL particles from the circulation and delivers the receptor-bound LDL to the endosomal system for degradation while the LDLr returns to the cell surface for recycling (Kong et al., 2006; Rudling et al.,

2002). The inhibition of hepatic cholesterol synthesis by ATV and GEM results in the depletion of hepatic intracellular stores of cholesterol. Consequently, a compensatory increase in the expression of cell surface LDLr occurs, resulting in an increased potential for the hepatic extraction of circulating plasma LDL cholesterol (Dong et al., 2011; Kong et al., 2006). The combined effect of ATV and GEM inhibited cholesterol synthesis and enhanced hepatic clearance of circulating LDL cholesterol by increasing LDLr may be responsible for the decrease in total cholesterol (Dong et al., 2011).

There were some inconsistencies in the effect of the two drugs on plasma triglycerides concentrations (Fig. 2.3). Neither drug alone significantly affected plasma triglycerides content in the FS experiment. This lack of drug effect may be attributed to the fact that the fish were fed during the entire period of the experiment, therefore the fish were able to compensate for any drug-induced loss of triglyceride by attaining it from food ingestion. The effects of the two drugs were more prominent when the fish were not fed, hence the reduction of plasma triglycerides in nearly all treated fish in the nFS and nFnS experiments. The absence of an ATV effect on plasma triglycerides was reported in human subjects treated with Fluvastatin (another statin drug) (Marz et al., 2001). Moreover, *in vitro* evidence using HepG2 cells treated with three different statins (simvastatin, lovastatin and ATV) reported an increase production and contents of triglyceride (Scharnagl et al., 2001).

The reduction of plasma triglyceride concentrations in the nFS experiment coincided with an increase in hepatic SREBP-1 mRNA transcript abundance. SREBPs are considered global physiological regulators of lipid synthesis, that are linked to a suite of biosynthetic pathways involved in cholesterol, fatty acid and triglyceride synthesis and storage (Shimano, 2009). Three members of the SREBP family are described in mammals: SREBP-1a, SREBP-

1c, and SREBP-2 each with specific functions. SREBP-1c regulates transcription of genes involved in fatty acids synthesis, whereas SREBP-1a and SREBP-2 are involved in transcription of genes involved in cholesterol biosynthesis (Horton et al., 2002). Moreover, mammalian SREBP-1c is mainly expressed in the liver, adipose tissue and brain, whereas SREBP-1a is highly expressed in tissues with rapidly growing cells such as intestines and spleen (Shimomura et al., 1997). The SREBP-1 primers used in this study were adopted from (Skiba-Cassy et al., 2009), and were designed to target shared regions of SREBP-1a and SREBP-1c. Therefore, the reduction in the plasma triglyceride concentration by GEM and A+G resulted in the increased hepatic SREBP-1 transcript abundance to compensate for these plasma changes.

Furthermore, the reduction of plasma triglyceride concentrations in the nFS experiment was coincidental with a significant elevation of liver PPARα transcript abundance in all treated-fish compared with the control (Fig. 2.4), even where plasma triglyceride concentrations were not reduced as in ATV-treated fish. Moreover, hepatic PPARγ mRNA levels were also increased in ATV + GEM-treated fish, whereas liver PPARβ mRNA was not affected by the two drugs. Peroxisome proliferator activated receptors (PPARs) are a family of cellular receptors mainly implicated in the regulation of lipid metabolism. Three different PPAR subtypes have been identified in mammals and other species, showing different tissue distribution patterns and regulation (Batista-Pinto et al., 2005; Ibabe et al., 2005; Ibabe et al., 2002; Ruyter et al., 1997; Schoonjans et al., 1996). PPARα mainly regulates lipid catabolism and is highly expressed in the liver (Cajaraville et al., 2003). PPARβ is widely distributed in rainbow trout (Mimeault, 2008) and brown trout (*Salmo trutta fario*) (Batista-Pinto et al., 2005). Its ubiquitous organ expression has made a

definitive functional assignment to PPARβ difficult. It is thought that this receptor is implicated in basic cellular functions, such as lipid synthesis and turnover, but it also participates in cell differentiation in mammals (Braissant and Wahli, 1998). Statins are reported to activate PPARα and PPARγ *in vivo* and *in vitro* (Martin et al., 2001; Paumelle et al., 2006). Moreover, in mammals fibrate drugs have been shown to bind preferentially to PPARα with weak binding to PPARβ and PPARγ (Desvergne and Wahli, 1999). These results suggest that the two drugs have similar effects in trout as in human.

Plasma lactate concentrations were significantly reduced in treated fish compared to the control in both the nFS and FS but not the nFnS experiments (Fig. 2.6). Stress is generally associated with increases in plasma and muscle lactate concentrations (Barton and Iwama, 1991). The elevation of lactate in the control fish was accompanied by an elevation in plasma cortisol levels (Fig. 2.7), which was only statistically different from the A+G- group. However, this elevation in cortisol in control fish was reversed in the experiment where fish were not stressed. Moreover, hematocrit in treated fish was significantly lower compared to the control fish, except when fish were not stressed (Fig. 2.8). A rise in hematocrit is a good indication of stress response in fish. This rise of hematocrit may be a strategy for increasing the oxygen carrying capacity of the blood during stress, which could be achieved by recruiting more red blood cells (Wells and Pankhurst, 1999). Furthermore, all fish in the nFS and FS experiments were subjected to the same stress protocol, yet only the control fish showed an increase in plasma cortisol concentration. This is an indication either that the reduction in cholesterol might have resulted in the treated fish lacking a proper stress response or that the treated fish were simply not responsive to the stress protocol. Moreover, most treated-fish in the nFnS experiments had significantly increased plasma cortisol

compared with the control fish that were injected with DMSO alone, which is indicative of stress that resulted from drug injections. Previously, it has been shown that the presence of pollutants or contaminants in the water results in elevated plasma cortisol levels in fish (Hontela et al., 1992). This effect possibly arises as a result of either a toxic action of the chemical stressor on the hypothalamus-pituitary-interrenal (HPI) axis or down-regulation of the HPI axis from continuous negative feedback by cortisol (Hontela, 1997).

Plasma creatinine concentration was significantly elevated in all treated fish compared with the control, except in the GEM-nFnS group. Elevation of plasma creatinine is an indicator kidney damage, since creatinine is a breakdown product of creatine phosphate that is produced primarily by the body skeletal muscle and excreted by the kidneys at a constant rate (Scown et al., 2009). Hence, any change in plasma creatinine concentration is used as a biomarker of glomerular filtration rate, with a rise in creatinine levels indicating nephron damage possibly due to the treatment used. Mean plasma creatinine concentrations in the control fish were similar to reported normal trout levels (Manera and Britti, 2006), validating our experimental approach. Furthermore, plasma protein concentration was elevated in most injected-fish; however, the significant increase was seen in the nFS experiment where ATV was injected alone or in combination with GEM (Fig. 2.9). A rise in plasma protein concentration may indicate an impairment in kidney function which can be demonstrated by a reduction in urine production (Scown et al., 2009). The mean protein concentration of all fish was within the levels reported in the literature, but lower in the control group compared to the treated groups where fish were stressed and fasted (Manera and Britti, 2006).

Plasma creatine kinase activity was significantly elevated in nearly all injected fish in the three experiments treatments, which is indicative of muscle damage (Fig. 2.10). An increase in creatine kinase levels is a biochemical indicator of statin-associated rhabdomyolysis (Farnier et al., 2003). The elevation of creatine kinase was coincidental with significantly elevated levels of white muscle (Fig. 2.11) and red muscle (Fig. 2.12) f-box 25 and f-box 32 (atrogin-1) mRNA abundance (Fig. 2.11). Moreover, muscle RING finger 1 (MuRF1) mRNA abundance in the white muscle was elevated although not significantly compared with the control (Fig. 2.11). In mammals, muscle degradation occurs primarily through increased protein degradation through the ubiquitin proteasome pathway. The degradation of proteins by the 26S proteasome is achieved by covalent linking a chain of ubiquitin molecules to the target proteins tagging it for degradation (Ciechanover, 1998). Three components are involved in the formation of ubiquitin-protein complexes: ubiquitin activation enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3) (Ciechanover, 1998). Reports suggest that two of the E3 ubiquitin ligases, muscle atrophy F-box (also called atrogin-1) and muscle RING finger 1(MuRF1) are key elements of regulation of ubiquitin-proteasome-mediated muscle protein degradation (Bodine et al., 2001; Seiliez et al., 2008). Furthermore, it has been reported that atrogin-1 is induced early during the degradation process, and the rise in atrogin-1 expression precedes the loss of muscle weight as a result of this degradation (Gomes et al., 2001). Moreover, animals lacking atrogin-1 are resistant to muscle degradation, suggesting that atrogin-1 targets key muscle proteins for destruction (Bodine et al., 2001). Atrogin-1, is an E3 ubiquitin ligase which is up-regulated in food-deprived fish (Cleveland and Burr, 2011; Cleveland and Evenhuis, 2010a; Cleveland et al., 2009). A second ubiquitin E3 ligase, F-box 25, plays an

important role in protein mobilization by ubiquitin proteasome (Maragno et al., 2006). In the current study mRNA transcript abundance of both members of E3 was up-regulated in treated fish, indicating that injecting ATV alone or in combination with GEM promotes muscle damage and possibly rhabdomyolysis in the trout. However, the third member of E3 (MuRF1) was not statistically up-regulated in the white muscle. In mammals, MuRF1 and atrogin-1 genes are consistently up-regulated in atrophying muscles (Bodine et al., 2001; Gomes et al., 2001). Taken together, results from plasma creatine kinase, creatinine and mRNA transcripts of E3 indicate that the two drugs, either alone or in combination may lead to rhabdomyolysis in fish.

Since cholesterol in the essential precursor of all steroid hormones, it is expected that changes in its levels will affect steroidogenesis (Eacker et al., 2008). For this reason one of the objectives of this study was to determine to what extent the reduction of cholesterol induced by ATV or in combination with GEM would lead to changes in sex steroids in trout. However, there was a lack of consistency in the results of sex steroids in the current study even though cholesterol levels were reduced in all experimental treatments. Plasma estradiol concentrations were elevated but not significant in the nFS group; however, there was a significant reduction in plasma estradiol in the GEM-treated group compared with control (Fig. 2.13). In the FS experiment, plasma estradiol concentration was significantly elevated in ATV-treated group compared with control, but there was no significant change in GEM or in A+G-treated fish (Fig. 2.13).

Mimeault and colleagues (2005) reported a decrease in plasma testosterone content in goldfish exposed to a pharmacological dose of GEM. Also, it was reported that stress resulted in a reduction of sex steroids in a variety of animals (Moore et al., 1991; Mosconi et

al., 1994). An inverse relationship between cortisol and sex steroid levels is reported during smoltification and sexual maturation in salmonid species (Pickering, 1987). Moreover, acute stress decreased sex steroids in male and female salmon (*Oncorhynchus nerka*) (Kubokawa et al., 1999). In the present study, a reduction in plasma testosterone concentrations was observed only in the nFnS experiment, suggesting that reducing cholesterol may under specific conditions lead to the reduction of sex steroids. In the same experiment, plasma estradiol concentrations trended to decrease in all treatments, but this trend was not statistically significant when compared with the control (Fig. 2.13). The unexpected rise in some of sex steroids when fish were stressed cannot be explained with the current end-points investigated.

2.5. Conclusions

The aim of the present study was to examine the effects of ATV and GEM alone and in combination on rainbow trout. Three experiments were conducted to achieve this goal and the results demonstrated that the two drugs had several detrimental effects on trout. Plasma cholesterol concentrations were reduced and generally this was associated with the failure of these fish to mount an appropriate stress response as assessed by plasma cortisol content and in some cases changes in sex steroids. Furthermore, fish treated with the two drugs had elevated levels of creatine kinase and elevated expression of atrogin-1 and f-box 25 transcript abundance, an indication of skeletal muscle damage and possibly rhabdomyolysis. Plasma creatinine levels were elevated in most injected fish, an indication of kidney damage. Taken together, these results support my original hypothesis and suggest that ATV and GEM exposure may challenge the stress response and the reproductive axis, and modify tissue

metabolism and muscle structure thus potentially impacting the wellbeing and the fitness of the trout.

CHAPTER 3

Exposure to Atorvastatin and Gemfibrozil affects cholesterol metabolism and steroid production in the zebrafish *Danio rerio*

3.1. Introduction

Cholesterol is an amphipathic molecule that is an essential structural component of eukaryote cell membranes (Burger et al., 2000; Norum et al., 1983; Robertson and Hazel, 1995; Simons and Ikonen, 2000; Williams and Hazel, 1995), of the outer layer of blood lipoproteins (LPs) (Faust et al., 1988; Siperstein, Marvin D., 1984), and a precursor of steroids including corticosteroids (cortisol), sex hormones (estrogen, testosterone), bile acids, and vitamin D (Espenshade and Hughes, 2007). Cholesterol plays a major regulatory role in several cellular processes including lipoprotein synthesis, cell growth, receptor function, gene expression and DNA replication (Morris et al., 1995; Norum et al., 1983; Russell, 1992). For these and other reasons, the synthesis and utilization of cholesterol must be tightly regulated to prevent over-accumulation and abnormal deposition within the body. Of particular importance clinically is the abnormal high deposition of cholesterol and cholesterol-rich LPs in the coronary arteries. Such deposition, eventually leading to atherosclerosis, is a leading contributory factor in diseases of coronary arteries in North America and is considered a leading cause of cardiovascular mortality and morbidity (Boden et al., 2007; Choy et al., 2000). It is estimated that each year 50% of mortality in the United States is caused by atherosclerosis or atherosclerosis-related diseases (Choy et al., 2000; Christian et al., 2011).

Statins or 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) inhibitors, are drugs used to treat hypercholesterolemia (Stancu and Sima, 2001). Statins are

also recommended for the treatment and prevention of other conditions including stroke, osteoporosis, progression of multiple sclerosis and Alzheimer's disease (Amarenco et al., 2004). According to IMS Health (Cavallucci, 2007), prescriptions for cholesterol reducers (statins) continue to increase and between 2004 and 2007, Atorvastatin (ATV) was the most prescribed drug in Canada. ATV targets the liver and inhibits HMGCoAR, the enzyme that converts 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) into mevalonate, a cholesterol precursor. ATV competes with the normal substrate for the active site of the enzyme, altering the conformation of the enzyme preventing HMGCoAR from attaining a functional structure. This results in an overall decrease of the amount of cholesterol produced by the liver (Blumenthal, 2000).

Gemfibrozil (GEM), a lipid lowering drug, belongs to the fibrate drug family (Corbelli et al., 2002). GEM elicits its biological effects by activating peroxisome proliferator activated receptors (PPARs), and primarily PPARα (Bishop-Bailey, 2000). As a consequence of PPAR activation, marked changes in gene expression of key enzymes involved in the synthesis of several apolipoproteins and lipoprotein metabolism occurs (Mandard et al., 2004; Skolness et al., 2012), ultimately resulting in the reduction of plasma triglyceride concentrations, activation of β-oxidation of fatty acids, down-regulation of lipid biosynthesis, and the elevation of cholesterol.

Hypercholesterolemia in teleost fishes seems to be a normal condition in contrast to the condition in mammals where this condition is associated with coronary heart disease and atherosclerosis (Larsson and Fange, 1977). Fish, and salmonids in particular, are susceptible to atherosclerotic lesion formation in coronary arteries which is attributed not to hypercholesterolemia *per se* but to growth rate and sexual maturity (Saunders et al., 1992).

In general, the plasma cholesterol concentration in most fish species is between 2- and 6-times higher than that of mammals (Babin and Vernier, 1989; Larsson and Fange, 1977).

Statins have few known side effects, but cerivastatin (another statin drug) was voluntarily removed from the US market because of its association with a higher than expected number of fatal cases of rhabdomyolysis (breakdown of skeletal muscles, with degradation products from these muscle cells causing further damage in the body). Rhabdomyolysis is a rare but potentially fatal complication of the combined use of fibrate and statin drugs (SoRelle, 2001).

There is little research reported on statin drugs in non-human organisms. Brain and colleagues (2006) demonstrated that ATV is phytotoxic in the higher aquatic plant *Lemna gibba* and found biochemical alterations in the pathway products downstream of HMGCoAR in these plants. Furthermore, significant disruptions occurred primordial germ cell migration during zebrafish (*Danio rerio*) development when ATV was used at pharmacological doses (10 μM) (Thorpe *et al.*, 2004). However, this effect may be related not to reduced cholesterol but to the reduction in prenylated protein production (isoprenoid addition to proteins, catalyzed by geranylgeranyl transferase 1), a downstream process blocked by statins. A similar requirement for protein prenylation is thought to be responsible for changes in early heart morphogenesis in zebrafish (D'Amico *et al.*, 2007) and cerebral hemorrhage in zebrafish embryos (Eisa-Beygi et al., 2012). Moreover, Hanai and colleagues (2007) demonstrated that statins induced marked induction of atrogin-1 expression in zebrafish an indication of skeletal muscle damage (rhabdomyolysis). In addition, Estey and colleagues (2008) demonstrated that HMGCoAR in fish can be inhibited both *in vitro* and *in vivo* when

treated with statin drugs and as shown in Chapter 2 of this thesis, statin treatment effects cholesterol and steroid production in rainbow trout (*Oncorhynchus mykiss*).

Fatal rhabdomyolysis with cerivastatin have been reported most frequently when used at high doses and particularly, when used in combination with GEM (SoRelle, 2001). It was reported by Metcalfe and colleagues (2004) that in Canadian wastewater treatment plant (WWTP) effluents where statins drugs were detected, fibrate drugs were present at comparable or higher concentrations. ATV is the most prevalent drug in the aquatic environment of the statin class. The concentrations of ATV were 15 ng/L and 44 ng/L and that of GEM 1500 ng/L and 2100 ng/L in surface waters and WWTP effluent, respectively (Metcalfe et al., 2004).

The main objective of this study was to mimic the actions of ATV and GEM in the environment. In the environment, fish “ingest” or encounter pharmaceuticals either through water exposure or through food. In this study, both venues of exposure will be assessed in zebrafish to better understand and investigate the impact of these two pharmaceuticals on a non-target species. Zebrafish are exposed to environmental stressors and must be able to mount an appropriate stress response. Additionally, zebrafish are continuous breeders and may breed daily (Westerfield, 2000), so presumably the maintenance of sex steroids levels are important for this reproductive activity. It is hypothesized that exposing zebrafish to ATV and GEM will challenge their cholesterol balance (availability vs utilization) and thus affect steroid production and those factors responsible for the maintenance of cholesterol homeostasis.

3.2. Materials and Methods

3.2.1. ATV and GEM water-borne exposure

Male and female zebrafish *Danio rerio* were purchased from a local supplier (Big Al's Aquarium Services, Ottawa, ON), sex identified (females have a large white belly and are thicker in the body than the males), and separated into ten different stand-alone glass tanks (21 cm x 23 cm x 35 cm (W, H, L) containing 10-L of aquarium water); the mass of the fish used was 0.5 to 0.9 g. Twenty male or female fish were placed into each tank (2 fish/L). Fish were exposed in the tank water to a nominal concentration of either 45 µg/L ATV (1000-times environmental concentration), 1.5 mg/L GEM (1000-times environmental concentration), or the combination of both drug concentrations (ATV + GEM; A+G). Two control tanks, one for males and one for females were treated with DMSO only; the final concentration of DMSO was less than 0.01%. Each tank was slowly aerated using an air stone, temperatures were maintained at 28 ^{o}C, and 5-L of tank water was removed and replaced every other day (static-renewal); water was dechloraminated City of Ottawa tapwater. Drugs were added daily for a period of 28 days, and the fish were not fed for the entire duration of the experiment. At the end of the experiment, the fish were subjected to a standardized air-exposure net stress test (Ramsay et al., 2009). At the end of the experiment, the fish were euthanized with an overdose of buffered tricaine methane sulfonate (4 g/L MS 222), blotted on tissue paper to remove excess water, flash frozen in liquid nitrogen, and placed into individual 2.0 mL plastic centrifuge tubes. The fish samples were stored at -80°C until analyzed. All experiments were subjected to review by the University of Ottawa Protocol Review Committee and adhere to the published guidelines of the Canadian Council on Animal Care for the use of animals in teaching and research.

3.2.2. ATV and GEM feeding experiment

Zebrafish were fed 1% body weight once per day a commercial zebrafish food (complete diet, Zeigler, Gardners, PA) containing ATV, GEM, and the combination of the two drugs (A+G) or food containing the carrier solvent (95% ethanol) for control. Zebrafish diet containing a human equivalent dose of ATV (0.53 μg/g) was formulated by addition of the appropriate amount of ATV (dissolved in ethanol) and zebrafish food to give a final concentration of 0.53 μg/g. The GEM diet was formulated as the ATV diet to give a final concentration of 16 μg/g. The combination of the two drugs (A+G) was formulated as above to give a combined concentration of the two drugs. The food was spread on an aluminum tray, sprayed with the drug and left in a fumehood to air dry overnight. The following day, the weight of the food was measured to validate that the food was dry and that no trace of ethanol remained in the food. The food was stored in glass vials covered with aluminum foil. The food and the drugs were prepared weekly. The fish were fed daily the food containing the drugs for 30 days. The fish eat all food allocated to them within 5 min and no food was left at the bottom of the tank. Furthermore, fish mass was assessed weekly by taking total weight of fish in each tank, and food assigned for each treatment was corrected accordingly. At the end of the experiment, only the fish allocated for biochemical analysis not the molecular biology endpoints were subjected to the standardized net stress test (Ramsay et al., 2009). At the end of the 30 day experiment, the fish were euthanized, flash frozen in liquid nitrogen, and stored in individual 2.0 mL plastic centrifuge tubes at -80°C as above.

3.2.3. Lipid and steroid extraction and estimates

Lipids and sex steroids were extracted using a modified Folch extraction method. Briefly, individually frozen zebrafish were ground in liquid N_2 using a mortar and pestle,

before homogenizing for 30 sec in 15 mL 2:1 chloroform/methanol (Folch) with a Polytron homogenizer (Kinematica, Luzern, Switzerland). An additional 5 mL Folch was added to the homogenized mixture and incubated at room temperature for 30 min; the mixture was vortexed every 10 min to ensure mixing of the tissues with the Folch media. At the end of incubation period, 5 mL 2M KCl buffered with 5 mM EDTA was added, the mixture vortexed and allowed to settle for 10 min. Once settled, the bottom phase (chloroform) was transferred to a clean glass tube and evaporated under a stream of N_2 gas. The evaporated samples were re-suspended in 1.0 mL ethylene glycol monomethyl ether (EGME), and stored at -80°C until analyzed. The extraction efficiency (based on radioactive isotope spiking into zebrafish before the commencing of the extraction procedure) was determined three times for each individual lipid or steroid, and the average reported. These efficiencies were: cholesterol (84%), cortisol (82.6%), estradiol (78.1%), testosterone (79.3%), and triglyceride (76.6%).

Cholesterol (catalog # C507-480) and triglycerides (catalog # T532-480) were assessed using a commercially available spectrophotometric assay (TECO Diagnostic, Anaheim, CA). In brief, 10 µL of the EGME-suspended tissue was added to 250 µL of the corresponding color reagent and incubated at 37 °C for 10 min (cholesterol) or 5 min (triglycerides). Absorbance was read at 520 nm using a microplate spectrophotometer (SpectraMAX Plus 384; Molecular Devices, Sunnydale, CA, USA) and the concentrations of unknown samples were calculated relative to a standard curve.

Cortisol was estimated using a ^{125}I radioimmunoassay (RIA) kit as per the manufacturer's instructions (MP Biomedicals, Orangeburg, NY, USA).

Plasma estradiol and plasma testosterone were determined using enzyme immunoassay (EIA) test kits for estradiol (catalogue # ESTRA-96) and testosterone (catalogue # TEST-96) as per the manufacturer's instructions (TECO Diagnostic). This assay is based on the competitive binding between estradiol and testosterone in the plasma and estradiol or testosterone-horseradish peroxidase conjugate for a constant amount of rabbit anti-estradiol or testosterone. The sensitivity of the kits as reported by the manufacturer is 10 and 50 pg/mL, for estradiol and testosterone, respectively.

3.2.4. RNA isolation

RNA was prepared using Trizol reagent, following the manufacturer's instructions (Gibco BRL, Burlington, ON). Briefly, 10-30 mg (liver and brain) or 80-100 mg (muscle) of frozen tissue, were suspended in 1 mL Trizol in 2 mL Safe-Lock Eppendorf® tubes and homogenized using a Polytron homogenizer. The homogenate was incubated at room temperature for 5 min, followed by the addition of 0.3 ml chloroform and then shaken vigorously by hand for about 15 sec. The homogenate was incubated at room temperature for 2-3 min, and then centrifuged at 12000 x g for 15 min at 4 °C. After centrifugation, the upper aqueous phase was removed carefully without disturbing the lower (Trizol) phase and placed in a new autoclaved 1.5 mL tube. RNA precipitation was performed by the addition of 0.5 mL isopropanol and 4 µL linear acrylamide (2 mg/mL) to the aqueous phase (Gaillard and Strauss, 1990) and mixed by flicking. The aqueous phase was incubated on dry ice for 30 min, and then centrifuged for at 12000 x g for 15 min at 4 °C. Following centrifugation, the supernatant was removed and the RNA pellet was rinsed with cold 75% ethanol. RNA pellets were vortexed and centrifuged at 7500 x g for 5 min at 4 °C; rinsing was repeated 3-times to ensure the removal of any contaminants. After rinsing, the supernatant was removed

and the RNA pellet was allowed to air dry for 10-15 min, then dissolved in an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water depending on the size of the pellet. The dissolved pellet was incubated at 65 °C for 10 min with vortexing every 2-3 min until dissolved. The quantity and quality of RNA was measured using a NanoDrop 2000 (ThermoScientific, USA). Only RNA with a 260/280 ratio greater than 1.9, and a 260/230 ratio greater than 2 was used for cDNA synthesis. RNA samples were stored at -80 °C.

3.2.5. Real-time quantitative RT-PCR

All transcript levels were assessed in duplicate using quantitative real-time RT-PCR (qPCR) and a MX3000P® Multiplex Quantitative PCR system (Stratagene, La Jolla, CA, USA). Data were analyzed using the MX3000P Software Package. Total RNA from selected tissues were used to synthesize cDNA. Briefly, 1 µg total RNA was DNase treated using DNase I amplification grade (Invitrogen, Burlington, ON, Canada). Following DNase treatment, first strand cDNA synthesis was undertaken using 300 ng random hexamer primers (Invitrogen), and 200 units of Superscript® II Reverse transcriptase (Invitrogen). A relative standard curve for each primer set used was constructed by pooling dilutions of cDNA of all samples (treated and control). In each run, a standard curve was generated that was used to calculate relative changes in mRNA abundance between control and treated samples. Genes were normalized to the expression of the house-keeping gene elongation factor 1-α (EF1-α) which did not change across treatments. Each qPCR reaction contained the following final concentrations: 10 µL Brilliant III Ultra-fast SYBR® Green QPCR Master Mix (Agilent Technologies, Mississauga, ON), 15 nM ROX reference dye, 500 nM gene-specific primers (forward and reverse), and 10 ng first-strand cDNA template, in a 20 µL reaction volume. The thermal cycling parameters were 3 min initial denaturation step at

95 °C, followed by 40 cycles of 95 °C for 20 s, and 60 °C for 20 s. Unless otherwise indicated, all primers were designed using OligoPerfect™ Designer (Life Technologies. Primers for SREBP-1 and SREBP-2 were from (Craig and Moon, 2011), those for PPARα, PPARβ, and PPARγ were from (Velasco-Santamaria et al., 2011), those for LDLR were from (Her et al., 2011) and those for CYP3A65 were from (Lister et al., 2009). All primers used are presented in Table 1.

Table 3.1. Primers used to assess mRNA abundance in liver, brain, and muscle of zebrafish, their sequences, amplicon size and GenBank accession number.

Gene	Primer	Sequence (5'→3')	Amplicon size (base pairs)	Accession number
EF1-α	Forward	CAA ACATGGGCTGGTTCAAG	245	AY422992
	Reverse	AGTGGTTACATTGGCAGGG		
HMGCR-1	Forward	CCAAGAGGATTGAGCCTGAC	207	NM_001079977
	Reverse	GATCCGGAGACCATTTCTGA		
HMGCR-2	Forward	GTACATCCGCTTTCAGTCTCAG	233	NM_001014292
	Reverse	AACACCTCTTTGACCACTCG		
SREBP-1	Forward	GACACTTCTCTGGACACTCTG	135	NM_001105129
	Reverse	ATCGAACAGCCCAAACTCC		
SREBP-2	Forward	GAGATAAAGTGGACCCCATCG	134	NM_001089466
	Reverse	CAGAAACTCCAGAACCCCAG		
PPAR-α	Forward	CATCTTGCCTTGCAGACATT	81	NM_001161333
	Reverse	CACGCTCACTTTTCATTTCAC		
PPAR-β	Forward	GCGTAAGCTAGTCGCAGGTC	204	AF342937
	Reverse	TGCACCAGAGAGTCCATGTC		
PPAR-γ	Forward	GGTTTCATTACGGCGTTCAC	250	DQ839547
	Reverse	TGGTTCACGTCACTGGAGAA		
Atrogin-1	Forward	GTCAGTCTGGGTCAAGTGTG	233	NM_200917
	Reverse	AAGAGGATGTGGCAGTGTG		
CYP3A65	Forward	CTTCGGCACCATGCTGAGAT	86	NM_001037438
	Reverse	AGATACCCCAGATCCGTCCATA		
LDLR	Forward	GCCAGCAAGGCCTGCAAAGC	Not Available [a]	NM_001030283
	Reverse	CTTCAGGCGGGGGATGACGC		

[a]See Her et al. (2011)

3.2.6. Tissue distribution of hmgcr transcripts

Two hmgcr transcripts are reported in the zebrafish (Thorpe et al., 2004), but to my knowledge, the tissue distribution of these transcripts has yet to be reported. The tissue distribution of hmgcr-1 and -2 was determined using four adult naïve (non-exposed) female zebrafish. Tissues from two females were combined to extract total RNA. Tissues (liver, gut, brain, spleen, heart, gonads, and skeletal muscle) were removed and snap frozen in liquid nitrogen and stored at -80 °C until analyzed. Elongation factor-1α (ELF-1α) mRNA expression was used as internal control for data normalization. Tissues were prepared as in 3.2.4 and assessed as in 3.3.5.

3.2.7. Statistical analysis

Experimental results are presented as the means ± standard error of the mean (SEM). Statistical analysis was conducted using SigmaPlot™ 11.0 (SPSS Corporation, Chicago, IL, USA). Log-transformed data were used wherever necessary to satisfy homogeneity of variance; data that failed the normality test after log transformation were assessed using a Kruskal-Wallis One-way ANOVA on ranks. Only non-transformed data are presented in the text figures and tables. Whenever appropriate, statistical significance was tested with either a One-way ANOVA followed by a Tukey test (for biochemical end-point) or a Student-Newman-Keuls test (for gene expression). A value of $p < 0.05$ was accepted as significant.

3.3. Results

3.3.1. Tissue distribution of hmgcr1 and hmgcr2

Thorpe and colleagues (2004) established that zebrafish hmgcr-1 transcripts are acquired after 4 dpf and that hmgcr-2 is a maternal transcript that is present in 4-cell stage embryos. Hmgcr-1 transcripts from adult zebrafish were detected in most tissues examined, except spleen, gonads, and skeletal muscle (Fig. 3.1). Liver and gut had the greatest abundance followed by brain and heart; gills had detectable levels but these were well below that of the brain and heart. Hmgcr-2 expression was detected in all tissue examined (Fig. 3.1). There were differences between tissues, with liver and gut transcript levels well below those observed in the brain or gonads.

3.3.2. Water-borne exposure

Whole-body cholesterol content was significantly reduced in all female drug-treated zebrafish compared with the control ($p < 0.001$) (Fig. 3.2. GEM-treated male fish did not show a significant reduction in whole-body cholesterol content compared with the control ($p = 0.08$), although both ATV and A+G were significantly different from the control ($p < 0.001$).

Whole-body triglycerides were significantly reduced in all female-treated zebrafish compared with the control (p = 0.003, 0.019, and < 0.001, for ATV, GEM and A+G, respectively) (Fig. 3.3); no significant interaction was observed between ATV and GEM ($p < 0.255$). No significant differences were noted with male-treated fish compared with the control (p = 0.375, 0.934, and 0.15, for ATV, GEM and A+G, respectively) (Fig. 3.3), although there was a significant difference when comparing GEM to A+G ($p = 0.043$).

Whole-body cortisol was significantly reduced only in ATV and A+G-treated female zebrafish but not in GEM-treated fish compared with the control (p = 0.002, .0499, and 0.001, for ATV, GEM and A+G, respectively) (Fig. 3.4). Moreover, a One-way ANOVA detected significance when comparing the GEM and A+G groups (p = 0.048) but not with ATV. In male zebrafish, whole-body cortisol was significantly reduced by all drug-treatments compared to the control ($p < 0.001$) (Fig. 3.4).

Whole-body testosterone content was significantly reduced only in the ATV and A+G-treated female fish but not in GEM-treated fish compared with the control ($p < 0.001$, < 0.001, and 0.266, for ATV, GEM and A+G, respectively) (Fig. 3.5). A One-way ANOVA detected significance when comparing GEM to ATV and A+G ($p < 0.001$). A similar pattern of whole-body testosterone was also seen in male-treated fish where the significant reduction was observed in ATV and (A+G), but not in the GEM-treatment (p = 0.008, < 0.001, and 0.983, for ATV, GEM and A+G, respectively) (Fig. 3.5). A One-way ANOVA detected significant differences when comparing GEM to ATV and A+G (p = 0.003, < 0.001, for ATV, and A+G, respectively).

Whole-body estradiol was significantly reduced in all drug-treated female fish compared with the control ($p < 0.001$ for all treatments) (Fig. 3.5), and the Two-way ANOVA indicated a significant interaction between ATV and GEM ($p < 0.001$). Changes were not as striking in drug-treated male fish with significant reductions in ATV and A+G, but not in GEM-treated fish (p = 0.017, 0.086, and 0.025, for ATV, GEM and A+G, respectively) compared with the control (Fig. 3.5).

3.3.3. Drug feeding experiment

The zebrafish in the previous water-borne exposure experiment were not fed during the entire 30 day experiment so to determine whether the route of administration and feeding may affect the fish response to the drugs, the drugs were sprayed on the food that was subsequently fed to the fish. Zebrafish were fed once per day for the entire experimental period.

Feeding generally resulted in lower control whole-body cholesterol contents in both females and males, but the pattern of change with drug treatment was similar between exposure routes. Cholesterol contents were significantly reduced in all drug-treated female fish compared with the control (p <0.001, 0.003, and 0.023, for ATV, GEM and A+G, respectively) (Fig. 3.6). Male-treated fish exhibited the same pattern as the females, where drug treatments significantly reduced cholesterol contents in all treated fish compared with the control (p = 0.024, <0.001, and 0.007, for ATV, GEM and A+G, respectively) (Fig. 3.6).

The reduction of whole-body cholesterol content coincided with an increase in liver SREBP-2 mRNA transcript abundance in both female (p <0.001, 0.006, and <0.001, for ATV, GEM and A+G, respectively) and male (p = 0.011, 0.002, and 0.01, for ATV, GEM and A+G, respectively) treated fish compared with the control (Fig. 3.7). Moreover, a One-way ANOVA detected a significant difference when comparing GEM to ATV and A+G (p = 0.002, 0.001, for ATV and A+G respectively) only in the females. Liver LDLr mRNA transcript abundance was not affected by the drugs in either females or males (Fig. 3.7).

The reduction of whole-body cholesterol and the up-regulation of SREBP-2 were concurrent with a significant elevation of liver hmgcr mRNA abundance in both females and males. Liver hmgcr-1 mRNA levels were significantly elevated in the female-treated fish

compared with the control except in GEM-treated fish (p <0.001, 0.750, and <0.001, for ATV, GEM and A+G, respectively) (Fig. 3.7). Moreover, a One-way ANOVA detected significant differences when comparing GEM to ATV and A+G ($p < 0.001$). Male zebrafish treated with ATV and A+G also demonstrated increased liver hmgcr-1 mRNA abundance, similarly GEM-treatment fish were not significantly different compared with the control (p <0.001, 0.131, and <0.001, for ATV, GEM and A+G, respectively) (Fig. 3.7). However, One-way ANOVA detected statistical significance when comparing GEM to ATV and ATV and GEM (p < 0.002, 0.001, for ATV and A+G, respectively). Liver hmgcr-2 mRNA abundance was significantly elevated in all female-treated fish compared with the control (p <0.001, 0.031, and 0.002, for ATV, GEM and A+G, respectively). In the male-treated fish, hmgcr-2 mRNA abundance was significantly elevated in all male-treated fish (ANOVA on ranks) (Fig 3.7).

Brain hmgcr mRNA abundance was also assessed as the brain was the one tissue that contained significant amounts of both forms (see Fig. 3.1). Male zebrafish showed no drug-treatment effects for either transcript. However for female-treated fish, brain hmgcr-1 mRNA abundance was significantly elevated in the ATV and A+G but not GEM-treated groups compared with the control (p <0.018, 0.338, and <0.008, for ATV, GEM and A+G, respectively) (Fig. 3.8). Moreover, a One-way ANOVA detected significant differences comparing GEM to ATV and A+G (p = 0.004, 0.003, for ATV an A+G, respectively). Brain hmgcr-2 mRNA abundance in females was significantly elevated only in ATV but not in GEM or A+G-treated fish compared with the control (p = <0.044, 0.461, and 0.061, for ATV, GEM and A+G, respectively).

Patterns of whole-body triglyceride content were generally similar between the water-borne vs feeding routes of administration of drugs especially in the female-treated group (compare Figs 3.3 and 3.9). A significant reduction in triglycerides was noted in all female-treated fish compared with the control (p <0.001, <0.001, and <0.001, for ATV, GEM and A+G, respectively) (Fig. 3.9). The male-treated fish exhibited a very different pattern from that of the females and from the water-borne route of administration; whole-body triglyceride content was significantly elevated (feed) rather than reduced (water-borne) in ATV and A+G-treated fish compared with the control (p <0.001 and <0.001, for ATV, and A+G, respectively) (Fig. 3.9). There was also a reduction of triglycerides in this group with GEM compared with control (p = 0.007) (Fig. 3.9) and the water-borne route of administration (Fig. 3.3). Moreover, One-way ANOVA detected statistical significance when comparing GEM to ATV and A+G (p <0.001, <0.001, for ATV and A+G, respectively).

Transcription factors linked to lipid regulation were also assessed in this experiment. Liver SREBP-1 mRNA abundance was generally elevated with drug-treatments in females but only statistically significant in the A+G treated group compared with the control (p = 0.055, 0.096, and 0.015, for ATV, GEM and A+G, respectively) (Fig. 3.10). For males, liver SREBP-1 mRNA transcripts were significantly elevated for all drug-treatments compared with the control (p = 0.012, 0.002, and 0.010, for ATV, GEM and A+G, respectively

Liver PPARα mRNA abundance was significantly elevated only in drug-treated females fish compared with the control (p <0.001, 0.002, and 0.036, for ATV, GEM and A+G, respectively), not in males (Fig. 3.10). Moreover, liver PPARβ mRNA abundance was unaffected by the drugs in both sexes (Fig. 3.10). However, liver PPARγ mRNA abundance

was significantly elevated in both female (*p* <0.001, 0.021, and <0.001, for ATV, GEM and A+G, respectively) and male (*p* <0.001, <0.001, and 0.002, for ATV, GEM and A+G, respectively) treated fish compared with the control (Fig. 3.10). A One-way ANOVA detected significant differences when comparing GEM to A+G and GEM in female (*p* = 0.008, 0.012, for ATV an A+G, respectively) but not male fish.

Skeletal muscle atrogin-1 mRNA abundance was generally elevated in both sexes although differences were most pronounced in the males (Fig. 3.11). Drug-treated females only demonstrated significant differences when treated with A+G compared with the control (*p* = 0.654, 0.05, and 0.043, for ATV, GEM and A+G, respectively). Male muscle atrogin-1 mRNA transcript levels were significantly elevated under all drug treatments compared with the control (*p* <0.001, 0.019, and 0.045, for ATV, GEM and A+G, respectively) (Fig. 3.11). Moreover, a One-way ANOVA detected statistical significance when comparing GEM to ATV and A+G in the males only (*p* <0.001, <0.001, for ATV an A+G respectively).

Hepatic CYP3A activities in mammals are involved in the metabolism of a range of endogenous substrates and xenobiotics, and CYP3A65 transcripts are induced by PCBs in zebrafish (Tseng et al., 2005). Transcript levels of CYP3A65 were generally elevated in female drug-treated fish but individual variation precluded significant differences compared with the control (Fig. 3.12). In the males, hepatic CYP3A65 transcripts were elevated but only significantly in the ATV and GEM groups but not in A+G-treated fish (*p* = 0.008, 0.005, and 0.179, for ATV, GEM and A+G, respectively) (Fig. 3.12).

Although the pattern of whole-body cortisol content was similar between the water-borne (Fig. 3.4) and the feeding (Fig. 3.13) experiments, values in the feeding experiment were generally higher. Whole-body cortisol content was significantly reduced in all female-

treated fish, except in GEM-treated group compared with the control ($p < 0.001$, 0.002, and 0.695, for ATV, GEM and A+G, respectively) (Fig. 3.13), and a One-way ANOVA detected significant differences when comparing GEM to ATV and A+G ($p = 0.004$, 0.034, for ATV an A+G, respectively). The males exhibited similar trend as the females although differences with the control are relatively less and a significant reduction was seen only in the ATV-treated fish ($p < 0.048$, 0.193, and 0.091, for ATV, GEM and A+G, respectively) (Fig. 3.13).

Whole-body testosterone and estradiol levels were generally similar although patterns of change with drug-treatment were different between the two experiments (water-borne, Fig. 3.5; feeding, Fig. 3.14). Testosterone content was significantly reduced in all drug-treated female fish compared to the control ($p < 0.001$, <0.001, and <0.001, for ATV, GEM and A+G, respectively). However, testosterone levels in males were unaffected by drug-treatments (Fig. 3.14). Whole-body estradiol was significantly reduced in all drug-treated female fish compared with the control ($p = 0.005$, 0.039, and 0.043, for ATV, GEM and A+G, respectively) but these differences were very much reduced compared to the water-borne experiment (Fig. 3.5). Estradiol contents were significantly reduced in ATV and A+G, but not in GEM-treated fish compared to the control ($p = 0.004$, 0.202, and 0.006, for ATV, GEM and A+G, respectively).

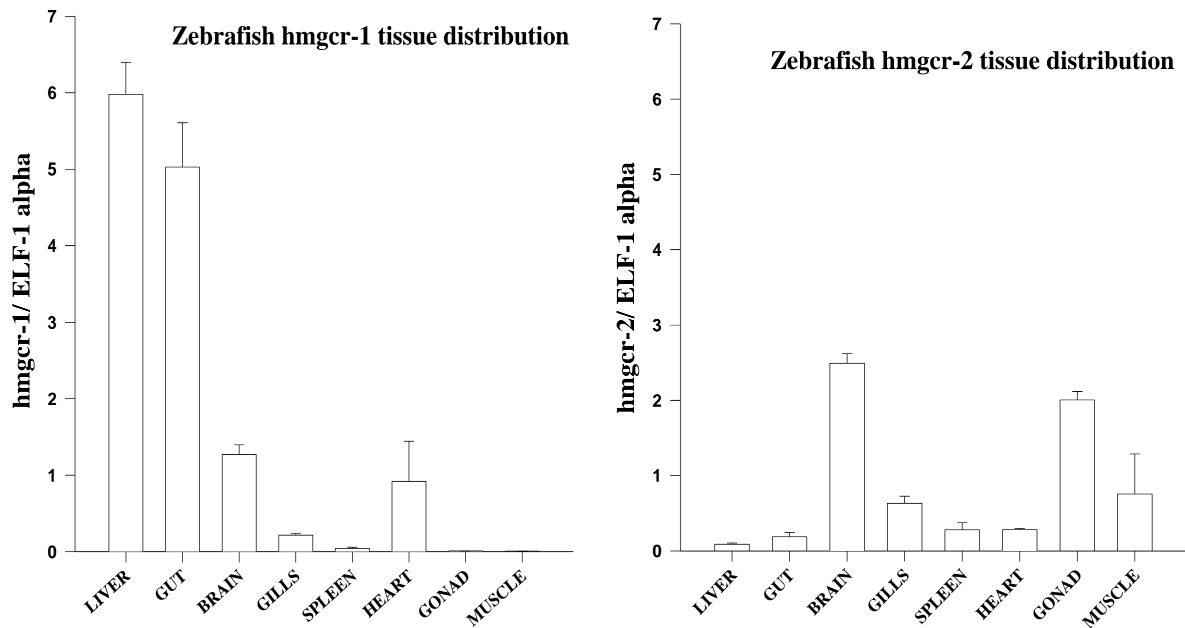


Figure 3.1. Tissue distribution of (A) hmgcr-1 and (B) hmgcr-2 mRNA abundance in various tissues of adult female zebrafish using qPCR, (n=2).

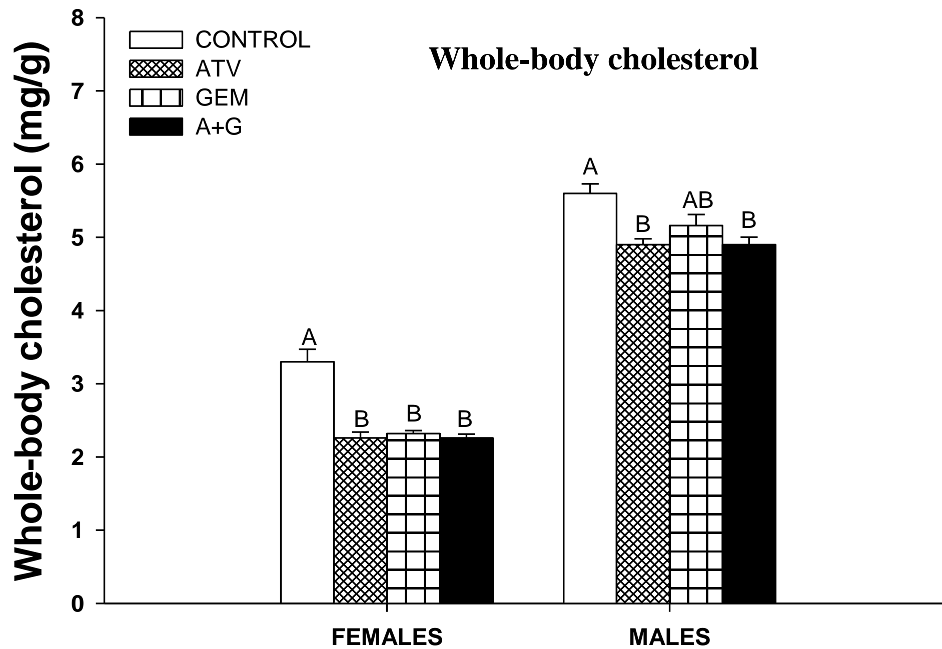


Figure 3.2. Effects of Atorvastatin (ATV, 45 μg/L), Gemfibrozil (GEM, 1.5 mg/L), and the combination of the two drugs (A+G) on whole-body cholesterol in male and female adult zebrafish following a 30 day water-borne exposure to the drugs. Control fish were exposed to DMSO. Data represent means + SEM; n = 10 fish per treatment group. Different letters denote statistical significance compared to the control ($p < 0.05$; One-Way ANOVA with a Tukey's post-hoc test).

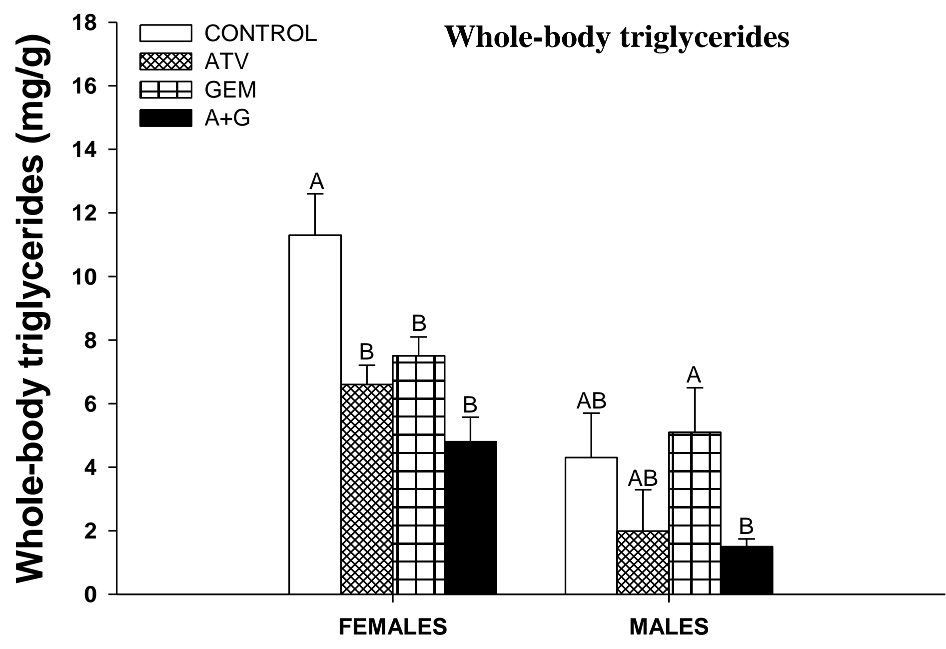


Figure 3.3. Effects of water-borne Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body triglyceride content of male and female adult zebrafish exposed for 30 days to the drugs. See Fig. 3.2 legend for details.

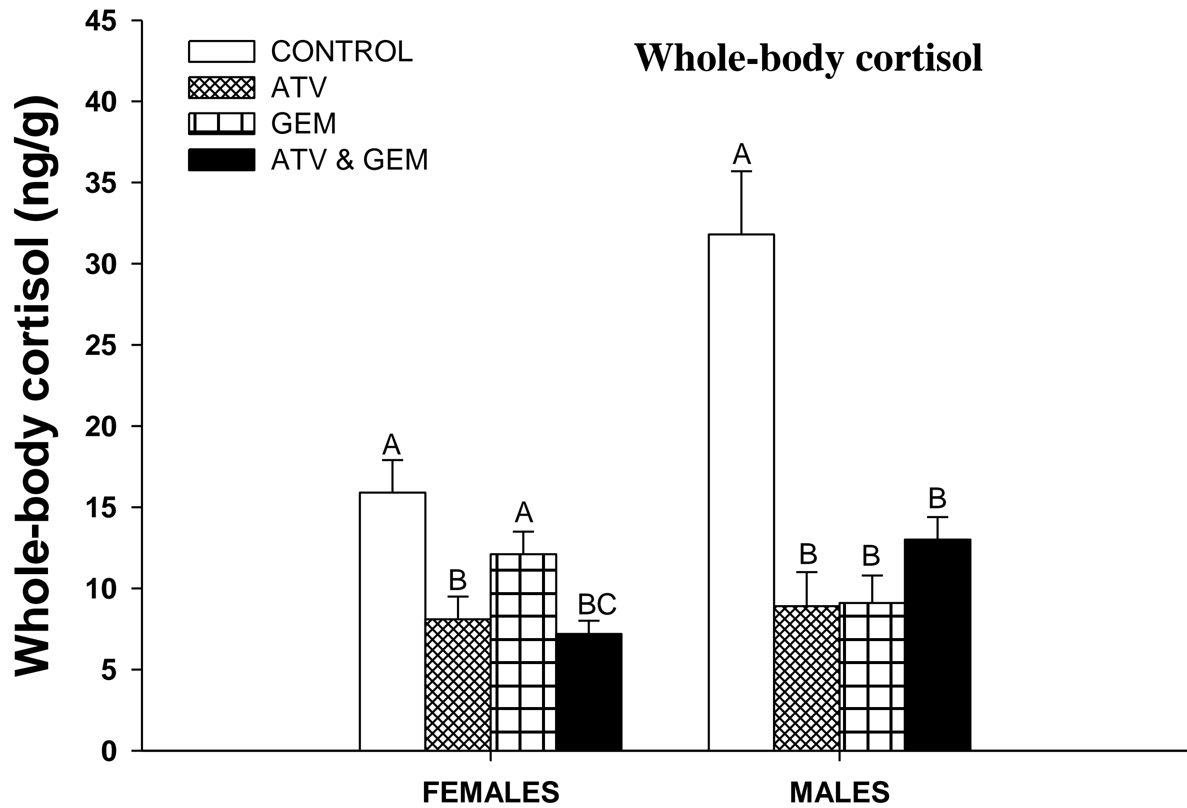
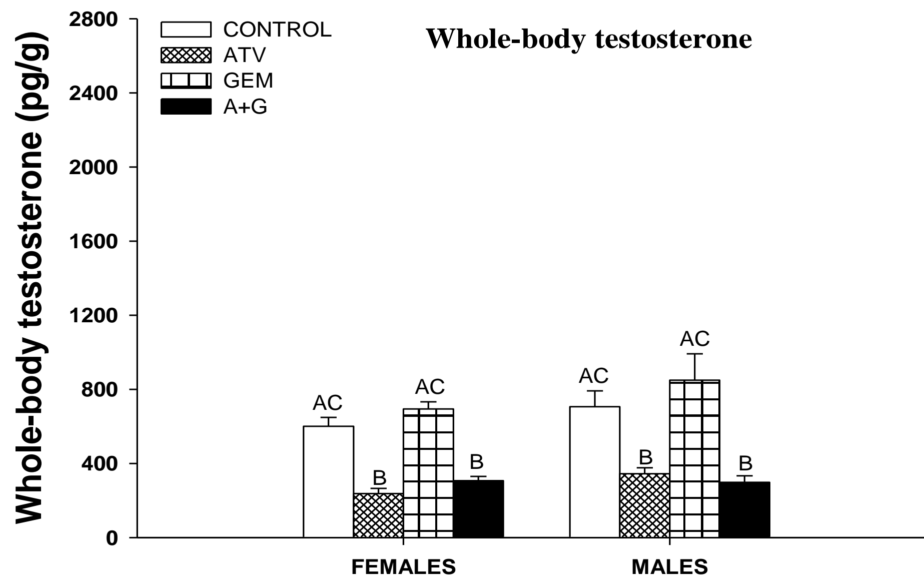


Figure 3.4. Effects of water-borne Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body cortisol content of male and female adult zebrafish exposed for 30 days to the drugs. See Fig. 3.2 legend for details.



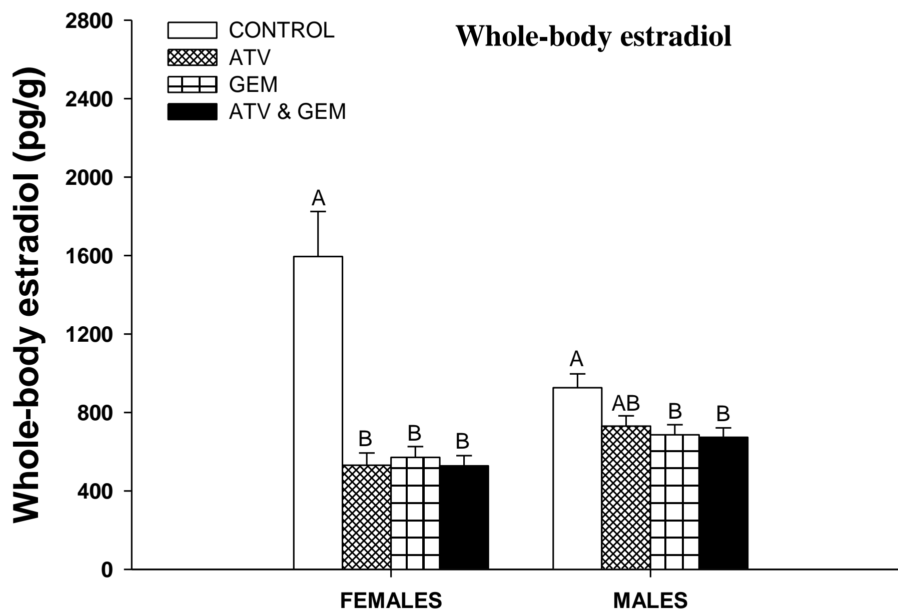


Figure 3.5. Effects of water-borne Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body testosterone and estradiol contents of male and female adult zebrafish exposed for 30 days to the drugs. See Fig. 3.2 legend for details.

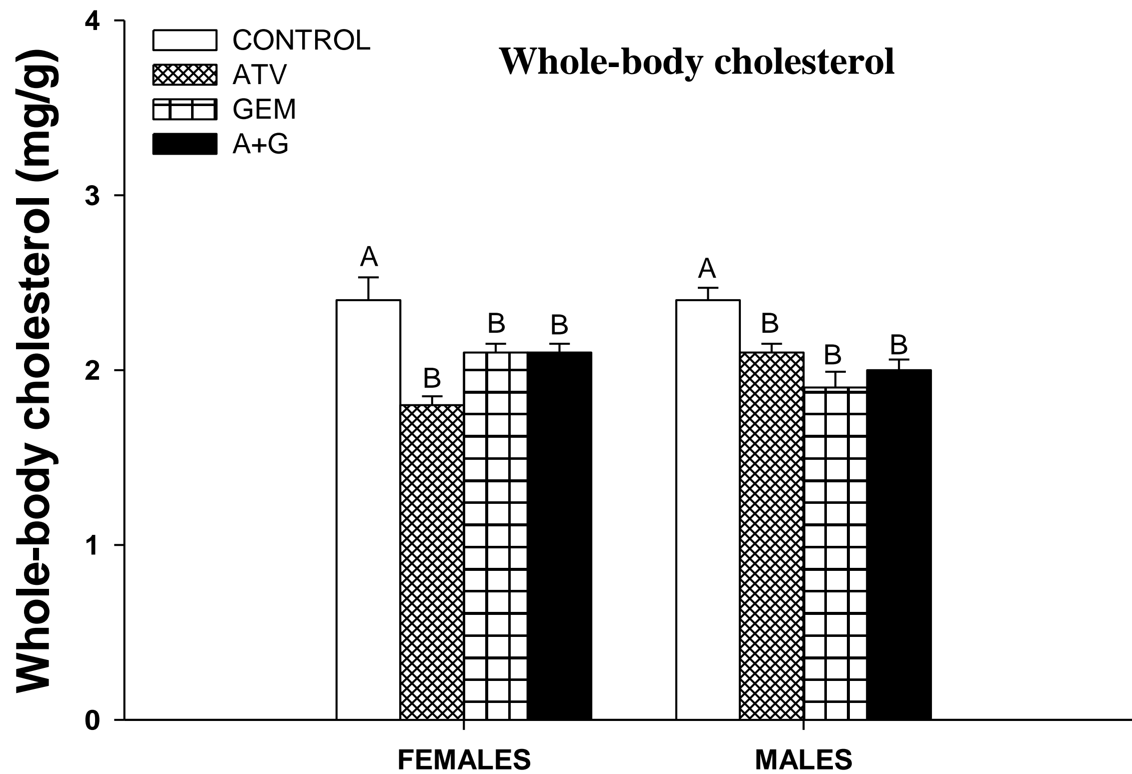


Figure 3.6. Effects of feeding Atorvastatin (ATV, 0.53 µg/g), Gemfibrozil (GEM, 16 µg/g), and the combination of the two drugs (A+G) on whole-body cholesterol contents in male and female adult zebrafish. Control fish were fed food sprayed with 95% ethanol. Fish received food at 1% body weight per day for 30 days. Data represent means + SEM; n = 10 fish per treatment group. Different letters denote statistical significance compared to the control ($p < 0.05$; One-Way ANOVA with Tukey's post-hoc test).

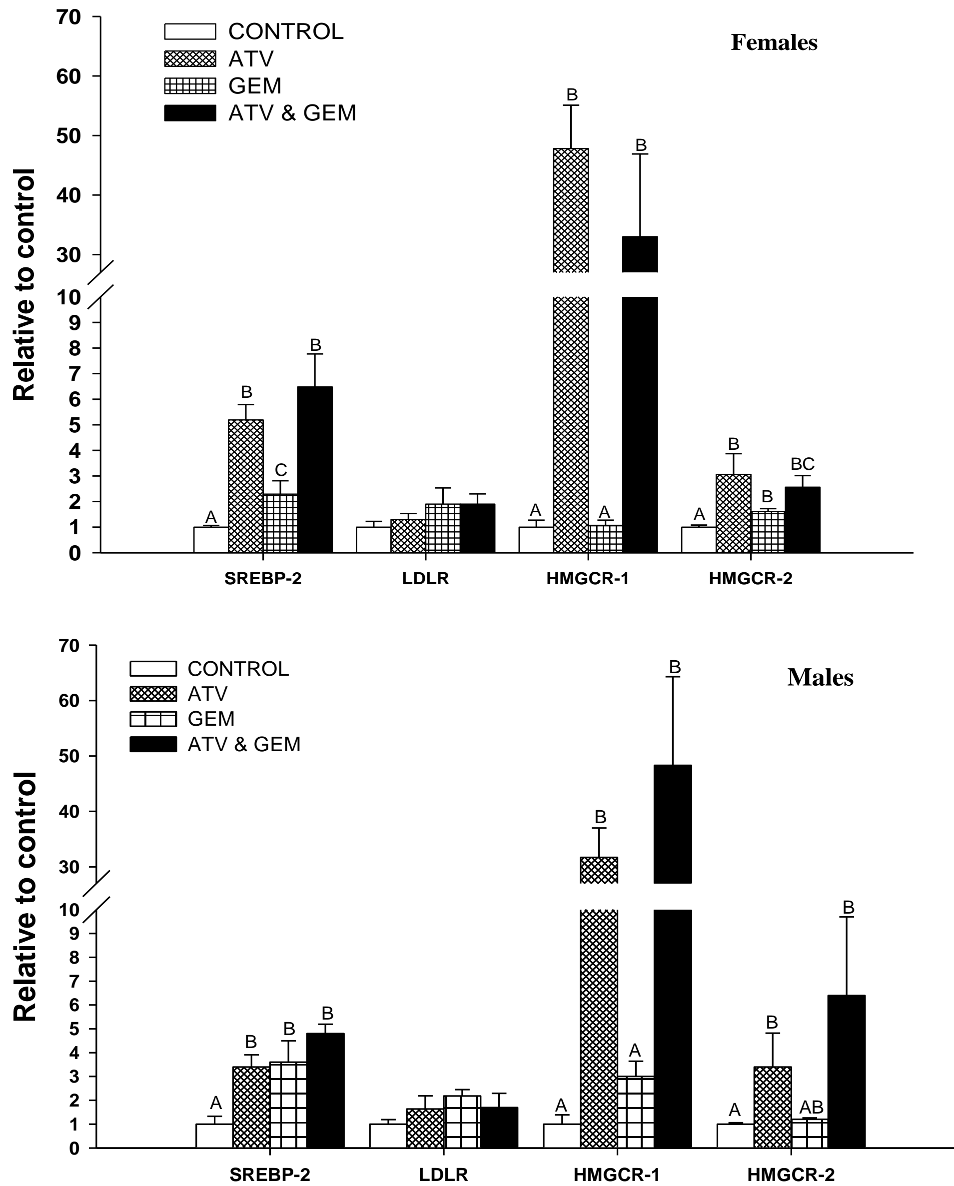
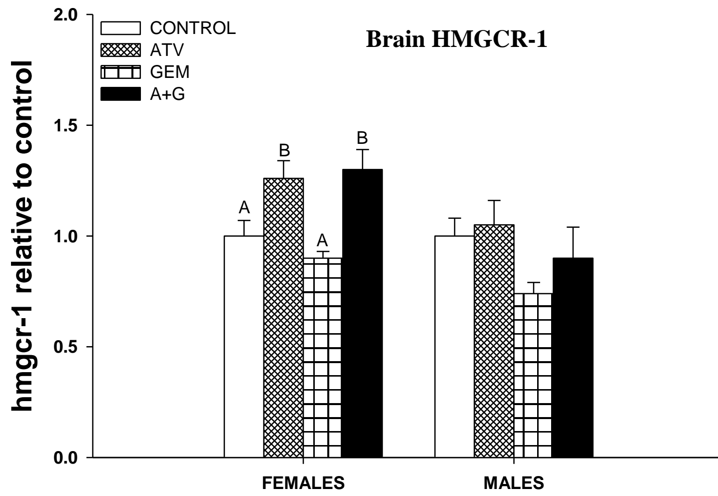


Figure 3.7. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on liver SREBP-2, LDLR and hmgcr-1 and -2 transcript abundance in male and female adult zebrafish. See Fig. 2.6 legend for details. Data represent means + SEM; n = 5 fish per treatment group). Different letters denote statistical significance compared with the control ($p < 0.05$; One-Way ANOVA with a Student-Newman-Keuls post-hoc test, SNK)



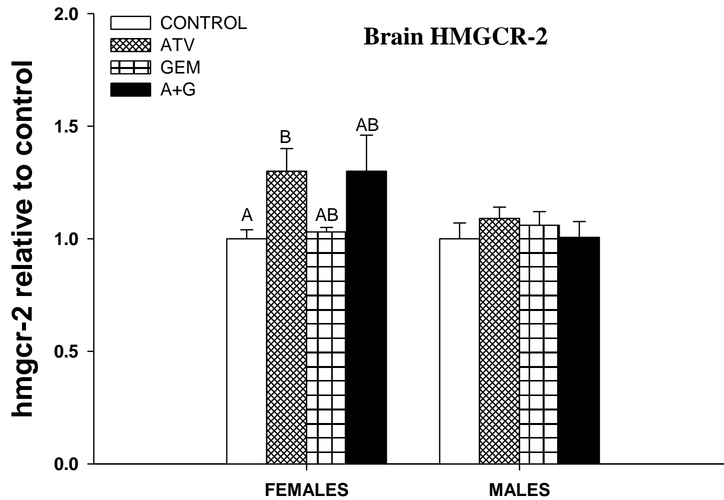


Figure 3.8. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on brain hmgcr-1 and -2 transcript abundance in male and female adult zebrafish. See Fig. 3.6 and 3.7 legends for details.

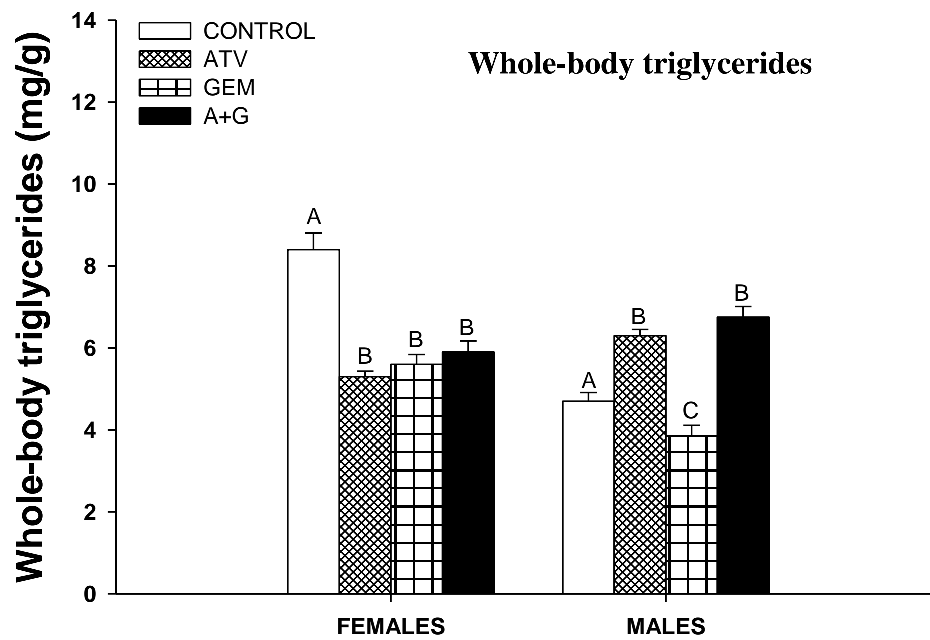


Figure 3.9. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body triglyceride content of male and female adult zebrafish. See Fig. 3.6 legend for details.

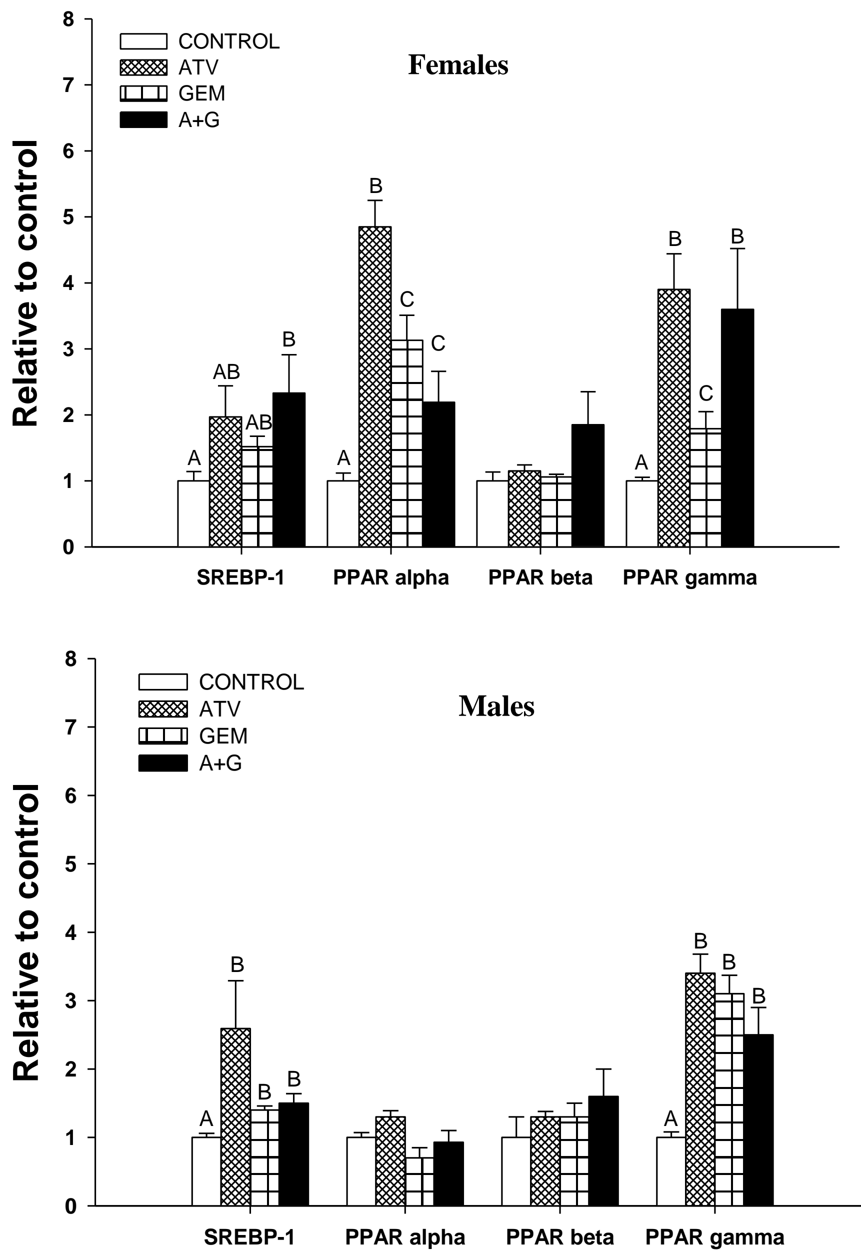


Figure 3.10. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on liver SREBP-1, PPARα, PPARβ and PPARγ transcript abundance in adult male and female zebrafish. See Fig. 3.7 legend for details.

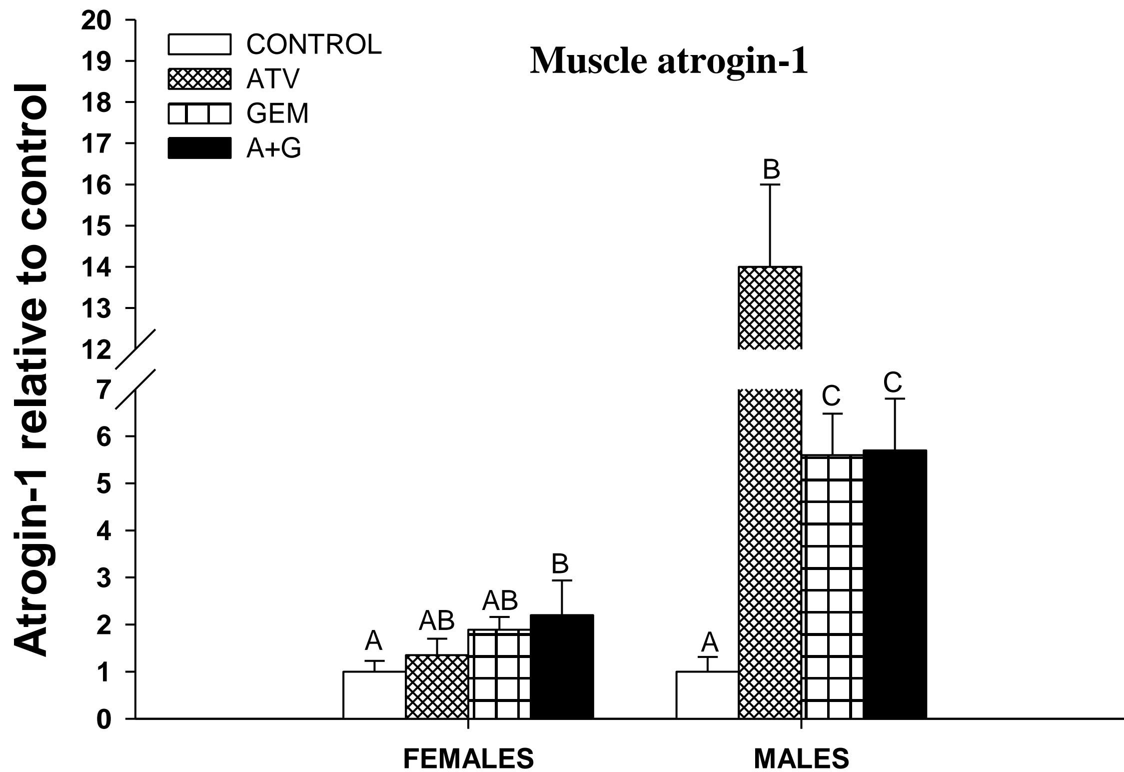


Figure 3.11. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on skeletal muscle atrogin-1 transcript abundance in adult male and female zebrafish. See Fig. 3.7 legend for details.

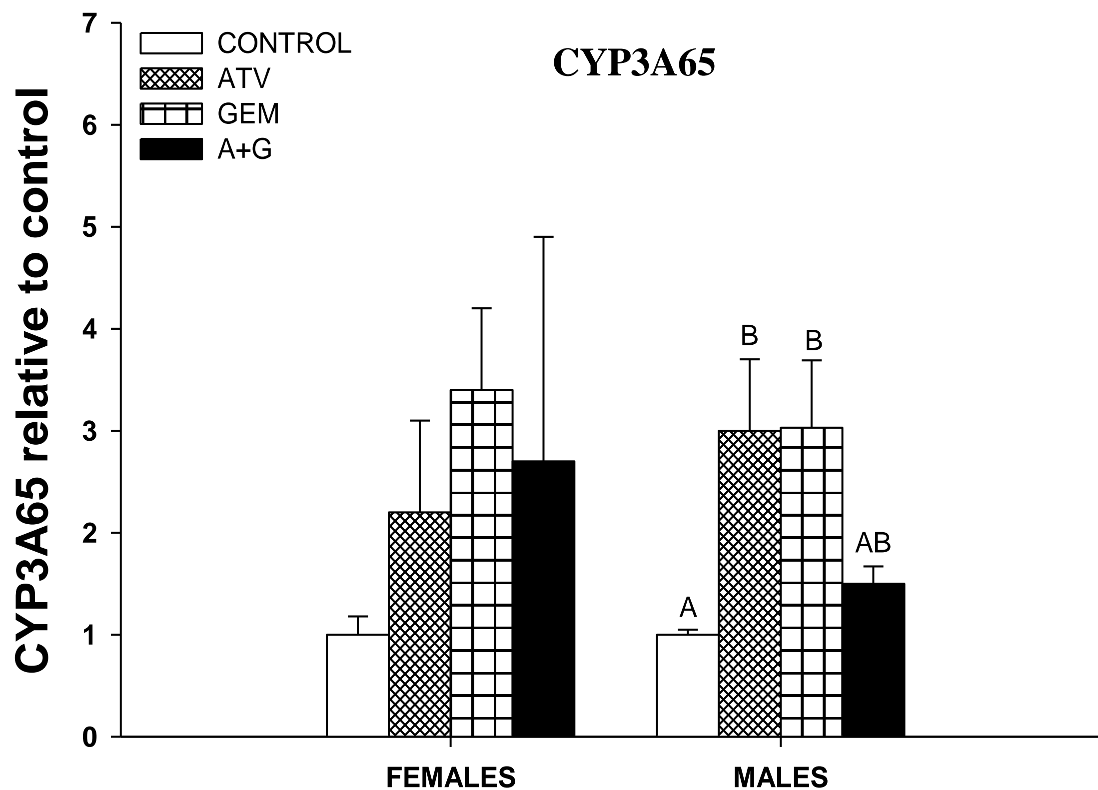


Figure 3.12. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on liver cyp3a65 transcript abundance in adult male and female zebrafish. See Fig. 3.7 legend for details.

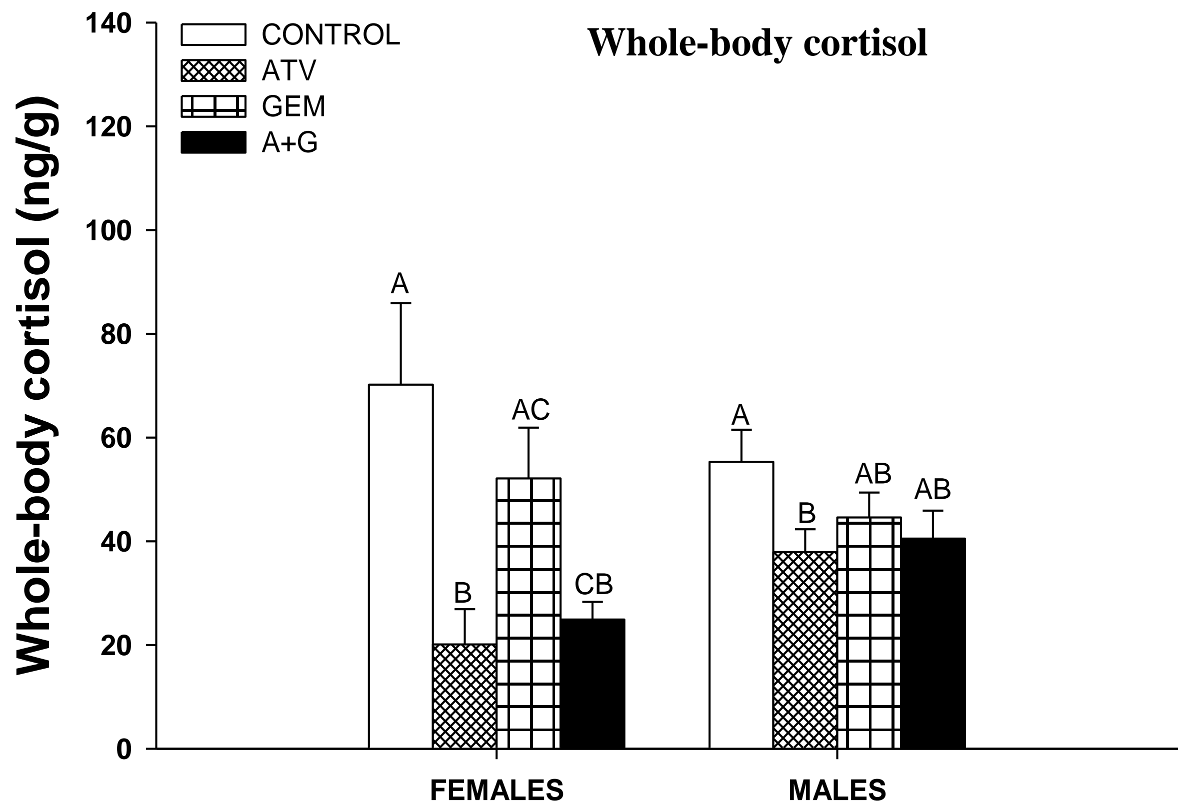


Figure 3.13. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body cortisol content in adult male and female zebrafish. See Fig. 3.6 legend for details.

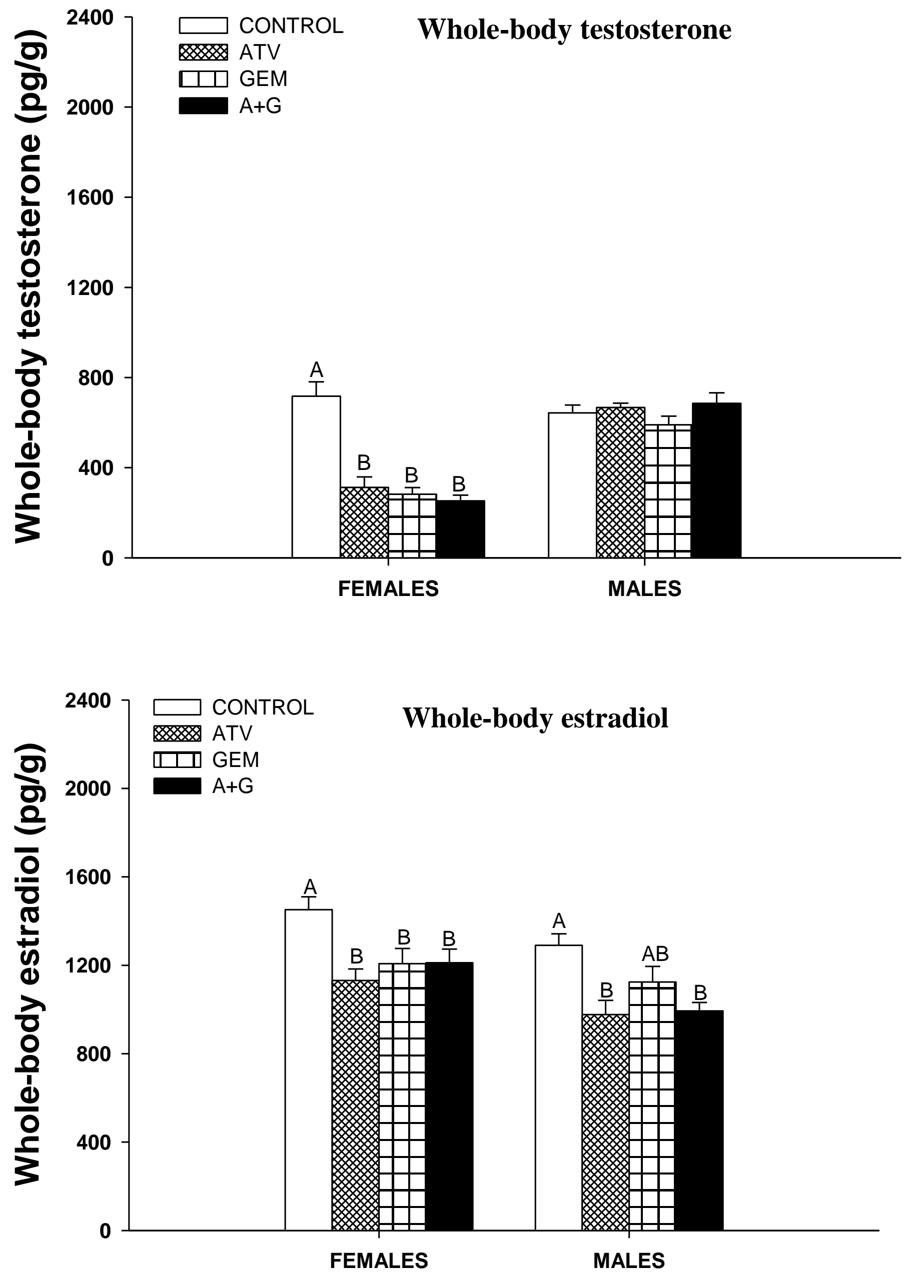


Figure 3.14. Effects of feeding Atorvastatin (ATV), Gemfibrozil (GEM), and the combination of the two drugs (A+G) on whole-body testosterone and estradiol contents in adult male and female zebrafish. See Fig. 3.6 legend for details.

3.4. Discussion

Exposing zebrafish either through food or in the tank water resulted in similar but not identical drug-induced biochemical and physiological responses. These similar responses support the ability of both ATV and GEM crossing the gut and the gill epithelia, respectively. Additionally, these similar findings justifies following the molecular endpoints only in the feeding experiment.

Whole-body cholesterol content was significantly reduced in all drug-exposed male and female adult zebrafish compared with the unexposed controls (Fig. 3.6). This effect was independent of drug delivery, except that cholesterol in the males-treated with GEM was not affected by this drug (Fig. 3.2). Furthermore, feeding male zebrafish bezafibrate (a fibrate drug) resulted in reduced plasma cholesterol after 7 and 21 days of exposure (Velasco-Santamaria et al., 2011). This reduction in cholesterol coincided with significant elevations in transcript abundance of both liver hmgcr forms (Fig. 3.7). The largest elevation was observed in hmgcr-levels in fish fed ATV or the combination of A+G with increases exceeding 30-50 fold compared with the control fish. Hmgcr-2 transcripts were up-regulated only about 3-fold (Fig. 3.7). These results are consistent with the results from the hmgcr tissue distribution (Fig. 3.1), where hmgcr-1 transcripts were highest in liver and hmgcr-2 was poorly expressed in the liver but highest in the heart compared to the rest of the tissues, suggesting that hmgcr-1 is more specific to liver where cholesterol and lipoproteins are synthesized as previously noted in mammals (D'Amico et al., 2007) and the rainbow trout (Estey et al., 2008). The precise roles of the two Hmgcr forms is not clear, although a study examining the two Hmgcr isoforms in humans reported that the Hmgcr-2 protein responded

to a range of statins less effectively than Hmgcr-1 (Karthik et al., 2012). The changes noted in transcript levels of the hmgcrs to the drugs were similar in both males and females.

Moreover, ATV is lipophilic and like other statins - depending on their lipophilicity - is known to cross the blood brain barrier (BBB) (Cibickova, 2011). Brain hmgcr-1mRNA transcript levels were significantly up-regulated in female zebrafish treated with ATV or the A+G combination compared with the control, but levels were not affected in GEM-treated fish (Fig. 3.8). Brain hmgcr-2 transcript abundance also increased in the females treated with ATV and the A+G combination but only significantly in the ATV-treated group (Fig. 3.8). Brain hmgcr-1 and -2 expression levels in male zebrafish were not significantly changed compared with the control (Fig. 3.8). The significance of these 2 forms in the fish brain is also unknown, but the mammalian brain contains the most cholesterol of all body organs and HMGCoAR is found in glial cells and is important for cholesterol homeostasis (Suzuki et al., 2010).

Furthermore, sterol regulatory element binding protein-2 (SREBP-2) mRNA transcript abundance was statistically up-regulated in all treated male and female zebrafish (Fig. 3.7). In mammals, hepatic SREBP-2 is a nuclear transcription factor that binds to the endoplasmic reticulum and is regulated by cholesterol content (Horton et al., 2002). SREBP-2 is regulated in a complex fashion in mammals (Brown et al., 2002; Sakai et al., 1996; Sun et al., 2007; Wang et al., 1994) but ultimately it regulates the transcription of HMGCoAR and LDLr, key proteins in cholesterol biosynthesis and uptake, respectively (Yokoyama et al., 1993). When cells are loaded with cholesterol, SREBP-2 translocation and processing is blocked and cholesterol synthesis declines (Sun et al., 2007). The present study shows that zebrafish hmgcrs mRNA transcript abundance in the liver is modulated by feeding zebrafish

ATV and GEM and these changes are correlated to changes in the levels of SREBP-2. These results support the involvement of zebrafish hmgcrs in cholesterol biosynthesis in the liver and its regulation by SREBP at the transcriptional level, as demonstrated in mammals.

Whole-body triglyceride content was significantly reduced in all female drug-treated fish regardless of the route of exposure (food, Fig. 3.9; water-borne, Fig. 3.3) lending credence to the fact that zebrafish could be affected by the drugs through feeding or exposure through water. In male zebrafish, whole-body triglyceride was significantly reduced in GEM-treated zebrafish, and significantly elevated in fish that were treated with ATV and with the combination of A+G when using feeding method (Fig. 3.9). Male zebrafish in ATV and A+G that were exposed through water showed a non-significant reduction in whole body triglyceride content compared with the control group as opposed to GEM-exposed group (Fig. 3.3). Possibly, the elevation of triglyceride in the A+G group resulted from the treatment of ATV and not GEM, since fish treated with GEM alone resulted in reduced whole-body triglyceride in both males and females, in both routes of exposure. The elevation of triglyceride in the ATV group was reported in a previous study performed to assess Fluvastatin (another statin drug) treatment in human subjects (Marz et al., 2001). Moreover, *in vitro* evidence from HepG2 cells treated with three different statins (including ATV) also noted an increase in triglyceride levels (Scharnagl et al., 2001). It seems that female zebrafish are more prone to the effect of ATV than the males when it comes to triglycerides, possibly due to the importance of these components for egg production.

PPARα mRNA transcript levels were not significantly different in any treated male fish compared with the control (Fig. 3.10), which was consistent with the triglyceride results.

PPARα mRNA levels in the females were significantly up-regulated in all treated fish compared with the control (Fig. 3.10). In zebrafish PPARα is predominantly expressed in the liver, kidney and pancreas, where it controls fatty acid metabolism (Ibabe et al., 2002). For instance, in response to endogenous fatty acids or exogenous synthetic ligands (such statins and fibrates), PPARα activates fatty acid catabolism through promoting triglyceride breakdown and fatty acid β-oxidation by inducing the expressions of acyl-CoA oxidase and carnitine palmitoyl transferase-1A (van Raalte et al., 2004; Wang and Wong, 2010). Moreover, statins are reported to up-regulate PPARα in other cell types, such as human HepG2 hepatoma cells (Martin et al., 2001) and mice peritoneal macrophages (Paumelle et al., 2006). The lack of changes in PPARα mRNA abundance in male zebrafish was reported previously in other fish species (goldfish) treated with GEM (Mimeault et al., 2006).

Moreover, PPARγ mRNA transcript abundance was significantly up-regulated in both male and female zebrafish in all treatment groups (Fig. 3.10). PPARγ in zebrafish, which is highly abundant in adipose tissues, the intestine, kidney, and the liver, triggers cellular differentiation, promotes lipid storage and modulates the action of insulin (Ibabe et al., 2005; Ibabe et al., 2002). Moreover, SREBP-1 mRNA abundance was significantly elevated in all male-treated zebrafish (Fig. 3.10). SREBP-1 mRNA abundance was elevated in all female-treated zebrafish, but only significantly in the (A+G)-treated group (Fig. 3.10). SREBP-1 activates transcription of genes involved in fatty acid and triglyceride synthesis, such as the genes encoding acetyl-CoA carboxylase, fatty acid synthase (Passeri et al., 2009). The elevation of male's SREBP-1 mRNA levels was unexpected, especially in the ATV and (A+G) groups since whole-body triglycerides were elevated in these two groups (Fig. 3.10).

Atrogin-1 mRNA abundance was significantly elevated in all male-treated fish (Fig. 3.11), and in all female-treated fish, but only significantly in (A+G)-treated group (Fig. 3.11). Atrogin-1 mRNA expression levels in males-treated with ATV were approximately 10-fold higher compared to the control, indicating that the impact of the drug is significant in the males as opposed to the females. Statin treatments induced marked induction of atrogin-1 expression in human skeletal muscle, cultured muscle cells, and zebrafish (Hanai et al., 2007); the authors demonstrated that, in the absence of atrogin-1, cells and animals are resistant to the toxic effects of statins, which led them to conclude that atrogin-1 mediates part of the effects of statins on muscle damage (Hanai et al., 2007).

Liver CYP3A65 mRNA abundance was significantly elevated in male-treated zebrafish (Fig. 3.12). In female zebrafish, liver CYP3A65 was elevated but not statistically compared with the control (Fig. 3.12). There is a lack of information regarding the impact that pharmaceuticals have on liver function. Zebrafish exposed to different fluoxetine concentrations did not change CYP3A65 mRNA transcripts, but there was a reduction of CYP3A65 mRNA transcripts exposed to 10 ng/L ethinylestradiol (EE2) (Lister et al., 2009). In the present study, CYP3A65 mRNA abundance in male livers was significantly up-regulated as opposed to that of females, suggesting that the CYP3A65 in the males are more sensitive to pharmaceuticals than the females. Sexually dimorphic rates of metabolism are reported for a killifish (*Fundulus heteroclitus*), where Hegelund and Celander (2003) found that CYP3A mRNA and protein expression was 2.5-folder higher in male killifish compared to females. This is in agreement with the finding in the current study with zebrafish. Even though CYP regulation in fish is not completely understood, CYPs are often under hormonal control and differences between males and females are commonly reported (Andersson and

Forlin, 1992; Gray et al., 1991). Moreover, these differences can be more apparent during breeding seasons (Devaux et al., 1998).

Whole-body testosterone content was significantly reduced in all female-treated fish but not in male-treated fish compared with the control when exposing the fish through food (Fig. 3.13); however, exposing the fish through water resulted in the reduction of whole-body testosterone in all exposed fish except when fish exposed to GEM alone (Fig. 3.5). Furthermore, whole-body estradiol content was significantly reduced in nearly all female and male-treated fish exposed through food (Fig. 3.14) or through water (Fig. 3.5). These results, suggest that female zebrafish are more sensitive to the drug treatment compared to the males, where the reduction of estradiol content being 33% of the control values in female fish exposed to A+G (through water). Velasco-Santamaria and colleagues (2011) reported a significant reduction in plasma 11-ketotestosterone (11-KT) in male zebrafish fed bezafibrate. The reduction of sex steroids might be a direct consequence of the reduction of cholesterol, since cholesterol is the precursor of sex steroids.

Whole-body cortisol was reduced in all female-treated fish (Fig. 3.14) but only statistically significant compared with the control in ATV and (A+G) group but the reduction was not significant when treated the fish with GEM alone. Furthermore, there was a similar reduction in whole-body cortisol in the male-treated fish (Fig. 3.14), but the reduction was only significant in the ATV treated male fish.

3.5. Conclusions

The present results suggest that lipids and steroids metabolism as well as several mRNA transcripts are affected in zebrafish as a consequence of the exposure and feeding of lipid lowering drugs. The reduction of cholesterol resulted in compromising the ability of

these fish to mount a suitable stress response by secreting cortisol in the circulation upon exposure to stressor. Furthermore, these fish may have had reduced reproduction potential as sex steroids were reduced in a manner consistent with the reduction of cholesterol. This study suggests that the wellbeing of the fish was affected as these fish showed a sign of skeletal muscle breakdown which an indication of rhabdomyolysis.

CHAPTER 4

The effects of atorvastatin on lipid metabolism in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes

4.1. Introduction

Cholesterol, generally considered as a major risk factor for arteriosclerosis in humans, is nonetheless a crucial component of all eukaryotic membranes, as well as being an important precursor of steroid hormones and bile acids. Cholesterol confers rigidity to lipid bilayers thus affecting their fluidity, permeability and thickness. It is essential for the functionality of the membranes and many membranes-associated proteins (Burger et al., 2000; Maxfield and Tabas, 2005). Body cholesterol originates from both the diet and from *de novo* synthesis, where the liver and intestines are the key organs active in cholesterol biosynthesis (Kruit et al., 2006). Cholesterol biosynthesis occurs through the mevalonate pathway that also generates important intermediates that are used in the synthesis of ubiquinone and isoprenylated proteins. The first steps of cholesterol synthesis involve the ultimate conversion of three molecules of acetyl-CoA into one 3-hydroxy-3-methylglutaryl-CoA (HMGCoA), which occurs in the cytoplasm (Goldstein and Brown, 1990). HMG-CoA is converted to mevalonate by HMG-CoA reductase (HMGCoAR; E.C. 1.1.1.88) that is associated with the endoplasmic reticulum. This is the rate limiting step, and is subject to complex regulation. High levels of cholesterol exert control by feedback inhibition and by stimulating HMGCoAR ubiquitination and degradation by the proteasome pathway (Song et al., 2005).

The cellular content of cholesterol must be maintained within narrow limits to prevent its over-accumulation and abnormal deposition within the body which may result in

arteriosclerosis. Arteriosclerosis is the leading contributory factor in coronary artery disease in North America and is considered one of the leading causes of cardiovascular death and morbidity (Boden et al., 2007; Choy et al., 2004).

Pharmaceuticals that modulate lipid metabolism are frequently prescribed in the developed world and are intended to decrease the concentration of cholesterol and triglycerides in the circulation. Drugs belonging to this therapeutic class can be divided into two main groups: statins and the group most frequently detected in the environment, fibrates (Miao and Metcalfe, 2003a).

Statins such as atorvastatin (ATV) act by inhibiting HMGCoAR an enzyme that is involved in feedback control of cholesterol synthesis. Statins specifically target the liver and compete with the normal substrate for the active site of the enzyme that result in conformation changes of the enzyme when it binds to the active site leading to the prevention of HMGCoAR from attaining a functional structure (Blumenthal, 2000). In response, the number of low density lipoprotein (LDL) receptors at the hepatocyte surface increases, thus lowering circulating LDL cholesterol (Blumenthal, 2000). Statins were found in untreated sewage samples at concentrations between 4 and 117 ng/L and in treated sewage samples at 1-59 ng/L (Miao and Metcalfe, 2003a; Miao and Metcalfe, 2003b). Additionally, statins were also detected in surface water at concentration of 15 ng/L (Metcalfe et al., 2004; Miao and Metcalfe, 2003a) and drinking water (Benotti et al., 2008).

Gemfibrozil (GEM), a lipid-regulating agent belongs to the fibrate class of pharmaceuticals that is specifically prescribed to reduce elevated triglyceride levels in the circulation. Fibrates activate specific transcription factors belonging to the nuclear receptor superfamily, known as peroxisome proliferator-activated receptors (PPARs) (Staels et al.,

1998). Upon activation as a consequence of GEM administration, changes in gene expression of key enzymes involved in the biosynthesis of several apoliproteins and lipoprotein metabolism are observed (Gervois et al., 2000; Staels et al., 1998) ultimately resulting in the reduction of plasma triglyceride concentration and the elevation of high density lipoprotein cholesterol (HDL) levels (Staels et al., 1998). There are three PPARs related to different cellular events. PPAR-α and PPAR-β play key roles in catabolism and storage of fatty acids while PPAR-γ plays an important role in cellular differentiation (Bishop-Bailey, 2000). The three PPAR isoforms found in mammals (α, β, and γ) have been identified in numerous fish species (Leaver et al., 2005; Leaver et al., 2007; Morash et al., 2008). GEM have been detected in several environmental sample at the concentrations of 1500 ng/L in the surface water and 2100 ng/L in WWTP effluent (Metcalfe et al., 2004).

Two isoforms of HMGCoAR enzyme have been identified in rainbow trout (*Oncorhynchus mykiss*) (Estey et al., 2008). mRNA abundance of HMGCoAR-1 was primarily seen in the liver, while HMGCoAR-2 mRNA was ubiquitously expressed in all tissues examined, but at much lower levels than HMGCoAR-1 (Estey et al., 2008).

The main aim of this study is to investigate the potential effects of ATV and GEM on the biosynthesis of cholesterol and other lipids *in vitro*, using freshly isolated rainbow trout hepatocytes. A comprehensive investigation of cholesterol dynamics and metabolism in the rainbow trout will be valuable in determining the effect of pharmaceuticals, in particular ATV and other related drugs, on fish. I hypothesize that exposing isolated rainbow trout hepatocytes to ATV will significantly reduce cholesterol and other lipids produced by these cells. Concurrently, the genes that are related to cholesterol and lipid homeostasis will be up-

regulated in order to compensate for the reduction of these lipids, especially when GEM is used in the combination with ATV.

4.2. Materials and Methods

4.2.1. Chemicals and standards

[1-^{14}C]-Acetic acid sodium salt (58.0 mCi/mmol) in aqueous solution was purchased from GE Healthcare (Amersham, UK), TLC mixed lipid standards were purchased from Nu-check prep, Inc. (Elysian, MN, USA). Atorvastatin was a gift from Pfizer Pharmaceutical Inc. All other chemicals and drugs were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise specified.

4.2.2. Hepatocytes preparation and isolation

Hepatocytes from juvenile rainbow trout (200-250 g) were isolated according to (Mommsen et al., 1994). Briefly, immature rainbow trout were killed with a lethal dose of benzocaine (200 mg/L). A blood sample was removed from the caudal vein with a 23-gauge needle attached to an EDTA-coated syringe; this step was performed to reduce the amount of blood in the fish hence to reduce the time and the volume of solution used to clear the blood from the liver. A ventral incision was made in the fish to expose the posterior cardinal vein. The vessel was nicked and a PE 50 cannula was inserted. The liver was first perfused (at 1.5 mL/min) with a modified Hanks' solution (**Solution 1**: 136.9 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 5.0 mM $NaHCO_3$, 10 mM HEPES, pH 7.63) to remove the blood remaining in the liver using a peristaltic pump (Gilson Minipuls^{TM}3 Peristaltic Pump, Mandel Scientific Co Inc, Guelph, ON Canada). After the liver was cleared, the perfusate was switched to **Solution 2**, consisting of 100 mL of Solution 1 supplemented with 15 mg of collagenase (from *Clostridium histolyticum*, type IV)

at room temperature. The liver was perfused for about 15 min (depending on the size of the liver) with occasional massaging to increase hepatocyte yield. After this step, the liver was carefully removed from the body cavity, the gall bladder carefully removed, and then the liver was finely chopped with a razor blade. The cell suspension was filtered through a series of two plankton nets (150 and 75 mm), rinsed three times with **Solution 3** consisting of 100 mL of Solution 1 supplemented with 1.5 mM $CaCl_2$, and 1% bovine serum albumin (BSA). After rinsing the hepatocytes were re-suspended in culture medium (100 mL **Solution 3** supplemented with 1.25 mL 100X MEM non-essential amino acids, 2.5 mL 50X MEM essential amino acids, and 1.0 mL 100X antibiotic-antimycotic solution; GIBCO, Burlington, Ont.) for 1 h before the start of the exposure. Cells were counted using a hemocytometer, weighed and viability evaluated using the Trypan Blue exclusion test (Mommsen et al., 1994). Only cell preparations with over 95% viability were used.

4.2.3. Exposure to ATV

Radiolabeled acetate was prepared fresh before each exposure as following: [^{14}C]-acetate was taken to dryness under a stream of N_2, and then dissolved in 0.5 M acetate to give an activity of 0.31 μCi/10 μL.

The effect of ATV on lipid synthesis was studied *in vitro* using freshly isolated trout hepatocytes (25-30 mg/mL) and the radiolabeled precursor acetate [^{14}C]-acetate. Hepatocytes were incubated with 0.31 μCi/mL [^{14}C]-acetate and treated with either 4.5 or 45 μg/L ATV; these concentrations of ATV represent 100- and 1000-times the reported environmental concentration (Metcalfe et al., 2004), respectively. Hepatocytes were pre-incubated with the different ATV concentrations (4.5 or 45 μg/L) for 30 min before the addition of [^{14}C]-acetate. The control hepatocytes were exposed to DMSO only (final

concentration was 0.01%). The hepatocytes were incubated for 3 or 6 h. The effect of ATV was evaluated by comparing the incorporation of ^{14}C-acetate into different lipid fractions between the control and ATV-treated hepatocytes. All the incubations were carried out in glass 20 mL scintillation vials, in a metabolic shaker (~60 strokes/min) at 13°C, in duplicate.

4.2.4. Non-Radioactive exposure

Another experiment was performed to investigate the combined effects of ATV and GEM on the abundance of selected mRNA transcripts that are involved in cholesterol biosynthesis [(hmgcr, srebps, and ldlr), biotransformation (cyp3a27), and peroxisomal proliferation-activated receptors (PPAR-α, PPAR-β, and PPAR-γ)]. Hepatocytes were exposed for a period of 3 or 6 h to 45 μg/L and 150 μg/L, ATV and GEM, respectively individually or in combination. At the end of exposure period, hepatocytes were collected, washed three times with PBS, and stored at -80°C until analyzed.

4.2.5. $^{14}CO_2$ collection

The $^{14}CO_2$ generated as a result of [^{14}C]-acetate metabolism in trout hepatocytes was collected according to the method of French et al. (1981) with minor modifications. Briefly, the hepatocyte incubation was terminated by the addition of 0.1 mL 70% $HClO_4$ through the cap. $^{14}CO_2$ was collected on Whatman glass fiber filter paper (Fisher Scientific) in a small plastic cup suspended above the reaction mixture and soaked with 0.2 mL hyamine hydroxide (injected through the cap). The stoppered scintillation vials were shaken for 1 h after which the filter papers were removed and counted in 10 mL Ultima Gold™ (PerkinElmer, Canada) liquid scintillation cocktail using a Tri-Carb® Liquid Scintillation Analyzer (PerkinElmer, Canada). The precipitate remaining at the bottom of the scintillation

vial following acidification was removed to a 2.0 mL conical plastic centrifuge tube and frozen until analyzed for radioactive glucose and glycogen.

4.2.6. Isolation of [^{14}C]-Glucose

The precipitated protein was removed from the incubation medium by centrifugation. The resulting supernatant was neutralized with 5 M K_2CO_3, and 0.5 mL of the supernatant was added to 4.5 mL 1 M glucose plus 1.5 g Amberlite MB3 mixed bed resin (Sigma-Aldrich) in a stoppered glass scintillation vials. The stoppered vials were shaken for 1 h, and then 2.0 mL was removed and centrifuged to remove any resin remaining. One mL of the resulting supernatant was counted in 10 mL of liquid scintillation cocktail and counted as above.

4.2.7. Determination of [^{14}C]-Glycogen

The pellet remaining following centrifugation was dissolved in 1.0 mL 2 M KOH by heating at 100 °C for 35 min. The solution was cooled, centrifuged and 1.0 mL supernatant was added to 2.05 mL cold 95% ethanol, and 0.5 mg of glycogen Oyster type II (Sigma-Aldrich) was added to aid the precipitation of the radioactive glycogen. The solution was left standing in the cold overnight, and then the precipitate was collected by centrifugation and washed twice with 3.0 mL cold 65% ethanol. The final precipitate was dissolved in 1.0 mL distilled H_2O and 0.5 mL added to 10 mL of liquid scintillation cocktail and counted as above.

4.2.8. Lipid isolation and fractionation

Lipid was extracted from the pellet using a modified chloroform/methanol extraction (Folch) (Folch et al., 1957) method. In brief, lipids were homogenized for 30 s in 5 mL 2:1 chloroform/methanol (Folch) with a Polytron homogenizer (Kinematica, Luzern,

Switzerland). The tubes were mixed and left to settle for 30 min in room temperature; the mixture was vortexed every 10 min to ensure mixing of hepatocyte pellet with Folch. At the end of the incubation period, 2 mL 2 M KCl buffered with 5 mM EDTA was added; the mixture was vortexed and allowed to settle for 10 min. Once settled, the bottom chloroform layer was transferred to a clean glass tube and evaporated under a stream of N_2 gas in a fumehood and the samples were re-suspended in 200 µL chloroform for use in thin-layer chromatography (TLC) to separate individual lipid classes (Bitman et al., 1981).

Samples were spotted (10 µl) on 20x20 cm Whatman LK5DF linear plates (Fisher Scientific), with (10 µL) a mixed lipid standard control (Nu-check prep, Inc., Elysian, MN, USA) run concurrently on each plate. Plates were run through two phases of development in separate chambers (Sharpe et al., 2006). Phase 1 consisted of chloroform: methanol: acetic acid (98:2:1) and the solvent was allowed to travel to 17 cm on the plate. Phase 2 consisted of hexane: ethyl ether: acetic acid (96:4:0.2) and was developed to the top of the plate. The plates were left to dry in a fumehood and areas corresponding to different lipids were identified by exposure to iodine vapor and marked. The plates were left in the fumehood until the disappearance of the iodine, and then the spots corresponding to the lipids of interest were scraped from the plates into scintillation vials and counted in 10 mL liquid scintillation cocktail as above.

4.2.9. RNA isolation

RNA was isolated from hepatocytes using Trizol reagent, following manufacturer's instructions (Gibco BRL, Burlington, ON) as reported in Chapter 2.

4.2.10. Real-time quantitative RT-PCR

Transcript abundance was assessed in duplicate using quantitative real-time RT-PCR and a MX3000P® Multiplex Quantitative PCR system (Stratagene, La Jolla, CA, USA). Data were analyzed using the MX3000P Software Package. Total RNA from the hepatocyte pellet was used to synthesize cDNA. Briefly, 1 μg total RNA was DNase treated using DNase I amplification grade (Invitrogen, Burlington, ON, Canada). Following the DNase treatment, first strand cDNA synthesis was prepared by the addition of 300 ng random hexamer primers (Invitrogen), and using 200 units of Superscript® II Reverse transcriptase (Invitrogen). A relative standard curve for each primer set used was constructed by pooling dilutions of cDNA of all samples (treated and control). In each run, a standard curve was generated that was used to calculate relative changes in mRNA abundance between control and treated samples. Genes were normalized to the expression of the house-keeping gene β-actin. Each PCR reaction contained the following final concentrations: 10 μL Brilliant III Ultra-fast SYBR® Green QPCR Master Mix (Agilent,), 15 nM ROX reference dye, 500 nM gene-specific primers (forward and reverse), and 10 ng first-strand cDNA template, in a 20 μL reaction volume. The thermal cycling parameters were a 3 min initial denaturation step at 95°C, followed by 40 cycles of 95°C for 20 s, and 60°C for 20 s. Unless otherwise indicated, all primers were designed with OligoPerfect™ Designer (Life technologies™). Primers for SREBP-1 were adopted from (Skiba-Cassy et al., 2009), and those for HMGCR-1 and -2 were adopted from (Estey et al., 2008); primers for CYP3A27 were adopted from (Ellesat et al., 2012). Primers for PPAR-α were adopted from (Kolditz et al., 2008), for PPAR-β from (Morash et al., 2008) and for PPAR-γ from (Prindiville et al., 2011). See Table 4.1 for details.

Table 4.1. Primers used to assess mRNA transcript abundance in hepatocytes from rainbow trout, their sequences, amplicon size and GenBank accession number.

Gene	Primer	Sequence (5'→3')	Amplicon size (base pairs)	Accession number
β-actin	Forward	GGCATCCTGACCCTGAAGTA	203	AJ438158
	Reverse	GGGGTGTTGAAGGTCTCAAA		
HMGCR-1	Forward	CTCTGGCCAGGTTACAGGAG	180-200	Not Available [a]
	Reverse	TCTACCAGGGCTTCAGTGCT		
HMGCR-2	Forward	TCCCTGAGCTCCAGGTTCTA	180-200	Not Available [a]
	Reverse	CATGGCTGAGCCCACTAGAT		
SREBP-1	Forward	GACAAGGTGGTCCAGTTGCT	59	Not Available [b]
	Reverse	CACACGTTAGTCCGCATCAC		
LDLR	Forward	CAGCGAAGGACTGGAGAAAC	108	AF542091
	Reverse	TTCAGCCCACTCTTCTCGAT		
PPAR-α	Forward	CTGGAGCTGGATGACAGTGA	192	AY494835
	Reverse	GGCAAGTTTTTGCAGCAGAT		
PPAR-β	Forward	CTGGAGCTGGATGACAGTGA	195	NM_001197207
	Reverse	GTCAGCCATCTTGTTGAGCA		
PPAR-γ	Forward	ATGAACAAGGACGGCACACT	310	NM_001197212
	Reverse	GCAGTAGCTTGGCGAACAG		
CYP3A27	Forward	GAACCGGAGGAAAGATGATG	139	U96077
	Reverse	GGACCAGGGATACCCATCTTA		

[a] See Estey et al. (2008)
[b] See Skiba-Cassy et al. (2009)

4.2.11. Statistical analysis

The untreated control samples were assigned a value of 100 percent. Treated samples were then compared with untreated samples at the same incubation time to create percentage values. Experimental results are presented as the means ± standard error of the mean (SEM). Statistical analyses were conducted using SigmaPlot™ 11.0 (SPSS Corporation, Chicago, IL, USA). Log-transformed data were used wherever necessary to satisfy homogeneity of variance; data that failed the normality test after log-transformation, a Kruskal-Wallis one way ANOVA on ranks was used. All data are presented in the text as non-transformed values. Whenever appropriate, statistical significance was tested with one-way ANOVAs followed by Tukey tests (^{14}C incorporation into different molecules) or Student-Newman-Keuls (for gene expression). A value of $p < 0.05$ was accepted as significant.

4.3. Results

Hepatocyte samples collected following incubation for 3 and 6 h or following scraping from a TLC plate were tested for radioactivity. The resulting disintegrations per minute (DPM) readings were converted to nmol ^{14}C per gram hepatocytes based on the nmol of [^{14}C]-acetate added to the incubate. In general there was low incorporation of radioactivity into the molecules assessed, possibly related to the low specific activity of [^{14}C]-acetate used and the short incubation period.

[^{14}C]-Acetate incorporated into [^{14}C]-cholesterol was significantly reduced at both time points and at both 4.5 and 45 µg/L ATV (Fig. 4.1). At 3 h, both ATV concentrations significantly reduced cholesterol radioactivity compared with the control untreated hepatocytes (p = <0.001, for both 4.5 and 45 µg/L ATV, One-way ANOVA. The results at 6

h were similar to the 3 h incubation (p = .004 and <0.001, for 4.5 and 45 μg/L ATV, respectively; One-way ANOVA).

The reduction of [^{14}C]-acetate incorporation into hepatocyte [^{14}C]-cholesterol at 3 h coincided with an up-regulation of hepatocyte LDL-r mRNA transcript abundance to the combination of the two drugs compared with the control (p = 0.099, 0.871, and 0.015, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 4.2). At 6 h, LDL-r mRNA transcript abundance was up-regulated in hepatocytes exposed to ATV compared with the control (p = 0.034, 0.663, and 0.093, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 4.2).

Concurrent with the significant reduction in cholesterol radioactivity and the significant up-regulation of LDL-r mRNA transcript abundance, hmgcr-1 mRNA transcript abundance was significantly up-regulated in hepatocytes exposed to ATV and A+G at 3 h compared with the control ($p < 0.05$, Kruskal-Wallis One Way ANOVA on Ranks) (Fig. 4.2). This statistically significant trend was absent in the hepatocytes exposed to the drugs for 6 h, even though a visible up-regulation was noted in Figure 4.2. Hmgcr-2 mRNA transcript abundance was unaffected by either drug (Fig. 4.2).

The significant reduction noted for [^{14}C] incorporation into [^{14}C]-cholesterol was also observed for [^{14}C] incorporation into cholesterol ester in hepatocytes exposed for 6 but not 3 h at 4.5 μg/L ATV compared with the control (p = 0.007 and 0.16, for 4.5 μg/L and 45 μg/L ATV, respectively; One-way ANOVA) (Fig. 4.3).

[^{14}C]-Acetate incorporation into the triglyceride fraction was not significantly affected by ATV at either dose or incubation time (p = .38 and 0.749, for 4.5 μg/L and 45 μg/L ATV, respectively; ANOVA on Ranks) (Fig. 4.4) and no interaction between dose and

time (p = 0.283) was observed. Regardless, SREBP-1 mRNA transcript abundance was significantly elevated in drug exposed hepatocytes compared with the control. At 3 h, SREBP-1 mRNA transcript abundance was significantly elevated in ATV and A+G treated hepatocytes compared with the control (p = 0.015, 0.251, and 0.049, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 4.5). Additionally, a One-way ANOVA detected statistical significance when comparing GEM-treated hepatocytes to the ATV and A+G treated groups (p = 0.003 and 0.014, for ATV and A+G, respectively). At 6 h there was a general trend for an increased abundance of SREBP-1 mRNA transcripts but this was significant only in the A+G-exposed hepatocytes compared with the control (p = 0.085, 0.126, and 0.028, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 4.5).

Furthermore, mRNA abundance for PPAR-α, PPAR-β, and PPAR-γ was not significantly affected by either drug at 3 h compared with the control, even though a visible trend was observed (Fig. 4.5). A One-way ANOVA did detect significant up-regulation of PPAR-γ mRNA abundance in all hepatocytes exposed to the drugs for 6 h compared with the control (p = 0.046, 0.018, and 0.049, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 4.5).

[^{14}C]-Acetate incorporation into phospholipids was not significantly affected by ATV at either dose or incubation time (p = .157 and 0.112, for 3 h and 6 h, respectively; ANOVA on Ranks) (Fig. 4.6).

The cytochrome P450 (CYP) system plays an important role in rainbow trout and other teleosts, as in mammals not only in the metabolism and excretion of endogenous and xenobiotic compounds but also in the biotransformation of certain xenobiotics to reactive metabolites (Buhler and Wang-Buhler, 1998). In particular, CYP3A enzymes are involved in

the metabolism of a wide variety of chemically diverse substances, including environmental pollutants, pesticides, therapeutic drugs, and steroids (Buhler and Wang-Buhler, 1998; Lee and Buhler, 2002). Transcript abundance of the mRNA for CYP3A27 was increased in all treated hepatocytes at both incubation time periods; however, statistical significance was only detected in hepatocytes exposed to the drugs for 6 h compared with the control (p = 0.029, 0.036, and 0.022, for ATV, GEM and A+G, respectively; One-way ANOVA) (Fig. 4.7).

[^{14}C]-Acetate released as [^{14}C]-carbon dioxide ($^{14}CO_2$) was not significantly affected by ATV at either dose or incubation time (p = .170 and 0.061, for 3 h and 6 h incubation, respectively; ANOVA on Ranks) (Fig. 4.8).

To investigate the effects of these drugs on carbohydrate status, [^{14}C]-acetate incorporation into glucose and glycogen was assessed. Generally, there was no effect of ATV on [^{14}C] incorporation into glucose at 3 h of incubation; however, at 6 h there was a visible trend, although not statistically significant, to increase glucose output (p = 0.815 and 0.706, for 3 h and 6 h incubation, respectively; ANOVA on Ranks) (Fig. 4.9). Similarly, [^{14}C] incorporation into glycogen showed trends but not significant differences at 3 and 6 h (p = 0.815 and 0.570, for 3 h and 6 h incubation, respectively; ANOVA on Ranks) (Fig. 4.10).

Finally, the distribution of [1-^{14}C]-acetate radioactivity among the various lipid fractions was assessed to better understand the lipid profile of rainbow trout liver and to estimate the normal lipid amounts produced by trout hepatocyte during a 6 h incubation time in the absence of drug exposure (Fig. 4.11). Data from control, unexposed hepatocytes at 6 h were used to calculate Figure 4.1. The results indicated that most of the radioactivity was

incorporated into the cholesterol and phospholipid fractions (76.9 and 45.3 nmol[^{14}C]-acetate incorporation/6 h/ g hepatocytes, respectively). The remaining fractions incorporated similar amounts of [^{14}C]-acetate.

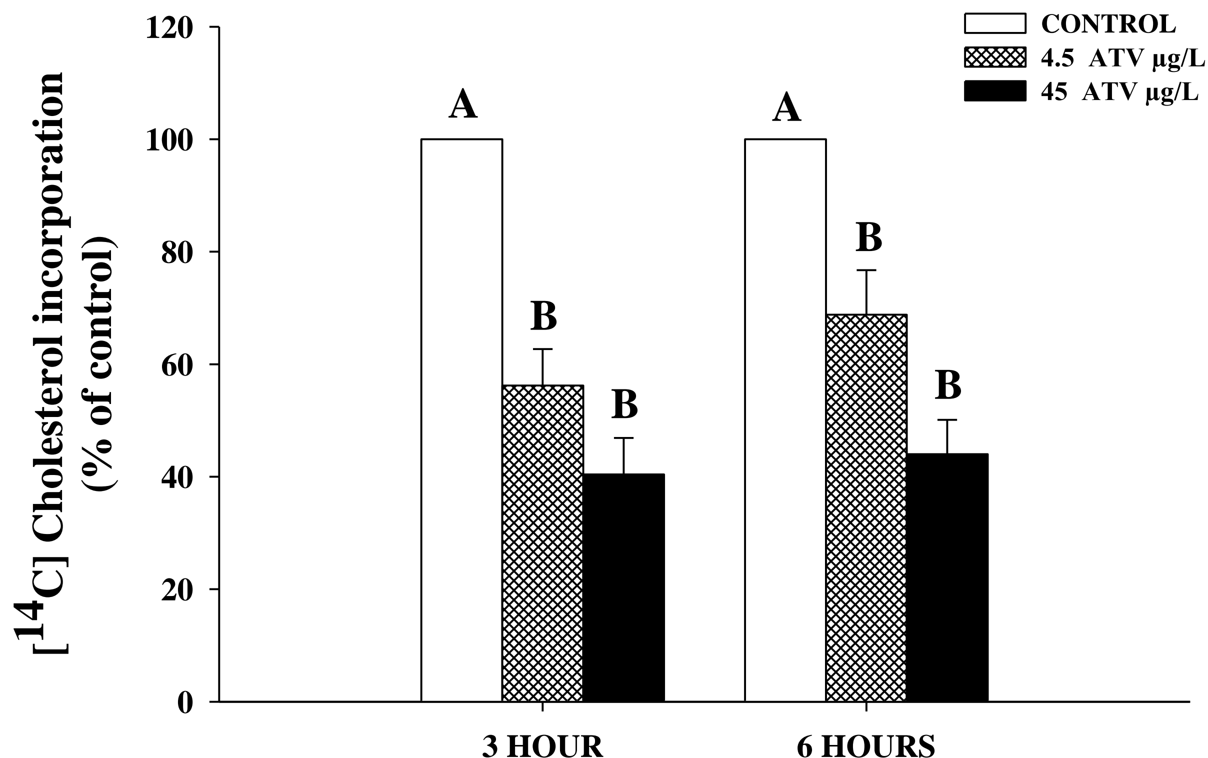


Figure 4.1. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-cholesterol. Hepatocytes were exposed for 3 and 6 h; control hepatocytes were exposed to DMSO (final concentration <0.01%). Data are presented relative to the control as means + SEM (n=5 for each exposure). Different letters denote significance differences compared with the control (p<0.05; One-Way ANOVA).

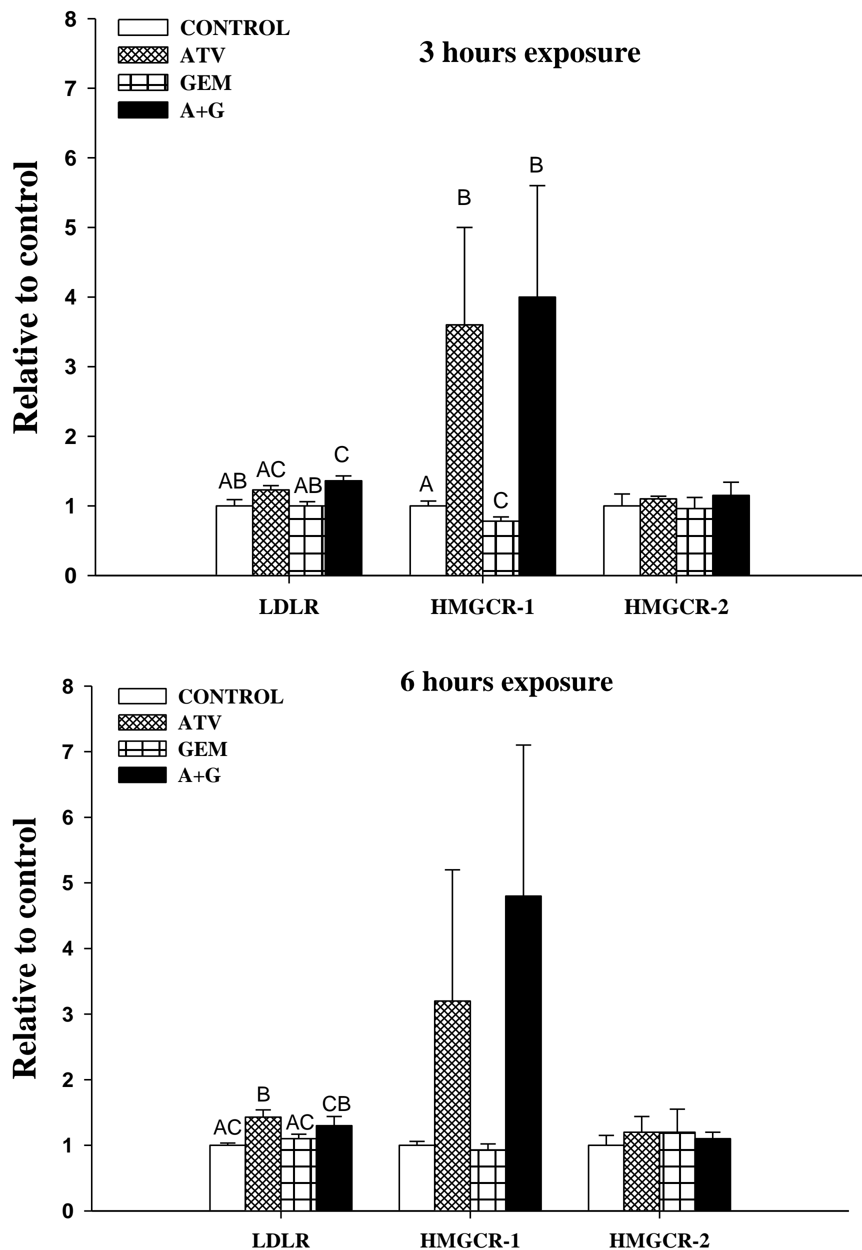


Figure 4.2. The effects of exposure to Atorvastatin (ATV, 45 μg/L), Gemfibrozil (GEM, 150 μg/L), and the combination of the two drugs (A+G) on trout hepatocyte LDLR, HMGCR-1 and HMGCR-2 transcript abundance at 3 and 6 h. Control hepatocytes were exposed to DMSO (final concentration <0.01%). Data are presented as relative values compared with the control and β-actin and represent means + SEM (n= 4 for each exposure). Different letters denote significant differences compared with the control at each time point ($p<0.05$; One-Way ANOVA).

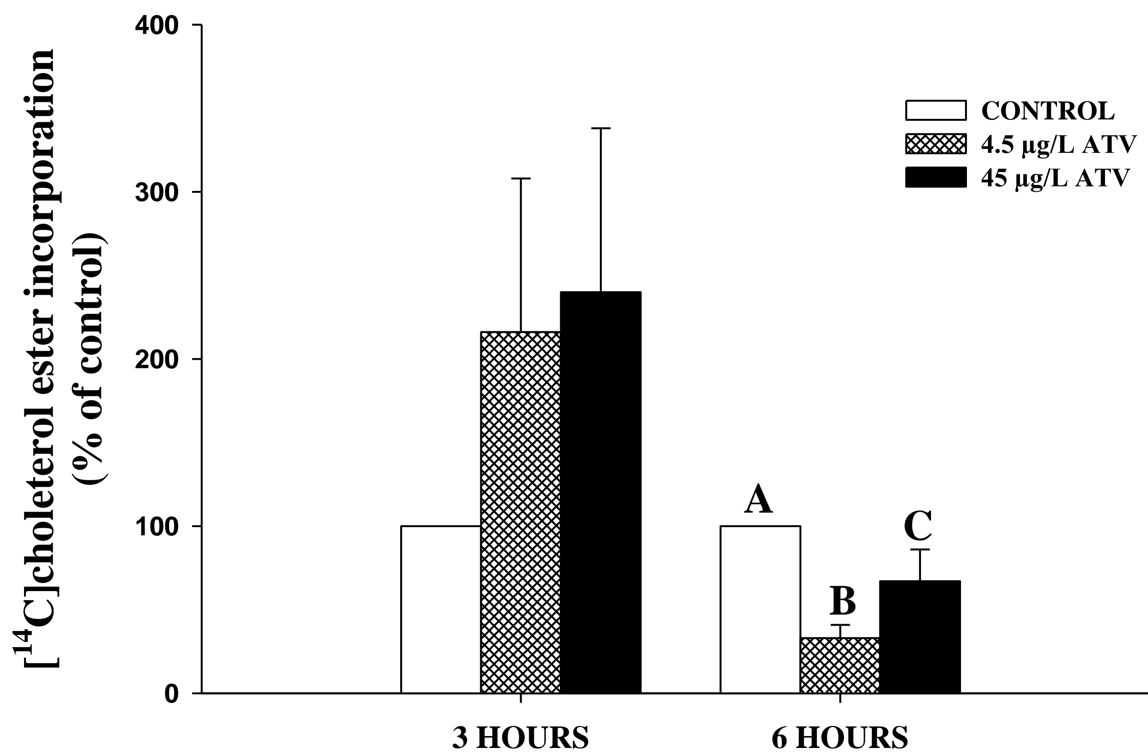


Figure 4.3. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-cholesterol ester at 3 and 6 h. See legend to Fig. 4.1 for details.

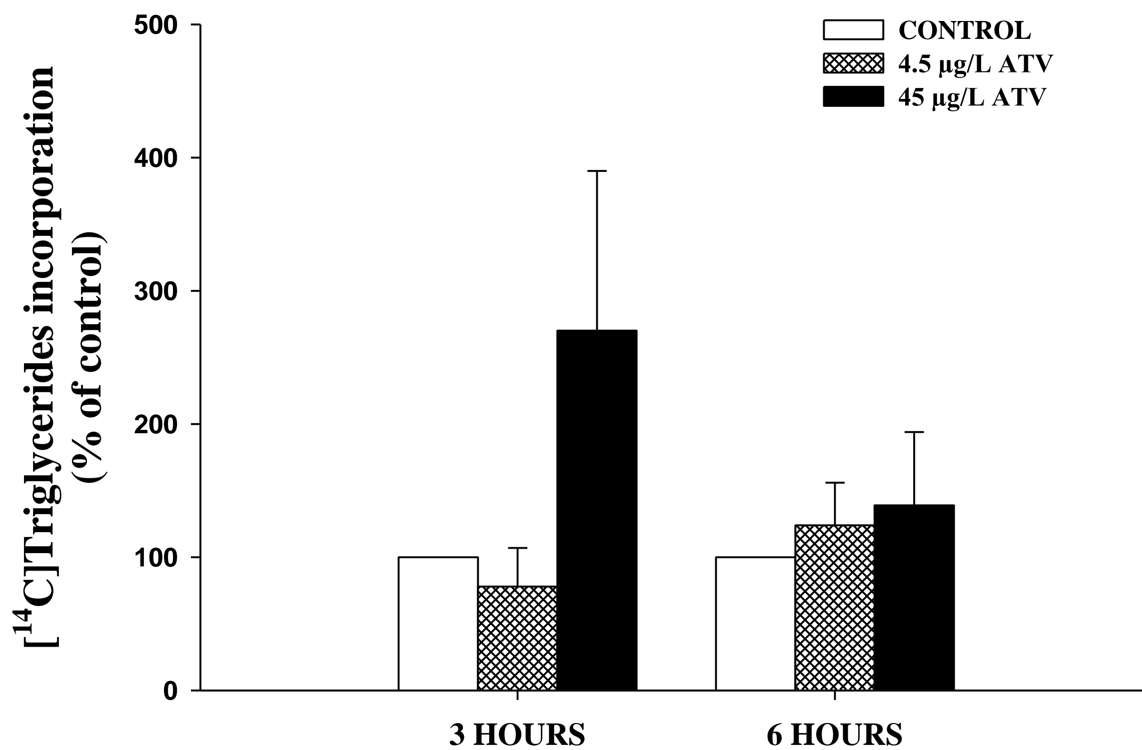
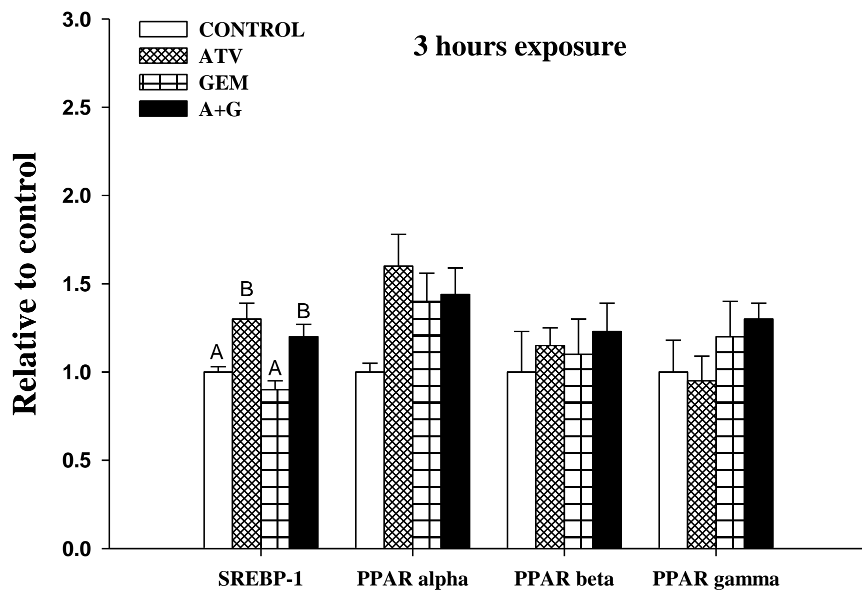


Figure 4.4. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-triglycerides at 3 and at 6 h. There were no statistical differences for any treatment or time. See legends in Fig. 4.1 for details.



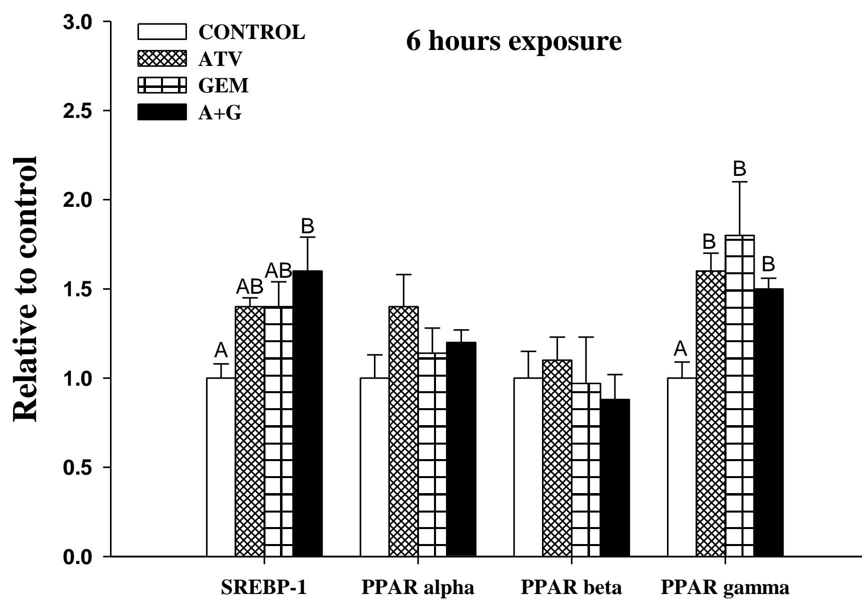


Figure 4.5. The effect of exposure of Atorvastatin (ATV, 45 μg/L), Gemfibrozil (GEM, 150 μg/L), and the combination of the two drugs (A+G) on hepatocytes SREBP-1, PPARα, PPARβ and PPARγ transcript abundance. See legends in Fig. 4.2 for details.

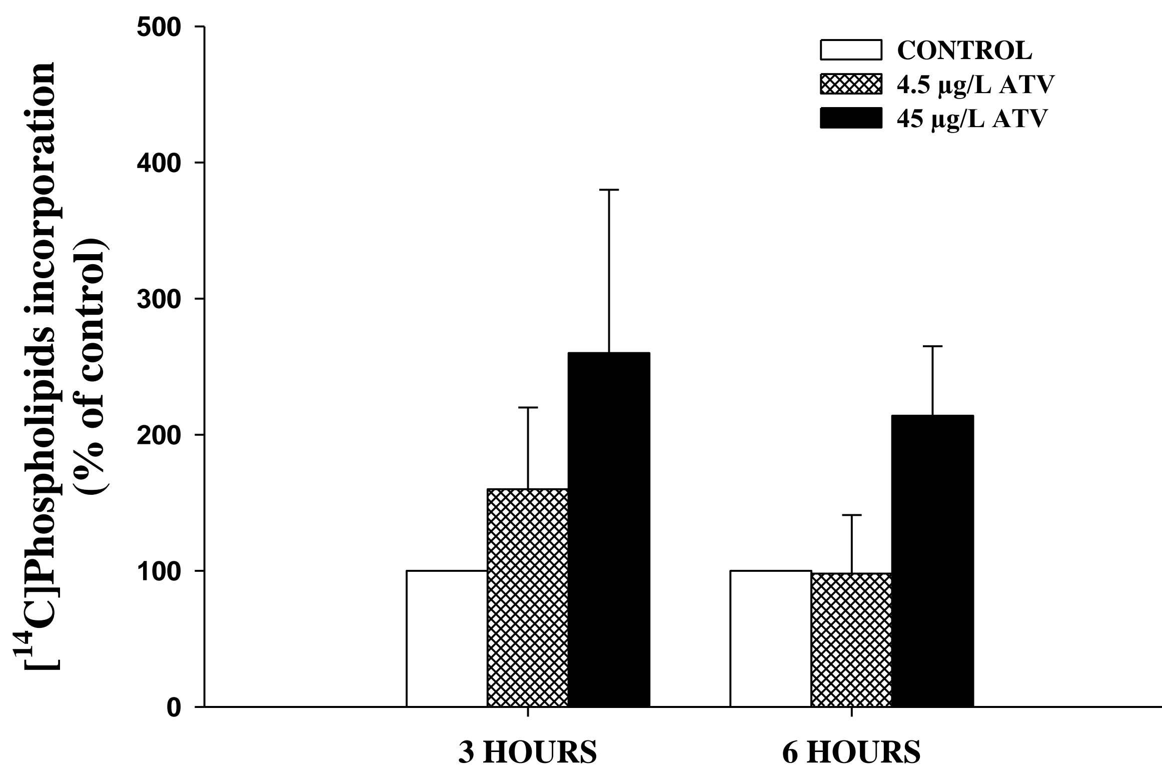


Figure 4.6. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-phospholipids at 3 and at 6 h. There were no statistical differences for any treatment or time. See legends in Fig. 4.1 for details.

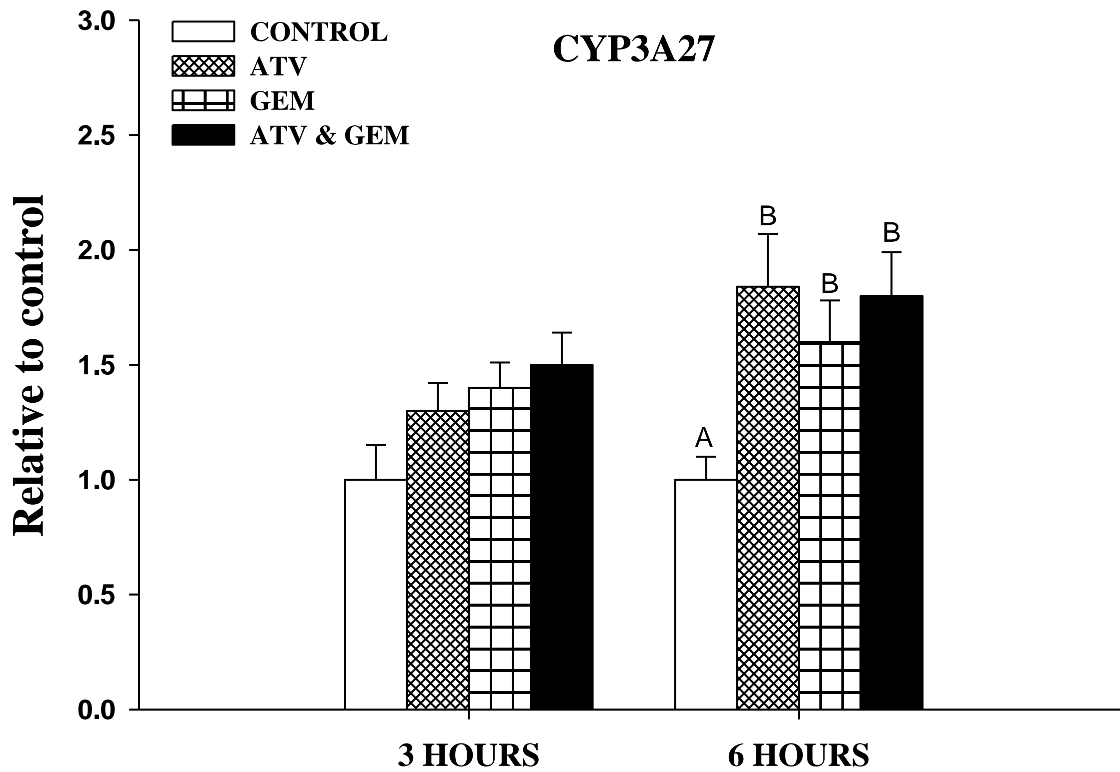


Figure 4.7. The effect of exposure of Atorvastatin (ATV, 45 µg/L), Gemfibrozil (GEM, 150 µg/L), and the combination of the two drugs (A+G) on hepatocytes CYP3A27 transcript abundance. See legends in Fig. 4.2 for details.

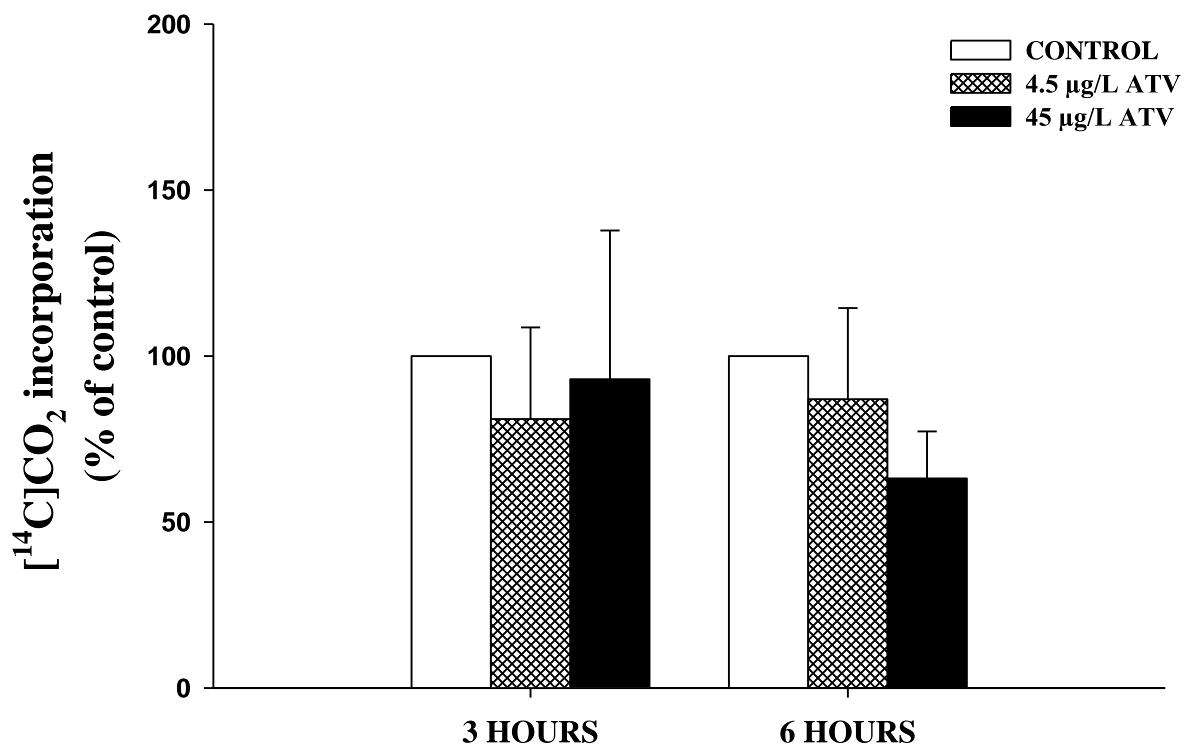


Figure 4.8. The effects of two atorvastatin (ATV) concentrations on hepatocyte $[^{14}C]$-acetate incorporation into $^{14}CO_2$ at 3 and at 6 h. There were no statistical differences for any treatment or time. See legends in Fig. 4.1 for details.

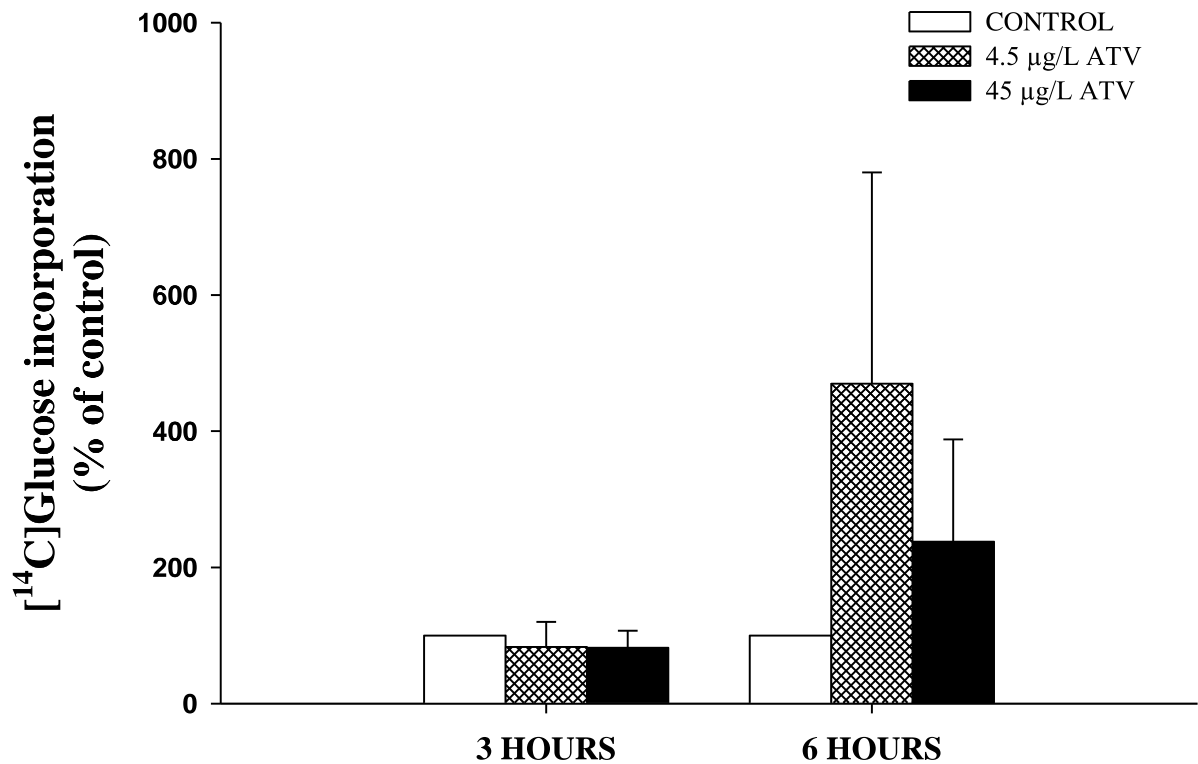


Figure 4.9. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-glucose at 3 and at 6 h. There were no statistical differences for any treatment or time. See legends in Fig. 4.1 for details.

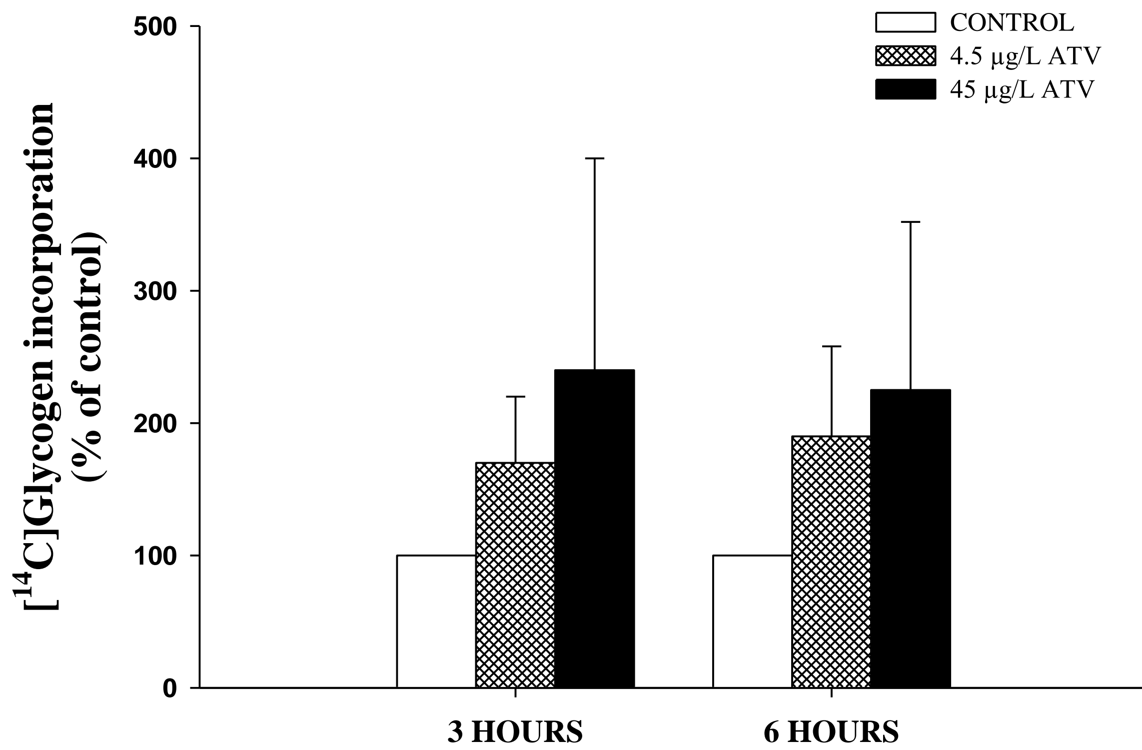


Figure 4.10. The effects of two atorvastatin (ATV) concentrations on hepatocyte [^{14}C]-acetate incorporation into [^{14}C]-glycogen at 3 and at 6 h. There were no statistical differences for any treatment or time. See legends in Fig. 4.1 for details.

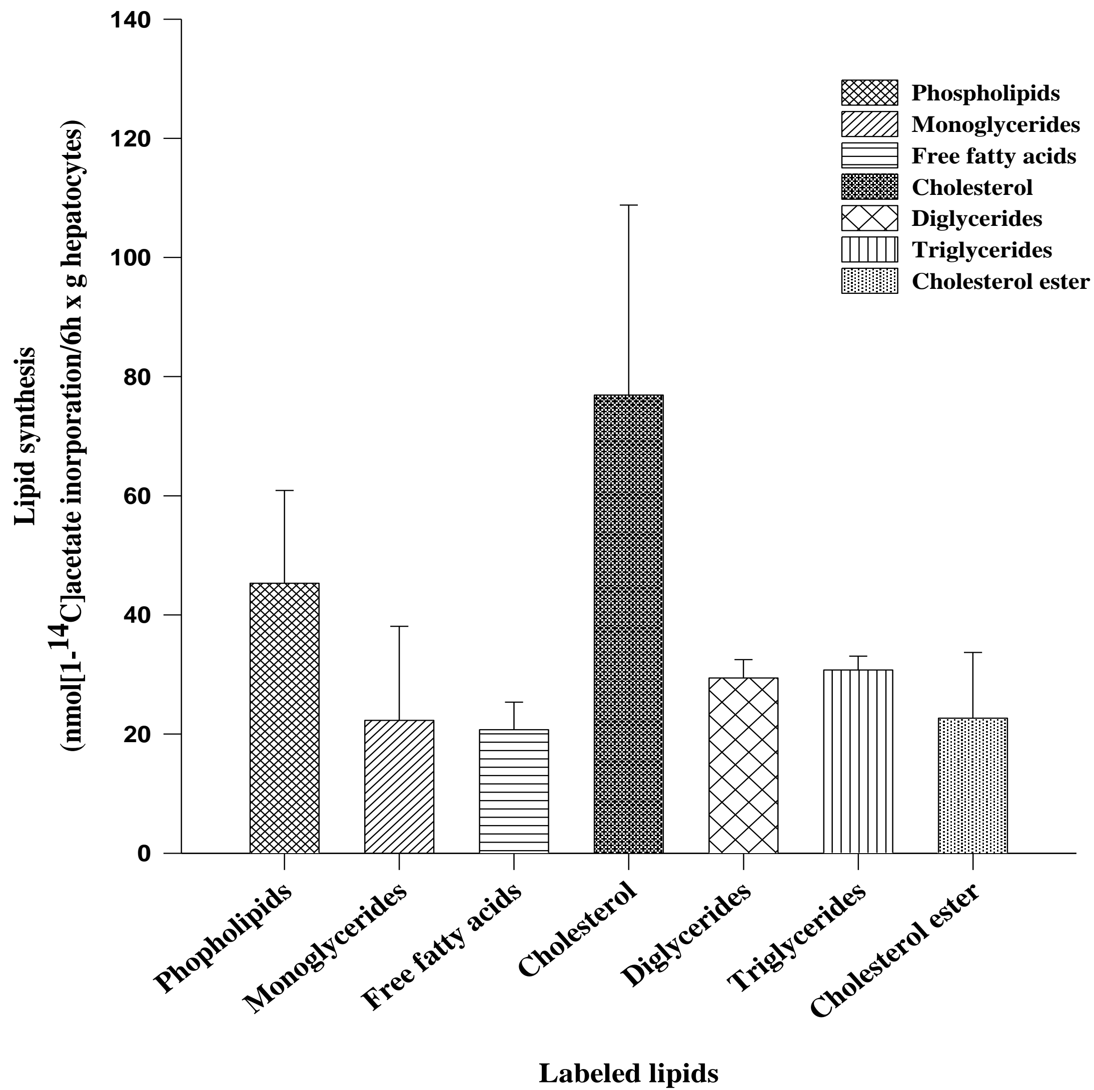


Figure 4.11. Total [^{14}C]-acetate incorporation into different lipid fractions by hepatocytes isolated from rainbow trout. Hepatocytes were incubated for 6 h in the presence of 0.31 µCi/mL [^{14}C]-acetate. Data represent mean (+ SEM, n=5).

4.4. Discussion

The aim of this study was to assess the potential effects of two lipid-lowering drug, ATV (a statin) and GEM (a fibrate) on the incorporation of [^{14}C]-acetate into the various lipid fractions of rainbow trout hepatocytes. Moreover, GEM was used in combination with ATV in a non-radioactive experiment to assess their effects on the gene expression related to lipid homeostasis. Previous *in vivo* studies summarized in Chapter 1 indicated that ATV has a significant impact on cholesterol synthesis (Estey et al., 2008) and GEM impacted both lipid and lipoprotein content (Prindiville et al., 2011) in rainbow trout. Using *in vitro* incubations of trout hepatocytes it is possible to better understand in a tissue that synthesize lipids, what factors may be resulting in these *in vivo* drug effects. As expected the HMGCoA reductase inhibitor ATV depressed the *de novo* synthesis of cholesterol and the cellular formation of new cholesterol esters in the hepatocytes (Figs 4.1 and 4.2). ATV statistically reduced the incorporation of [^{14}C]-acetate into cholesterol by more than 30% compared with the control. These results are in agreement with those Scharnagl et al. (2001). This group reported that the three statin drugs, ATV, lovastatin, and simvastatin, significantly decreased cellular cholesterol in human HepG2 cells by more than 30% compared to the control. Moreover, the reduced synthesis of cholesterol ester from [^{14}C]-acetate by ATV in trout hepatocytes at 6 h collaborates results reported by Isusi et al. (2000) using freshly isolated rat hepatocytes exposed to different ATV concentrations. Furthermore, statins are reported to act as direct inhibitors of cholesterol esterification in rabbit intestinal microsomes (Fellermann et al., 1992). As far as I am aware, the present study provides the first evidence for the *in vitro* effect of a HMGCoA reductase inhibitor (ATV) on the synthesis of cholesterol esters by trout hepatocytes.

Concomitant with the reduction in cholesterol synthesis, the mRNA transcript abundance of LDL-r, hmgcr-1 and SREBP-1 were all significantly increased in hepatocytes exposed to both drugs (Fig. 4.2, and Fig. 4.5). In human, cholesterol depletion by statins resulted in the activation of the transcription factor sterol regulatory element-binding protein (SREBP), which in turn induced the transcription of genes involved in cholesterol production and uptake from the systemic circulation (Friesen and Rodwell, 2004; Morikawa et al., 2005). SREBP transcripts are reported in rainbow trout liver (Skiba-Cassy et al., 2009) and several Atlantic salmon (*Salmo salar*) tissues (Minghetti et al., 2011). An increase in SREBP mRNA was accompanied by an increased abundance of HMGCoAR mRNA in Atlantic salmon SHK-1 cells treated with the fatty acid docosahexaenoic acid (Minghetti et al., 2011), an observation which would support a similar feedback-regulation of hmgcr in fish as that found in humans (Minghetti et al., 2011). From the results of my study, it would appear that rainbow trout hmgcr transcription in response to ATV may be regulated in a similar manner as in humans. The findings of my study are contrary to those previously reported by Estey et al. (2008) and Ellesat et al. (2012); both studies found that statin drugs affect the rainbow trout HMGCoAR system in a manner distinct from that of mammals. But it must be emphasized that Ellesat et al. (2012) investigated hmgcr in gills rather than the liver of rainbow trout. Furthermore, hmgcr mRNA in humans was found to be increased following the administration of two different statins, pravastatin and simvastatin, twice daily over a 3 week period (Rudling et al., 2002), a very different design than noted here.

Low-density lipoprotein receptor (LDLr) mRNA abundance was statistically induced by the two drugs either alone or in combination compared with the control hepatocytes (Fig. 4.2). This significant elevation of LDLr mRNA is consistent with the reduction of

cholesterol synthesis induced by ATV. *In vivo* hepatic LDLr mediates the uptake of LDL particles from the circulation and delivers the receptor-bound LDL to the endosomal system for degradation while the LDLr returns to the cell surface for recycling (Kong et al., 2006; Rudling et al., 2002). The inhibition of hepatic cholesterol synthesis by ATV results in the depletion of hepatic intracellular stores of cholesterol. Consequently, a compensatory increase in the expression of cell surface LDLr occurs, resulting in an increased potential for the hepatic extraction of circulating plasma LDL cholesterol (Dong et al., 2011; Kong et al., 2006). The combined effects of ATV and GEM to inhibit cholesterol synthesis and enhance hepatic clearance of circulating LDL cholesterol by increasing LDLr may be partially responsible for the decrease in total cholesterol (Dong et al., 2011).

The incorporation of [^{14}C]-acetate into triglycerides and phospholipids was not affected by a 3 or 6 h treatment with ATV (Fig. 4.4 and 4.6). The lack of ATV effects on triglycerides synthesis is not surprising since *in vitro* evidence from human HepG2 cells treated with three different statins (including ATV) also noted a similar trend (Scharnagl et al., 2001).

ATV and GEM did not have a significant impact on hepatocytes PPARs at the 3 h incubation time; the only significant change was detected at 6 h where PPAR-γ mRNA abundance was increased by ATV and GEM alone or in combination (Fig. 4.5). PPARs are transcription factors regulating a range of cellular processes such lipid metabolism and inflammation, both of which are found to be up-regulated following statin exposure in mammals (Lee et al., 2010). The two genes *ppar-α* and *ppar-β* in rainbow trout have sequence homologies of 77 and 82% to the corresponding human genes (Liu et al., 2005). Differences in *ppar* gene expression profiles were reported earlier between human cells and

rainbow trout cells following GEM exposure (Forman et al., 1997; Prindiville et al., 2011), which explains the lack of consistency on the effect of the two drugs on the PPAR gene expression between *in vivo* and *in vitro* exposure.

Carbon dioxide production is an indication of the metabolic functioning of the cells, more specifically oxidative respiration. $^{14}CO_2$ production was not significantly different from the control over time or across treatments, which indicates that exposure to ATV had little impact on oxidative respiration in the hepatocytes (Fig. 4.8). This was expected since there is no indication that ATV targets mitochondrial metabolism *per se*.

Most pharmaceuticals need to be metabolized for efficient excretion. They are converted to more hydrophilic and thus more readily excreted metabolites through biotransformation, which usually includes catabolic cytochrome P450 (CYP) enzyme activities within Phase 1 reactions. Phase 1 reactions are typically mediated by members of CYP1, CYP2, CYP3 and CYP4 gene families (Schlenk et al., 2008). Members of CYP3A subfamily are the most dominant CYP forms expressed in the liver and intestine of mammals and other vertebrates, including fish (Hegelund and Celander, 2003). In the present study, there was a general elevation in CYP3A27 mRNA transcript abundance in trout hepatocytes exposed to ATV and GEM and significant differences were detected at 6 h (Fig. 4.7). Studies in fish show that fish are less responsive to the induction of CYP3A protein and/or mRNA levels by pharmaceuticals compared to mammals (Schlenk et al., 2008). For instance, pregnenolone 16α-carbonitrile treatment resulted in a 60% increase in CYP3A protein levels in rainbow trout (Celander et al., 1989), and an 80% increase in CYP3A mRNA in zebrafish (Bresolin et al., 2005). An indication that fish are able to metabolize drugs but less effective compared with the mammals.

There were no significant changes in ^{14}C-acetate incorporation into either glucose or glycogen (Fig. 4.9 and 4.10). There is no report on the effect of ATV on glucose metabolism by hepatocytes. The absence of an effect of ATV on glucose and glycogen synthesis suggests this drug is ineffective in modifying carbohydrate metabolism at least at the concentrations and times used in this study. Therefore, the reduction of cholesterol was the result of the inhibition of HMGCoAR and probably not changes in general hepatocyte metabolism.

The incorporation of ^{14}C-acetate into each lipid class estimates the contribution of each class to total lipid production by hepatocytes in a 6 h period in the absence of drug exposure (Fig. 4.11). What this analyzes demonstrates is the relative importance of cholesterol production by the hepatocytes. Incorporation into the cholesterol fraction was higher than any other lipid class. Hypercholesterolemia in rainbow trout is well documented achieving values 12-times higher than reported in mammals (Babin and Vernier, 1989). The precise role of hypercholesterolemia in salmonids is not clear, but the reduction of these values by ATV may play a critical role in the health and welfare of these fish. Furthermore, the cholesterol levels generated by hepatocytes may further indicate the importance of cholesterol acting as precursor of many steroids including bile acids (Larsson and Fange, 1977). Moreover, phospholipids and triglycerides are also manufactured in relatively large quantities compared with other lipid classes. This is consistent with the fact that phospholipids are used in every cell of the body, and would thus require constant synthesis and production (Hill, 1982).

Conclusions

Exposure of rainbow trout hepatocytes to ATV and GEM resulted in the reduction of cholesterol synthesis and increases in transcripts of genes involved in cholesterol synthesis (*hmgcr*, *ldlr*, and *srebp*). Similar results were obtained when injecting juvenile rainbow trout with these two drugs (see Chapter 3). The results of the present study illustrate that even after a few hours (3-6 h) of exposure, ATV can have a profound effect on cholesterol metabolism, and when combined with GEM, the two drugs affects genes related to lipid synthesis.

CHAPTER 5

General conclusions and perspectives

Pharmaceutical and Personal Care Products (PPCPs) represent an extraordinarily diverse group of chemicals used in veterinary medicine, agricultural practice, human health and cosmetic care (Fent et al., 2006). Large quantities of pharmaceuticals including anti-inflammatory drugs, beta-blockers, antibiotics, lipid regulators are sold and consumed worldwide for treatment or diagnosis of diseases (Fent et al., 2006). As a consequence, a variety of pharmaceutical compound residues are now detected in wastewater and surface water in the range of ng/L to µg/L quantities (Halling-Sörensen et al., 1998; Kolpin et al., 2002). Although many pharmaceuticals have relatively short environmental half-lives, many assume the quality of persistent pollutants as they are continuously introduced into the environment via sewage treatment facilities and water runoff (Daughton and Ternes, 1999).

Despite the mode of action of most pharmaceuticals being well known in humans and mammals in general, their toxicological mechanisms of action on non-target organisms remains relatively unknown (Fent et al., 2006). Existing data on PPCP residues in aquatic systems indicate that they unlikely to pose a risk in terms of acute toxicity, but data regarding chronic toxicity in aquatic organisms, and in particular fish, is lacking (Fent et al., 2006). Therefore, this thesis was undertaken to examine the potential effects of human pharmaceuticals that are found in the aquatic environment on non-target fish species. Statins drugs, specifically atorvastatin (ATV), and fibrate drugs, specifically gemfibrozil (GEM), were chosen because of their widespread use, occurrence and apparent environmental persistence to assess their effects in two model fish species, the rainbow trout, (*Oncorhynchus mykiss*), and the zebrafish, (*Danio rerio*). Furthermore, it was reported by

Metcalfe et al. (2004) that in Canadian WWTP effluents where statin drugs were detected, fibrate drugs were present at comparable or higher concentrations. Therefore, it was logical to use the combination of the two drugs to better assess their effects on fish species, since fetal rhabdomyolysis with cerivastatin (another statin drug) is reported most frequently when used at high doses and particularly, when used in combination with GEM (SoRelle, 2001).

The life history of fish and some features of their physiology potentially make them especially susceptible to pharmaceutical uptake and effects (Erickson et al., 2008). Uptake of pharmaceuticals into fish may occur via both dermal and gill surfaces for water-borne or sediment associated pharmaceuticals, orally through the diet, or maternally by the transfer of contaminants through the lipid reserves of their eggs (Erickson et al., 2008). Pharmaceuticals are generally designed to have low toxicity; however, there is the potential for unintended side effects (Cunningham, 2004). This is perhaps particularly important in non-target fish species, where side effects or even pharmacological effects may differ compared with those in humans and laboratory test mammals due to differences in physiologies and biochemistries (Wolf and Wolfe, 2005). For example, fish have a lower capacity to metabolize xenobiotics compared with mammals, especially during early life stages (Andersson and Forlin, 1992; Wolf and Wolfe, 2005).

5.1. Hypothesis tested

As stated in section 1.9, pp 25-26, this thesis tested the following general hypothesis:

General Hypothesis: Cholesterol metabolism and production are modified by statin and fibrates drugs which will impact the physiological role of steroids in fish.

Prediction 1: Rainbow trout and zebrafish exposed to a statin (ATV) and a fibrate (GEM) drug will decrease their plasma cholesterol and lipid contents compromising the ability of the fish to mount a proper stress response as indicated by a reduction in circulating cortisol content upon exposure to a stressor. Moreover, these fish will have reduced reproductive capacity as assessed by a reduction in the content of sex steroids the secretion of which also depends upon the availability of cholesterol as a precursor.

Prediction 2: Trout and zebrafish exposed to the combination of ATV and GEM will demonstrate biomarkers of muscle damage (rhabdomyolysis) as reported in mammals when exposed to the combination of the two drugs.

Prediction 3: HMGCR-1 and -2 mRNA abundances will increase with exposure to ATV.

The research presented in this thesis was designed to test the hypothesis that exposing fish to ATV and GEM, alone or in combination will lead to unfavorable consequences. Four different exposure approaches were applied to investigate the effects of the two drugs on the two fish species *in vivo* and on rainbow trout hepatocytes *in vitro*. In addition, the tissue distribution of the two hmgcrs isotypes was established in zebrafish, as a tissue distribution in this species was not reported previously.

5.2. Effects of ATV and GEM on cholesterol synthesis and related genes

The results of the thesis were generally supportive of the hypothesis that the two drugs will decrease plasma cholesterol (Chapter 2), whole-body cholesterol (Chapter 3), and de novo cholesterol synthesis (Chapter 4). Thus, both *in vivo* and *in vitro* exposure to ATV and GEM in trout and zebrafish lead to consequences not unlike results seen in mammals. First, there was a nearly 30% reduction in plasma cholesterol content in trout injected with a

human equivalent dose of ATV (0.29 μg/g) and 75% of the human equivalent dose of GEM (6.35 μg/g), alone or in combination (Chapter 2). Similar results were obtained when exposing zebrafish to the two drugs, through food (0.53 μg/g and 16 μg/g for ATV and GEM, respectively) or water-borne (45 μg/L and 1.5 mg/L for ATV and GEM, respectively) (Chapter 3). Moreover, ATV (45 μg/L) reduced the incorporation of [14C]-acetate into [14C]-cholesterol by more than 30% compared with the control, in rainbow trout hepatocytes (Chapter 4). The reduction in cholesterol observed in each experimental set-up was concurrent with the up-regulation of HMGCR-1 mRNA abundance. These results are consistent with results from my HMGCR tissue distribution (Figure 3.1) and that reported for rainbow trout (Estey et al., 2008), demonstrating that HMGCR-1 transcripts were highest in liver and hmgcr-2 was poorly expressed in the liver but highest in the heart compared to the rest of the tissues. Thus, HMGCR-1 is more specific to the liver where cholesterol and lipoproteins are synthesized as previously noted in mammals (D'Amico et al., 2007). HMGCR-2 mRNA abundance was not affected by the two drugs in trout liver (Chapter 2) nor in the trout hepatocytes (Chapter 4); however, there was 3-fold increase in HMGCR-2 transcripts in zebrafish fed the two drugs alone or in combination. The different responses of HMGCR-2 to ATV and GEM exposure between rainbow trout and zebrafish provide evidence that fish species, as with mammalian species, respond differently to the two drugs. At this time what is responsible for these differences are not known.

Furthermore, LDLr mRNA abundance was statistically elevated by ATV and GEM alone or in combination in rainbow trout in vivo (Chapter 2) and in vitro (hepatocytes, Chapter 4). In contrast, the elevation of LDLr mRNA abundance in zebrafish was not significant (Chapter 3). However, the elevation of LDLr mRNA was consistent with the

reduction of cholesterol in all the experiments. In addition, SREBP mRNA abundance was increased in most of the experiments across the 3 studies. SREBPs are considered global physiological regulators of lipid synthesis, that are linked to a suite of biosynthetic pathways involved in cholesterol, fatty acids and triglyceride synthesis and storage (Shimano, 2009).

Moreover, the two drugs reduced plasma triglyceride concentrations in rainbow trout (Chapter 2) except in the fed-stressed (FS) group, reduced whole-body cholesterol in zebrafish feeding regime (Chapter 3), and reduced whole-body triglyceride in all the females in the water-borne exposure system (Chapter 3), but did not have a detectable effect on rainbow trout hepatocytes (Chapter 4). The reduction of triglyceride in the corresponding experiments coincided with an increase in hepatic PPARα mRNA transcript abundance even where plasma triglyceride concentrations were not reduced (Chapter 3). PPARα mainly regulates lipid catabolism and is highly expressed in the fish liver (Cajaraville et al., 2003). Similar to PPARα, PPARγ transcript levels were also elevated in most of the experiments as a result of drug exposure. However, PPAR-β mRNA transcript was not affected by either drug, as none of the experimental regimes detected any significant effects. PPARβ is widely distributed in fish species (Batista-Pinto et al., 2005; Ibabe et al., 2005; Mimeault, 2008). Therefore, its ubiquitous organ expression has made a definitive functional assignment to PPARβ rather difficult. Statins are reported to activate PPARα and PPARγ *in vivo* and *in vitro* (Martin et al., 2001; Paumelle et al., 2006). Moreover, in mammals fibrate drugs have been shown to bind preferentially to PPARα with weak binding to PPARβ and PPARγ (Desvergne and Wahli, 1999). These results suggest that the two drugs have similar effects in fish as in human. Furthermore, since ATV and GEM mediate changes in PPARs in the two fish species, monitoring hepatic PPAR mRNA levels might render a good toxicological

biomarker of drug exposure across freshwater species. However, it should be noted that the results from fibrate drug exposures conflict with results from (Prindiville et al., 2011); these authors failed to find any effects of GEM on PPARs when IP injections of 100 mg GEM/kg were performed on rainbow for a total of 5 times over 15 days.

Furthermore, investigations of the effects of the two drugs on HMGCRs prompted an analysis of their tissue distribution in zebrafish as HMGCRs tissue distribution is not yet established in this fish species. Hmgcr-1 transcripts from adult zebrafish were detected in most tissues examined, except spleen, gonads, and skeletal muscle (Chapter 3). Liver and gut had the greatest abundance followed by brain and heart; gills had detectable levels but these were well below that of the brain and heart. Hmgcr-2 expression was detected in all tissue examined (Chapter 3). HMGCR tissue distribution was consistent with their function, where HMGCR-1 transcript were highest in the liver and HMGCR-2 was poorly expressed in the liver but highest in the heart compared to the rest of the tissues, suggesting that hmgcr-1 is more specific to liver where cholesterol and lipoproteins are synthesized as previously noted in mammals (D'Amico et al., 2007) and the rainbow trout (Estey et al., 2008).

5.3. Effects of ATV and GEM on cortisol and sex steroid production

I predicted that ATV and GEM could reduce endogenous steroid hormone production through their inhibitory effects on cholesterol synthesis, since cholesterol is the essential precursor of all steroid hormones. Results from this study provided some support that exposing rainbow trout and zebrafish to ATV and GEM reduced sex steroid and cortisol production. However, inconsistencies were noted in the results of sex steroids, particularly in the IP experiments with rainbow trout (Chapter 2) where a stress protocol was conducted. The only consistent results from Chapter 2 were the reduction of plasma estradiol and

testosterone in the nFnS (non-fed, not stressed) group by the two drugs (Figs. 2.13 and 2.14), however significant reduction was only detected in plasma testosterone concentration. Data from the zebrafish experiments (Chapter 3) were more conclusive with respect to the effect of ATV and GEM on female sex steroids, particularly when ATV was present in the exposure. Whole-body testosterone content was significantly reduced in all female-treated fish but not in male-treated fish compared with the control when exposing the fish through food (Fig. 3.13); however, exposing the fish through the tank water resulted in the reduction of whole-body testosterone in all exposed fish except when fish exposed to GEM alone (Fig. 3.5). Furthermore, whole-body estradiol content was significantly reduced in nearly all female and male-treated fish exposed through both the food (Fig. 3.14) and tank water (Fig. 3.5). This reduction of sex steroids might be a direct consequence of the reduction of cholesterol, since cholesterol is the precursor of sex steroids.

The work presented in this thesis provides evidence that *in vivo* exposure to ATV and GEM can compromise the ability of the fish to mount a stress response (increase in cortisol). Rainbow trout IP injected with 0.29 μg ATV/g or 6.35 μg GEM/g, alone or in combination resulted in reduced plasma lactate concentrations in the nFS (non-fed, stressed) and FS (fed, stressed) experiments but not in nFnS experiment compared with the control fish under the same experimental regime (Fig. 2.6). Stress is generally associated with increases in plasma and muscle lactate concentrations (Barton and Iwama, 1991). Moreover, fish in nFS and FS experiments had reduced plasma cortisol and hematocrit values when compared with the control fish. Furthermore, water-borne exposure to 45 μg ATV/L or 1.5 mg GEM/L, or the combination of the two drugs resulted in reduction of whole-body cortisol content of all males and females zebrafish (Fig. 3.4). Equally, all females and males zebrafish fed 0.53 μg

ATV/g or 16 μg GEM/g, or the combination of the two drugs had reduced whole-body cortisol content compared with the controls (Fig. 3.14). Since, all fish in the nFS, FS, and zebrafish experiments were subjected to the same stress protocol, depending on fish species, yet only control fish showed an increase in hematocrit and plasma cortisol concentration, this indicates either that the reduction in cholesterol might have resulted in treated fish lacking proper stress response or that treated fish were simply not responsive to the stress protocol. Further studies with potential inhibitors of cortisol production would assist in distinguishing between these two possibilities.

5.4. Effects of ATV and GEM on muscle and kidney

Rhabdomyolysis in human is a rare, serious side effect of statin monotherapy and of statin-fibrate combination therapy (Jennie et al., 2004). Previously it was reported that lovastatin induced the expression of atrogin-1, a key gene involved in skeletal muscle atrophy, in humans with statin myopathy, in zebrafish embryos, and *in vitro* in murine skeletal muscle cells (Hanai et al., 2007). The results of this thesis demonstrated that exposing fish to ATV and GEM, alone or in combination may lead to muscle damage. Plasma creatine kinase activity was significantly elevated in nearly all injected fish (Chapter 3), a biochemical indicator of statin-associated rhabdomyolysis (Farnier et al., 2003). Furthermore, the elevation of creatine kinase was coincidental with significantly elevated abundance of white muscle (Fig. 2.11) and red muscle (Fig. 2.12) f-box 25 and f-box 32 (atrogin-1) mRNA transcripts (Fig. 2.11). Furthermore, feeding zebrafish either 0.53 μg ATV/g or 16 μg GEM/g, alone or in combination resulted in significant elevation of atrogin-1 mRNA abundance in all male zebrafish and in the female treated with the combination of ATV and GEM. Taken together, results from rainbow trout (Chapter 2) and

zebrafish (Chapter 3) unequivocally indicate that ATV and GEM, either alone or in combination promote muscle damage and possibly rhabdomyolysis in fish. Whether such effects modify the structure of muscle or the performance of the fish is not known at this time and should be further investigated.

Furthermore, results from this thesis demonstrate that fish have the ability to metabolize ATV and GEM. Feeding male zebrafish the two drugs resulted in a significant elevation of liver CYP3A65 mRNA abundance, although the increase was not significant in the female zebrafish (Chapter 3). Moreover, there was a general elevation in CYP3A27 mRNA transcript abundance in trout hepatocytes exposed to ATV and GEM and significant differences were detected at 6 h (Chapter 4). There is a lack of information regarding the impact of pharmaceuticals on liver function of fish. Adding to this, rainbow trout injected with the two drugs, either alone or in combination had significantly elevated plasma creatinine concentrations compared with the controls, except in the GEM-nFnS. Elevation of plasma creatinine is an indicator of kidney damage, since creatinine is a breakdown product of creatine phosphate that is produced primarily by the body skeletal muscle and excreted by the kidneys at a constant rate (Scown et al., 2009).

5.5. Summary

Despite the use of pharmaceuticals by humans for many years, only in the last decade or so has the issue of pharmaceuticals in the environment received significant attention (Fent et al., 2006). It is now clear that a wide range of human pharmaceuticals including analgesics, antibiotics, steroids, cardiovascular drugs and various drugs used to treat mental illness, are all present in sewage treatment plants effluents (at low concentration) that ultimately enter rivers (Fent et al., 2006). Therefore, aquatic wildlife including fish will be

exposed, probably continuously throughout their lives, to a complex mixture of pharmaceuticals, albeit at low concentrations. In this thesis I attempted to establish whether two lipid lowering drugs, either alone or in combination affected two fish species. The work presented in this thesis, although at drug concentrations well above those found in the environment (Metcalfe et al., 2004), provides evidence that *in vivo* and *in vitro* exposure to ATV and GEM can lead to unfavorable effects in fish including potential rhabdomyolysis, liver dysfunction, kidney damage, stress response impairment and reduced sex steroids. One aspect of exposure that merits consideration is the duration and time these mixtures are present in the life cycle or the fish. In the experiments of this thesis, sexually mature zebrafish and sexually immature (juvenile) rainbow trout were exposed to these drugs for 30 days prior the assessment of the effects. However, wild fish could presumably be exposed to ATV and GEM throughout their lives. Whether such prolonged exposure can lead to effects at lower concentrations compared to the higher concentrations that were used in this thesis remains unknown. Also, whether the effects noted here have any longer term impacts of wild fish species with respect to reproduction, predator-prey and other performance traits, remains unknown.

Finally, the results of this thesis demonstrate a point of more general relevance to the issue of pharmaceuticals in the environment. Although the results of this thesis were unable to unequivocally demonstrate in fish all the therapeutic effects of ATV and GEM seen in humans, reproductive, muscle damage and many other effects were observed, which are side effects of the two drugs in mammals. Perhaps what are side effect (i.e. not the main, intended, effect) in humans or other mammals could be the major effects, in non-target organisms such as fish.

References

Al-Ansari, A. M., Saleem, A., Kimpe, L. E., Sherry, J. P., McMaster, M. E., Trudeau, V. L. and Blais, J. M. (2010). Bioaccumulation of the pharmaceutical 17 alpha-ethinylestradiol in shorthead redhorse suckers (Moxostoma macrolepidotum) from the St. Clair River, Canada. *Environmental Pollution* **158**, 2566-2571.

Amarenco, P., Labreuche, J., Lavalle´e, P. and Touboul, P.-J. (2004). Statins in Stroke Prevention and Carotid Atherosclerosis Systematic Review and Up-to-Date Meta-Analysis. *Stroke* **35**, 2902-2909.

Andersson, T. and Forlin, L. (1992). Regulation of the cytochrome P450 enzyme system in fish. *Aquatic Toxicology (Amsterdam)* **24**, 1-19.

Babin, P. J. (1987). Plasma lipoprotein and apolipoprotein distribution as a function of density in the rainbow trout (Salmo gairdneri). *Biochemical Journal* **246**, 425-429.

Babin, P. J. and Vernier, J.-M. (1989). Plasma lipoproteins in fish. *Journal of Lipid Research* **30**, 467-490.

Baillie, T. A., Cayen, M. N., Fouda, H., Gerson, R. J., Green, J. D., Grossman, S. J., Klunk, L. J., Leblanc, B., Perkins, D. G. and Shipley, L. A. (2002). Drug metabolites in safety testing. *Toxicology and Applied Pharmacology* **182**, 188-196.

Barton, B. A. (2002). Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology* **42**, 517-525.

Barton, B. A. and Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases* **1**, 3-26.

Batista-Pinto, C., Rodrigues, P., Rocha, E. and Lobo-Da-Cunha, A. (2005). Identification and organ expression of peroxisome proliferator activated receptors in brown trout (Salmo trutta f. fario). *Biochimica et Biophysica Acta* **1731**, 88-94.

Bellosta, S., Ferri, N., Bernini, F., Paoletti, R. and Corsini, A. (2000). Non-lipid-related effects of statins. *Annals of Medicine* **32**, 164-176.

Benotti, M. J., Trenholm, R. A., Vanderford, B. J., Holady, J. C., Stanford, B. D. and Snyder, S. A. (2008). Pharmaceuticals and Endocrine Disrupting Compounds in U.S. Drinking Water. *Environmental Science & Technology* **43**, 597-603.

Bishop-Bailey, D. (2000). Peroxisome proliferator-activated receptors in the cardiovascular system. *British Journal of Pharmacology* **129**, 823-834.

Bitman, J., Wood, D. L. and Ruth, J. M. (1981). Two-stage, one-dimensional Thin Layer Chromatographic method for separation of lipid classes. *Journal of Liquid Chromatography & Related Technologies* **4**, 1007 - 1021.

Blumenthal, R. S. (2000). Statins: Effective antiatherosclerotic therapy. *American Heart Journal* **139**, 577-583.

Boden, W. E., O'Rourke, R. A., Teo, K. K., Hartigan, P. M., Maron, D. J., Kostuk, W., Knudtson, M., Dada, M., Casperson, P., Harris, C. L. et al. (2007). The evolving pattern of symptomatic coronary artery disease in the United States and Canada: Baseline characteristics of the Clinical Outcomes Utilizing Revascularization and Aggressive Drug Evaluation (COURAGE) trial. *American Journal of Cardiology* **99**, 208-212.

Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K. M., Nunez, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E., Dharmarajan, K. et al. (2001). Identification of ubiquitin ligases required for skeletal muscle atrophy. *SCIENCE* **294**, 1704-1708.

Boston, P. F., Dursun, S. M., Zafar, R. and Reveley, M. A. (1996). Serum cholesterol and treatment-resistance in schizophrenia. *Biological Psychiatry* **40**, 542-543.

Brain, R. A., Reitsma, T. S., Lissemore, L. I., Bestari, K., Sibley, P. K. and Solomon, K. R. (2006). Herbicidal Effects of Statin Pharmaceuticals in *Lemna gibba*. *Environmental Science & Technology* **40**, 5116-5123.

Braissant, O. and Wahli, W. (1998). Differential expression of peroxisome proliferator-activated receptor-alpha, -beta, and -gamma during rat embryonic development. *Endocrinology* **139**, 2748-2754.

Bresolin, T., de Freitas Rebelo, M. and Celso Dias Bainy, A. (2005). Expression of PXR, CYP3A and MDR1 genes in liver of zebrafish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **140**, 403-407.

Brown, A. J., Sun, L., Feramisco, J. D., Brown, M. S. and Goldstein, J. L. (2002). Cholesterol Addition to ER Membranes Alters Conformation of SCAP, the SREBP Escort Protein that Regulates Cholesterol Metabolism. *Molecular Cell* **10**, 237–245.

Brown, M. S. and Goldstein, J. L. (1980). Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *Journal of Lipid Research* **21**, 505-517.

Brown, M. S. and Goldstein, J. L. (1997). The SREBP Pathway: Regulation of Cholesterol Metabolism by Proteolysis of a Membrane-Bound Transcription Factor. *Cell* **89**, 331–340.

Buhler, D. R. and Wang-Buhler, J.-L. (1998). Rainbow trout cytochrome P450s: purification, molecular aspects, metabolic activity, induction and role in environmental monitoring. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* **121**, 107-137.

Bullen, W. W., Miller, R. A. and Hayes, R. N. (1999). Development and validation of a high-performance liquid chromatography tandem mass spectrometry assay for atorvastatin, ortho-hydroxy atorvastatin, and para-hydroxy atorvastatin in human, dog, and rat plasma. *Journal of the American Society for Mass Spectrometry* **10**, 55-66.

Burger, K., Gimpl, G. and Fahrenholz, F. (2000). Regulation of receptor function by cholesterol. *Cellular and Molecular Life Sciences* **57**, 1577-1592.

Cajaraville, M. P., Cancio, I., Ibabe, A. and Orbea, A. (2003). Peroxisome proliferation as a biomarker in environmental pollution assessment. *Microscopy Research and Technique* **61**, 191-202.

Cavallucci, S. (2007). What's topping the charts in prescription drugs this year?: http://www.imshealth.com/vgn/images/portal/cit_40000873/13/31/8286270612-TOP200-07-final.pdf.

Celander, M., Ronis, M. and Förlin, L. (1989). Initial characterization of a constitutive cytochrome P-450 isoenzyme in rainbow trout liver. *Marine Environmental Research* **28**, 9-13.

Chang, J. T., Staffa, J. A., Parks, M. and Green, L. (2004). Rhabdomyolysis with HMG-CoA reductase inhibitors and gemfibrozil combination therapy. *Pharmacoepidemiology and Drug Safety* **13**, 417-426.

Chapman, M. J., Goldstein, S., Mills, G. L. and Leger, C. (1978). Distribution and characterization of the serum lipoproteins and their apoproteins in the rainbow trout (*Salmo gairdnerii*). *Biochemistry* **17**, 4455-4464.

Choy, P. C., Mymin, D., Zhu, Q., Dakshinamurti, K. and O, K. (2000). Atherosclerosis risk factors: The possible role of homocysteine. *Molecular and Cellular Biochemistry* **207**, 143–148.

Choy, P. C., Siow, Y. L., Mymin, D. and O, K. (2004). Lipids and atherosclerosis. *Biochemistry and Cell Biology* **82**, 212-224.

Christian, J. B., Bourgeois, N., Snipes, R. and Lowe, K. A. (2011). Prevalence of Severe (500 to 2,000 mg/dl) Hypertriglyceridemia in United States Adults. *American Journal of Cardiology* **107**, 891-897.

Christopher-Stine, L. (2006). Statin myopathy: an update. *Current Opinion in Rheumatology* **18**, 647–653.

Cibickova, L. (2011). Statins and their influence on brain cholesterol. *Journal of Clinical Lipidology* **5**, 373-379.

Ciechanover, A. (1998). The ubiquitin-proteasome pathway: On protein death and cell life. *EMBO (European Molecular Biology Organization) Journal* **17**, 7151-7160.

Cilla, D. D., Jr., Gibson, D. M., Whitfield, L. R. and Sedman, A. J. (1996a). Pharmacodynamic effects and pharmacokinetics of atorvastatin after administration to normocholesterolemic subjects in the morning and evening. *Journal of Clinical Pharmacology* **36**, 604-609.

Cilla, D. D., Jr., Whitfield, L. R., Gibson, D. M., Sedman, A. J. and Posvar, E. L. (1996b). Multiple-dose pharmacokinetics, pharmacodynamics, and safety of atorvastatin, an inhibitor of HMG-CoA reductase, in healthy subjects. *Clinical Pharmacology and Therapeutics* **60**, 687-695.

Clarke, P. R. and Hardie, D. G. (1990). Regulation of HMG-CoA reductase: identification of the site phosphorylated by the AMP-activated protein kinase in vitro and in intact rat liver. *EMBO (European Molecular Biology Organization) Journal* **9**, 2439-2446.

Cleveland, B. M. and Burr, G. S. (2011). Proteolytic response to feeding level in rainbow trout (Oncorhynchus mykiss). *Aquaculture* **319**, 194-204.

Cleveland, B. M. and Evenhuis, J. P. (2010a). Molecular characterization of atrogin-1/F-box protein-32 (FBXO32) and F-box protein-25 (FBXO25) in rainbow trout (Oncorhynchus mykiss): Expression across tissues in response to feed deprivation. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **157**, 248-257.

Cleveland, B. M. and Evenhuis, J. P. (2010b). Molecular characterization of atrogin-1/F-box protein-32 (FBXO32) and F-box protein-25 (FBXO25) in rainbow trout (Oncorhynchus mykiss): Expression across tissues in response to feed deprivation. *Comparative Biochemistry and Physiology Part B Biochemistry & Molecular Biology* **157**, 248-257.

Cleveland, B. M., Weber, G. M., Blemings, K. P. and Silverstein, J. T. (2009). Insulin-like growth factor-I and genetic effects on indexes of protein degradation in response

to feed deprivation in rainbow trout (Oncorhynchus mykiss). *Am J Physiol Regul Integr Comp Physiol* **297**, R1332-1342.

Corbelli, J. C., Bullano, M. F., Willey, V. J., Cziraky, M. J., Corbelli, M. E. and Waugh, W. (2002). Effects of gemfibrozil conversion to fenofibrate on lipids in patients on statin therapy. *The American journal of cardiology* **90**, 1388-1391.

Corcoran, J., Winter, M. J. and Tyler, C. R. (2010). Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish. *Critical Reviews in Toxicology* **40**, 287-304.

Corrdy, J. M., Hooper, N. M. and Turner, A. J. (2006). The involvement of lipid rafts in Alzheimer's disease (Review). *Molecular Membrane Biology* **23**, 111-122.

Corsini, A., Bellosta, S., Baetta, R., Fumagalli, R., Paoletti, R. and Bernini, F. (1999). New insights into the pharmacodynamic and pharmacokinetic properties of statins. *Pharmacology and Therapeutics* **84**, 413-428.

Craig, P. M. (2013). Why trout? In *Trout: From Physiology to Conservation*, eds. S. Polakof and T. W. Moon). New York: Nova Publishers. pp. 1-7.

Craig, P. M. and Moon, T. W. (2011). Fasted Zebrafish Mimic Genetic and Physiological Responses in Mammals: A Model for Obesity and Diabetes? *Zebrafish* **8**, 109-117.

D'Amico, L., Scott, I. C., Jungblut, B. and Stainier, D. Y. R. (2007). A Mutation in Zebrafish hmgcr1b reveals a role for Isoprenoids in vertebrate heart-tube formation. *Current Biology* **17**, 252–259.

Daughton, C. G. and Ternes, T. A. (1999). Pharmaceuticals and Personal Care Products in the Environment: Agents of Subtle Change? *Environmental Health Perspectives* **Vol. 107, Supplement 6**, 907-938.

Desvergne, B. and Wahli, W. (1999). Peroxisome proliferator-activated receptors: Nuclear control of metabolism. *Endocrine Reviews* **20**, 649-688.

Devaux, A., Flammarion, P., Bernardon, V., Garric, J. and Monod, G. (1998). Monitoring of the chemical pollution of the river RhÃ´ne through measurement of DNA damage and cytochrome P4501a induction in chub (Leuciscus cephalus). *Marine Environmental Research* **46**, 257-262.

Dong, B., Wu, M., Cao, A., Li, H. and Liu, J. (2011). Suppression of Idol expression is an additional mechanism underlying statin-induced up-regulation of hepatic LDL receptor expression. *International Journal of Molecular Medicine* **27**, 103-110.

Eacker, S. M., Agrawal, N., Qian, K., Dichek, H. L., Gong, E.-Y., Lee, K. and Braun, R. E. (2008). Hormonal regulation of testicular steroid and cholesterol homeostasis. *Molecular Endocrinology* **22**, 623-635.

Eisa-Beygi, S., Hatch, G., Noble, S., Ekker, M. and Moon, T. W. (2012). The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) pathway regulates developmental cerebral-vascular stability via prenylation-dependent signalling pathway. *Developmental Biology* **373**, 258-266.

Ellesat, K., Holth, T., Wojewodzic, M. and Hylland, K. (2012). Atorvastatin up-regulate toxicologically relevant genes in rainbow trout gills. *Ecotoxicology*, 1-16.

Endo, A. (1992). The discovery and development of HMG-CoA reductase inhibitors. *Journal of Lipid Research* **33**, 1569-1582.

Endo, A., Kuroda, M. and Tsujita, Y. (1976). ML-236A, ML-236B, AND ML-236C, new inhibitors of cholesterogenesis produced by Penicillium citrinium. *The journal of antiobiotics* **29**, 1346-8.

Erickson, R. J., Nichols, J. W., Cook, P. M. and Ankley, G. T. (2008). Bioavailability of Chemical Contaminants in Aquatic Systems. In *The Toxicology of Fishes*, eds. R. T. D. Giulio and D. E. Hinton). New York: CRC Press. pp. 9-54.

Espenshade, P. J. and Hughes, A. L. (2007). Regulation of sterol synthesis in Eukaryotes. *Annual Review of Genetics* **41**, 401-427.

Estey, C., Chen, X. and Moon, T. W. (2008). 3-Hydroxy-3-methylglutaryl coenzyme A reductase in rainbow trout: Effects of fasting and statin drugs on activities and mRNA transcripts. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **147**, 386-398.

Farmer, J. A. (2001). Learning from the cerivastatin experience. *The Lancet* **358**, 1383-1385.

Farnier, M., Bortolini, M., Salko, T., Freudenreich, M.-O., Isaacsohn, J. L., Troendle, A. J. and Gonasun, L. (2003). Frequency of creatine kinase elevation during treatment with fluvastatin in combination with fibrates (bezafibrate, fenofibrate, or gemfibrozil). *The American journal of cardiology* **91**, 238-240.

Farrell, A. P. and Munt, B. (1983). Cholesterol levels in the blood of Atlantic salmonids. *Comparative Biochemistry and Physiology Part A: Physiology* **75**, 239-242.

Faust, J. R., Trzaskos, J. M. and Gaylor, J. L. (1988). Cholesterol biosynthesis. In *Biology of Cholesterol*, (ed. P. L. Yeagle). Boca Raton, Florida: CRC Press, Inc. pp. 19-38.

Fellermann, K., Reimann, F. M., Herold, G. and Stange, E. F. (1992). Mevinolin, a competitive inhibitor of hydroxymethylglutaryl coenzyme A reductase, suppresses enterocyte esterification of exogenous but not endogenous cholesterol. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **1165**, 78-83.

Fent, K., Weston, A. A. and Caminada, D. (2006). Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology* **76**, 122-159.

Folch, J., Lees, M. and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *THE JOURNAL OF BIOLOGICAL CHEMISTRY* **226**, 497-509.

Forman, B. M., Chen, J. and Evans, R. M. (1997). Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors Î± and Î´. *Proceedings of the National Academy of Sciences* **94**, 4312-4317.

French, C. J., Mommsen, T. P. and Hochachka, P. W. (1981). Amino acid utilisation in isolated hepatocytes from rainbow trout. *European Journal of Biochemistry* **113**, 311-317.

Friesen, J. and Rodwell, V. (2004). The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. *Genome Biology* **5**, 248.

Froehlicher, M., Liedtke, A., Groh, K. J., Neuhauss, S. C. F., Segner, H. and Eggen, R. I. L. (2009). Zebrafish (Danio rerio) neuromast: Promising biological endpoint linking developmental and toxicological studies. *Aquatic Toxicology* **95**, 307-319.

Gaillard, C. and Strauss, F. (1990). Ethanol precipitation of DNA with linear polyacrylamide as carrier. *Nucleic Acids Research* **18**, 378.

Gertler, F. B., Chiu, C. Y., Richter-Mann, L. and Chin, D. J. (1988). Developmental and metabolic regulation of the Drosophila melanogaster 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Molecular and Cellular Biology* **8**, 2713-2721.

Gervois, P., Torra, I. P., Fruchart, J.-C. and Staels, B. (2000). Regulation of lipid and lipoprotein metabolism by PPAR activators. *Clinical Chemistry and Laboratory Medicine* **38**, 3-11.

Geslin, M. and Auperin, B. (2004). Relationship between changes in mRNAs of the genes encoding steroidogenic acute regulatory protein and P450 cholesterol side chain cleavage in head kidney and plasma levels of cortisol in response to different kinds of acute stress in the rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **135**, 70-80.

Gilmour, K. M., Kirkpatrick, S., Massarsky, A., Pearce, B., Saliba, S., Stephany, C.-É. and Moon, T. W. (2012). The influence of social status on hepatic glucose metabolism in rainbow trout Oncorhynchus mykiss. *Physiological and Biochemical Zoology* **85**, 309-320.

Goldstein, J. L. and Brown, M. S. (1990). Regulation of the mevalonate pathway. *Nature* **343**, 425-430.

Goldstein, J. L., DeBose-Boyd, R. A. and Brown, M. S. (2006). Protein Sensors for Membrane Sterols. *Cell* **124**, 35-46.

Goldstein, J. L., Rawson, R. B. and Brown, M. S. (2002). Mutant Mammalian Cells as Tools to Delineate the Sterol Regulatory Element-Binding Protein Pathway for Feedback Regulation of Lipid Synthesis. *Archives of Biochemistry and Biophysics* **397**, 139-148.

Gomes, M. D., Lecker, S. H., Jagoe, R. T., Navon, A. and Goldberg, A. L. (2001). Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 14440-14445.

Gray, E. S., Woodin, B. R. and Stegeman, J. J. (1991). Sex Differences in Hepatic Monooxygenases in Winter Flounder (Pseudopleuronectes americanus) and Scup (Stenotomus chrysops) and Regulation of P450 Forms by Estradiol *Journal of Experimental Zoology* **259**, 330-342.

Halling-Sörensen, B., Nielsen, S. N., Lanzky, P. F., Ingerslev, F., Lutzhoft, H. C. H. and Jorgensen, S. E. (1998). Occurrence, Fate and Effects of Pharmaceutical Substances in the Environment- A Review. *Chemosphere* **36**, 357-393.

Hamelin, B. A. and Turgeon, J. (1998). Hydrophilicity/lipophilicity: Relevance for the pharmacology and clinical effects of HMG-CoA reductase inhibitors. *Trends in Pharmacological Sciences* **19**, 26-37.

Hampton, R. Y. (2002). Proteolysis and Sterol Regulation. *Annual review of cell and developmental biology* **18**, 345–378.

Hanai, J.-i., Cao, P., Tanksale, P., Imamura, S., Koshimizu, E., Zhao, J., Kishi, S., Yamashita, M., Phillips, P. S., Sukhatme, V. P. et al. (2007). The muscle-specific ubiquitin ligase atrogin-1/MAFbx mediates statin-induced muscle toxicity. *The Journal of Clinical Investigation* **117**, 3940-3951.

Hazel, J. R. and Prosser, C. L. (1979). Incorporation of 1-14C-Acetate into Fatty Acids and Sterols by Isolated Hepatocytes of Thermally Acclimated Rainbow Trout (Salmo gairdneri). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **134**, 321-329.

Hegelund, T. and Celander, M. C. (2003). Hepatic versus extrahepatic expression of CYP3A30 and CYP3A56 in adult killifish (Fundulus heteroclitus). *Aquatic Toxicology* **64**, 277-291.

Henderson, R. J. and Tocher, D. R. (1987). The lipid composition and biochemistry of freshwater fish. *Progress in Lipid Research* **26**, 281-347.

Her, G. M., Hsu, C.-C., Hong, J.-R., Lai, C.-Y., Hsu, M.-C., Pang, H.-W., Chan, S.-K. and Pai, W.-Y. (2011). Overexpression of gankyrin induces liver steatosis in zebrafish (Danio rerio). *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1811**, 536-548.

Hermann, M., Christensen, H. and Reubsaet, J. L. E. (2005). Determination of atorvastatin and metabolites in human plasma with solid-phase extraction followed by LC-tandem MS. *Analytical and bioanalytical chemistry* **382**, 1242-1249.

Hill, A. J., Teraoka, H., Heideman, W. and Peterson, R. E. (2005). Zebrafish as a Model Vertebrate for Investigating Chemical Toxicity. *Toxicological Sciences* **86**, 6-19.

Hille, S. (1982). A literature review of the blood chemistry of rainbow trout, Salmo gairdneri Rich. *Journal of Fish Biology* **20**, 535-569.

Holdgate, G. A., Ward, W. H. J. and McTaggart, F. (2003). Molecular mechanism for inhibition of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by rosuvastatin. *Biochemical Society Transactions* **31**, 528-531.

Hontela, A. (1997). Endocrine and physiological responses of fish to xenobiotics: Role of glucocrticosteriod hormones. *Reviews in Toxicology* **1**, 1-46.

Hontela, A. (2005). Adrenal toxicology: Environmental pollutants and the HPI axis. In *Biochemistry and Molecular Biology of Fishes*, vol. 6 eds. T. P. Mommsen and T. W. Moon). Amsterdam: Elsevier pp. 331-363.

Hontela, A., Rasmussen, J. B., Audet, C. and Chevalier, G. (1992). Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. *Archives of Environmental Contamination and Toxicology* **22**, 278-283.

Horton, J. D., Goldstein, J. L. and Brown, M. S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *The Journal of Clinical Investigation* **109**, 1125–1131.

Hua, X., Wu, J., Goldstien, J. L., Brown, M. S. and Hobbs, H. H. (1995). Structure of the Human Gene Encoding Sterol Regulatory Element Binding Protein-1 (SREBF1) and Localization of SREBF1 and SREBF2 to Chromosomes 17p11.2 and 22q13. *Genomics* **25**, 667-673.

Ibabe, A., Bilbao, E. and Cajaraville, M. P. (2005). Expression of peroxisome proliferator-activated receptors in zebrafish (*Danio rerio*) depending on gender and developmental stage. *Histochemistry and Cell Biology* **123**, 75-87.

Ibabe, A., Grabenbauer, M., Baumgart, E., Fahimi, H. D. and Cajaraville, M. P. (2002). Expression of peroxisome proliferator-activated receptors in zebrafish (Danio rerio). *Histochemistry and Cell Biology* **118**, 231-239.

Ishingami, M., Honda, T., Takasaki, W., Iked, T., Komai, T., Ito, K. and Sugiyama, Y. (2001). A comparison of the effects of 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) Reductase inhibitors on the CYP3A4-Dependent oxidation Mexazolam in vitro. *DRUG METABOLISM AND DISPOSITION* **29**, 282–288.

Istvan, E. S. and Deisenhofer, J. (2001). Structural mechanism for statin inhibition of HMG-CoA reductase. *SCIENCE* **292**, 1160-1164.

Isusi, E., Aspichueta, P., Liza, M., Hernandez, M. L., Diaz, C., Hernandez, G., Martinez, M. J. and Ochoa, B. (2000). Short- and long-term effects of atorvastatin, lovastatin and simvastatin on the cellular metabolism of cholesteryl esters and VLDL secretion in rat hepatocytes. *Atherosclerosis* **153**, 283-294.

Jacobsen, W., Kuhn, B., Soldner, A., Kirchner, G., Sewing, K.-F., Kollman, P. A., Benet, L. Z. and Christians, U. (2000). Lactonization Is the Critical First Step in the Disposition of the 3-Hydroxy-3-Methylglutaryl-Coa Reductase Inhibitor Atorvastatin. *DRUG METABOLISM AND DISPOSITION* **28**, 1369-1378.

Jeffrey, J. D., Esbaugh, A. J., Vijayan, M. M. and Gilmour, K. M. (2012). Modulation of hypothalamic-pituitary-interrenal axis function by social status in rainbow trout. *General and Comparative Endocrinology* **176**, 201-210.

Jennie, T. C., Judy, A. S., Mary, P. and Lanh, G. (2004). Rhabdomyolysis with HMG-CoA reductase inhibitors and gemfibrozil combination therapy. *Pharmacoepidemiology and Drug Safety* **13**, 417-426.

Jones, O. A. H., Voulvoulis, N. and Lester, J. N. (2001). Human pharmaceuticals in the aquatic environment a review. *Environmental Technology* **22**, 1383-1394.

Kantola, T., Kivisto, K. T. and Neuvonen, P. J. (1998). Effect of itraconazole on the pharmacokinetics of atorvastatin. *Clin Pharmacol Ther* **64**, 58-65.

Kluytmans, J. H. F. M. and Zandee, D. I. (1974). Lipid metabolism in the northern pike (*Esox Lucius L.*)-3. In vivo incorporation of 1-14C-acetate in the lipids. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **48**, 641-649.

Kolditz, C., Borthaire, M., Richard, N., Corraze, G., Panserat, S., Vachot, C., Lefevre, F. and Medale, F. (2008). Liver and muscle metabolic changes induced by dietary energy content and genetic selection in rainbow trout (Oncorhynchus mykiss). *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **294**, R1154-R1164.

Kong, W.-J., Liu, J. and Jiang, J.-D. (2006). Human low-density lipoprotein receptor gene and its regulation. *Journal of Molecular Medicine (Berlin)* **84**, 29-36.

Kruit, J. K., Groen, A. K., van Berkel, T. J. and Kuipers, F. (2006). Emerging roles of the intestine in control of cholesterol metabolism. *World Journal of Gastroenterology* **12**, 6429-6439.

Kubokawa, K., Watanabe, T., Yoshioka, M. and Iwata, M. (1999). Effects of acute stress on plasma cortisol, sex steroid hormone and glucose levels in male and female sockeye salmon during the breeding season. *Aquaculture* **172**, 335-349.

Larsson, A. and Fange, R. (1977). Cholesterol and free fatty acids (FFA) in the blood of marine fish. *Comparative Biochemistry and Physiology* **57B**, 191-196.

Lau, Y. Y., Okochi, H., Huang, Y. and Benet, L. Z. (2006). Multiple Transporters Affect the Disposition of Atorvastatin and Its Two Active Hydroxy Metabolites: Application of in Vitro and ex Situ Systems. *Journal of Pharmacology and Experimental Therapeutics* **316**, 762-771.

Leaver, M. J., Boukouvala, E., Antonopoulou, E., Diez, A., Favre-Krey, L., Ezaz, M. T., Bautista, J. M., Tocher, D. R. and Krey, G. (2005). Three peroxisome proliferator-activated receptor isotypes from each of two species of marine fish. *Endocrinology* **146**, 3150-3162.

Leaver, M. J., Ezaz, M. T., Fontagne, S., Tocher, D. R., Boukouvala, E. and Krey, G. (2007). Multiple peroxisome proliferator-activated receptor beta subtypes from Atlantic salmon (*Salmo salar*). *Journal of Molecular Endocrinology* **38**, 391-400.

Lee, C.-H., Olson, P. and Evans, R. M. (2003). Minireview: Lipid Metabolism, Metabolic Diseases, and Peroxisome Proliferator-Activated Receptors. *Endocrinology* **144**, 2201-2207.

Lee, J., Hong, E. M., Koh, D. H., Choi, M. H., Jang, H. J., Kae, S. H. and Choi, H. S. (2010). HMG-CoA Reductase Inhibitors (Statins) Activate Expression of PPAR alpha/PPAR gamma and ABCA1 in Cultured Gallbladder Epithelial Cells. *Digestive Diseases and Sciences* **55**, 292-299.

Lee, S.-J. and Buhler, D. R. (2002). Functional properties of a rainbow trout CYP3A27 expressed by recombinant baculovirus in insect cells. *DRUG METABOLISM AND DISPOSITION* **30**, 1406-1412.

Lennernas, H. (2003). Clinical pharmacokinetics of atorvastatin. *Clinical Pharmacokinetics* **42**, 1141-1160.

Lewis, G. F. and Rader, D. J. (2005). New Insights Into the Regulation of HDL Metabolism and Reverse Cholesterol Transport. *Circulation Research* **96**, 1221-1232.

Liao, J. K. and Laufs, U. (2005). Pleiotropic effects of statins. *Annual Review of Pharmacology and Toxicology* **45**, 89-118.

Liscum, L., Finer-Moore, J., Stroud, R. M., Luskey, K. L., Brown, M. S. and Goldstein, J. L. (1985). Domain structure of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a glycoprotein of the endoplasmic reticulum. *J. Biol. Chem.* **260**, 522-530.

Lister, A., Regan, C., Van Zwol, J. and Van Der Kraak, G. (2009). Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: A mechanistic evaluation. *Aquatic Toxicology* **95**, 320-329.

Liu, G., Moon, T. W., Metcalfe, C. D., Lee, L. E. J. and Trudeau, V. L. (2005). A teleost in vitro reporter gene assay to screen for agonists of the peroxisome proliferator-activated receptors. *Environmental Toxicology and Chemistry* **24**, 2260-2266.

Luskey, K. L. and Stevens, B. (1985). Human 3-hydroxy-3-methylglutaryl coenzyme A reductase. Conserved domains responsible for catalytic activity and sterol-regulated degradation. *Journal of Biological Chemistry* **260**, 10271-7.

Lűtjohann, D. (2007). Brain cholesterol and suicidal behaviour. *International Journal of Neuropsychopharmacology* **10**, 153–157.

MacCrimmon, H. R. (1971). World Distribution of Rainbow Trout (Salmo gairdneri). *Journal of the Fisheries Research Board of Canada* **28**, 663-704.

Mandard, S., Muller, M. and Kersten, S. (2004). Peroxisome proliferator-activated receptor alpha target genes. *CMLS Cellular and Molecular Life Sciences* **61**, 393-416.

Manera, M. and Britti, D. (2006). Assessment of blood chemistry normal ranges in rainbow trout. *Journal of Fish Biology* **69**, 1427-1434.

Maragno, A. L. G. C., Baqui, M. M. A. and Gomes, M. D. (2006). FBXO25, an F-box protein homologue of atrogin-1, is not induced in atrophying muscle. *Biochimica et Biophysica Acta* **1760**, 966-972.

Martin, G., Duez, H., Blanquart, C., Berezowski, V., Poulain, P., Fruchart, J.-C., Najib-Fruchart, J., Glineur, C. and Staels, B. (2001). Statin-induced inhibition of the

Rho-signaling pathway activates PPARalpha and induces HDL apoA-I. *Journal of Clinical Investigation* **107**, 1423-1432.

Marx, J. (2001). Bad for the Heart, Bad for the Mind? *SCIENCE* **294** 508-509.

Marz, W., Scharnagl, H., Abletshauser, C., Hoffmann, M. M., Berg, A., Keul, J., Wieland, H. and Baumstark, M. W. (2001). Fluvastatin lowers atherogenic dense low-density lipoproteins in postmenopausal women with the atherogenic lipoprotein phenotype. *Circulation* **103**, 1942-1948.

Maxfield, F. R. and Tabas, I. (2005). Role of cholesterol and lipid organization in disease. *Nature* **438**, 612-621.

Metcalfe, C., Miao, X.-S., Hua, W., Letcher, R. and Servos, M. (2004). Pharmaceuticals in the Canadian Environment. In *Pharmaceutical in the Environment, Sources, Fate, Effects, and Risks*, (ed. K. Kummerer). Berlin: Springer. pp. 67-90.

Metcalfe, C. D., Miao, X.-S., Koenig, B. G. and Struger, J. (2003). Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in lower Great Lakes, Canada. *Environmental Toxicology and Chemistry* **22**, 2881–2889.

Miao, X.-S. and Metcalfe, C. D. (2003a). Determination of cholesterol-lowering statin drugs in aqueous samples using liquid chromatography-electrospray ionization tandem mass spectrometry. *Journal of Chromatography A* **998**, 133-141.

Miao, X.-S. and Metcalfe, C. D. (2003b). Determination of pharmaceuticals in aqueous samples using positive and negative voltage switching microbore liquid chromatography/electrospray ionization tandem mass spectrometry. *Journal of Mass Spectrometry* **38**, 27-34.

Miller, D. B. and Spence, J. D. (1998). Clinical pharmacokinetics of fibric acid derivatives (Fibrates). *Clinical Pharmacokinetics* **34**, 155-162.

Miller, W. L. (1988). Molecular Biology of Steroid Hormone Synthesis. *Endocrine Reviews* **9**, 295-318.

Mimeault, C. (2008). Human Pharmaceuticals in the Environment: Effects of Fibrate Drugs on Two Fish Species In *Department of Biology*, vol. PhD, pp. 168. Ottawa: University of Ottawa.

Mimeault, C., Trudeau, V. L. and Moon, T. W. (2006). Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-activated receptor beta (PPARβ) mRNA levels in male goldfish (*Carassius auratus*). *Toxicology* **228**, 140–150.

Mimeault, C., Woodhouse, A. J., Miao, X.-S., Metcalfe, C. D., Moon, T. W. and Trudeau, V. L. (2005). The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, Carassius auratus. *Aquatic Toxicology* **73**, 44–54.

Minghetti, M., Leaver, M. J. and Tocher, D. R. (2011). Transcriptional control mechanisms of genes of lipid and fatty acid metabolism in the Atlantic salmon (Salmo salar L.) established cell line, SHK-1. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1811**, 194-202.

Miserez, A. G., Cao, G., Prosbst, L. C. and Hobbs, H. H. (1997). Structure of the human gene encoding sterol regulatory element binding protein 2 (SREBF2). *Genomics* **40**, 31-40.

Mo, H. and Elson, C. E. (2004). Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. *Experimental Biology and Medicine (Maywood)* **229**, 567-585.

Mommsen, T. P., Moon, T. W. and Walsh, P. J. (1994). Hepatocyes: isolation, maintenance and utilization. In *Biochemistery and molecular biology of fishes*, vol. 3 eds. P. W. Hochachka and T. P. Mommsen), pp. 355-373. Amsterdam: Elsevier Science B.V

Mommsen, T. P., Vijayan, M. M. and Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries* **9**, 211-268.

Moore, M. C., Thompson, C. W. and Marler, C. A. (1991). Reciprocal changes in corticosterone and testosterone levels following acute and chronic handling stress in the tree lizard, Urosaurus ornatus. *General and Comparative Endocrinology* **81**, 217-226.

Morash, A. J., Kajimura, M. and McClelland, G. B. (2008). Intertissue regulation of carnitine palmitoyltransferase I (CPTI): Mitochondrial membrane properties and gene expression in rainbow trout (Oncorhynchus mykiss). *Biochimica et Biophysica Acta* **1778**, 1382-1389.

Morikawa, S., Murakami, T., Yamazaki, H., Izumi, A., Saito, Y., Hamakubo, T. and Kodama, T. (2005). Analysis of the global RNA expression profiles of skeletal muscle cells treated with statins. *Journal of Atherosclerosis and Thrombosis* **12**, 121-131.

Morris, T. J., Palm, S. L., Furcht, L. L. and Buchwald, H. (1995). Effect of Lovastatin alone and as an adjuvant chemotherapeutic agent on hepatoma tissue culture-4 cell growth. *Annals of Surgical Oncology* **2**, 266-274.

Mosconi, G., Carnevali, O., Facchinetti, F., Neri, I. and Polzonetti-Magni, A. (1994). Opioid Peptide Modulation of Stress-Induced Plasma Steroid Changes in the Frog Rana esculenta. *Hormones and Behavior* **28**, 130-138.

Mück, W. (2000). Clinical Pharmacokinetics of Cerivastatin. *Clinical Pharmacokinetics* **39**, 99-116.

Murdock, D. K., Murdock, A. K., Murdock, R. W., Olson, K. J., Frane, A. M., Kersten, M. E., Joyce, D. M. and Gantner, S. E. (1999). Long-term safety and efficacy of combination gemfibrozil and HMG-CoA reductase inhibitors for the treatment of mixed lipid disorders. *American Heart Journal* **138**, 151-155.

Nakamura, I., Evans, J. C., Kusakabe, M., Nagahama, Y. and Young, G. (2005). Changes in steroidogenic enzyme and steroidogenic acute regulatory protein messenger RNAs in ovarian follicles during ovarian development of rainbow trout (Oncorhynchus mykiss). *General and Comparative Endocrinology* **144**, 224-231.

Norum, K. R., Berg, T., Helgerud, P. and Drevon, C. A. (1983). Transport of cholesterol. *Physiological Reviews* **63**, 1343-1400.

Oaks, J. L. and Gilbert, M. (2004). Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* **427**, 630-633.

Olson, K. R. (1992). Blood and extracellular fluid volume regulation: role of the rennin – angiotensin system, kallikrein–kinin system and atrial natriuretic peptides. In *Fish Physiology, Part B The Cardiovascular System*, vol. XII eds. W. S. Hoar D. J. Randall and A. P. Farrell). San Diego: Academic press. pp. 135-232.

Olsson, A. G., McTaggart, F. and Raza, A. (2002). Rosuvastatin: A Highly Effective New HMG-CoA Reductase Inhibitor. *Cardiovascular Drug Reviews* **20**, 303–328.

Passeri, M. J., Cinaroglu, A., Gao, C. and Sadler, K. C. (2009). Hepatic steatosis in response to acute alcohol exposure in zebrafish requires sterol regulatory element binding protein activation. *Hepatology* **49**, 443-452.

Paumelle, R., Blanquart, C., Briand, O., Barbier, O., Duhem, C., Woerly, G., Percevault, F., Fruchart, J.-C., Dombrowicz, D., Glineur, C. et al. (2006). Acute antiinflammatory properties of statins involve peroxisome proliferator-activated receptor-alpha via inhibition of the protein kinase C signaling pathway. *Circulation Research* **98**, 361-369.

Pederson, R. (1988). Cholesterol biosynthesis, storage, and mobilization in steroidogenic organs. In *Biology of Cholesterol*, (ed. P. L. Yeagle), pp. 39-69. Boca Raton, Florida: CRC Press, Inc.

Pfizer. (1996). Lipitor® (Atorvastatin Calcium) Tablets. http://www.pfizer.com/files/products/uspi_lipitor.pdf.

Pfizer. (2010). LOPID® (Gemfibrozil Tablets, USP). http://www.pfizer.com/files/products/uspi_lopid.pdf.

Pfizer®. (1996). Lipitor® (Atorvastatin Calcium) Tablets. http://www.pfizer.com/files/products/uspi_lipitor.pdf.

Prindiville, J. S., Mennigen, J. A., Zamora, J. M., Moon, T. W. and Weber, J.-M. (2011). The fibrate drug gemfibrozil disrupts lipoprotein metabolism in rainbow trout. *Toxicology and Applied Pharmacology* **251**, 201-208.

Prueksaritanont, T., Zhao, J. J., Ma, B., Roadcap, B. A., Tang, C., Qiu, Y., Liu, L., Lin, J. H., Pearson, P. G. and Baillie, T. A. (2002). Mechanistic studies on metabolic interactions between gemfibrozil and statins. *Journal of Pharmacology and Experimental Therapeutics* **301**, 1042-1051.

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L. and Schreck, C. B. (2009). Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture* **297**, 157-162.

Robertson, J. C. and Hazel, J. R. (1995). Cholesterol content of trout plasma membranes varies with acclimation temperature. *Am J Physiol Regul Integr Comp Physiol* **269**, R1113-1119.

Robinson, I., Junnqua, G., Coillie, R. V. and Thomas, O. (2007). Trends in the detection of pharmaceutical products, and their inpact and mitigation in water and wastewater in North america. *Anal Bioanal Chem* **387**, 1143-1151.

Roitelman, J., Olender, E. H., Bar-Nun, S., Dunn, W. A. J. and Simoni, R. D. (1992). Immunological evidence for eight spans in the membrane domain of 3- hydroxy-3-methylglutaryl coenzyme A reductase: implications for enzyme degradation in the endoplasmic reticulum. *Journal of Cell Biology* **117**, 959-973.

Rudling, M., Angelin, B., Stahle, L., Reihner, E., Sahlin, S., Olivecrona, H., Bjorkhem, I. and Einarsson, C. (2002). Regulation of hepatic low-density lipoprotein receptor, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and cholesterol 7alpha-hydroxylase mRNAs in human liver. *Journal of Clinical Endocrinology and Metabolism* **87**, 4307-4313.

Russell, D. W. (1992). Cholesterol biosynthesis and metabolism. *Cardiovascular Drugs and Therapy* **6**, 103-110.

Ruyter, B., Andersen, O., Dehli, A., Farrants, A.-K. O., Gjoen, T. and Thomassen, M. S. (1997). Peroxisome proliferator activated receptors in Atlantic salmon (Salmo salar): Effects on PPAR transcription and acyl-CoA oxidase activity in hepatocytes by peroxisome proliferators and fatty acids. *Biochimica et Biophysica Acta* **1348**, 331-338.

Sakai, J., Duncan, E. A., Rawson, R. B., Hua, X., Brown, M. S. and Goldstein, J. L. (1996). Sterol-Regulated Release of SREBP-2 from Cell Membranes Requires Two Sequential Cleavages, One Within a Transmembrane Segment. *Cell* **85**, 1037–1046.

Sakakura, Y., Shimano, H., Sone, H., Takahashi, A., Inoue, K., Toyoshima, H., Suzuki, S. and Yamada, N. (2001). Sterol Regulatory Element-Binding Proteins Induce an Entire Pathway of Cholesterol Synthesis. *Biochemical and Biophysical Research Communications* **286**, 176-183.

Saunders, R. L., Farrell, A. P. and Knox, D. E. (1992). Progression of coronary arterial lesions in atlantic salmon (*Salmo salar*) as a function of growth rate. *Canadian Journal of Fisheries and Aquatic Sciences* **49**, 878-884.

Schachter, M. (2005). Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update. *Fundamental & Clinical Pharmacology* **19**, 117-125.

Scharnagl, H., Schinker, R., Gierens, H., Nauck, M., Wieland, H. and März, W. (2001). Effect of atorvastatin, simvastatin, and lovastatin on the metabolism of cholesterol and triacylglycerides in HepG2 cells. *Biochemical Pharmacology* **62**, 1545-1555.

Scheen, A. J. (2012). Pharmacokinetic evaluation of atorvastatin and sitagliptin in combination for the treatment of type 2 diabetes. *Expert Opinion on Drug Metabolism & Toxicology* **8**, 745-758.

Schlenk, D., Celander, M., Gallagher, E. P., George, S., James, M., Kullman, S. W., Hurk, P. v. d. and Willett, K. (2008). Biotrasformation in Fishes. In *The Toxicology of Fishes*, eds. R. T. D. Giulio and D. E. Hinton). Boca Raton, FL: CRC Press, Tylor & Francis Group. pp. 153-205.

Schoonjans, K., Staels, B. and Auwerx, J. (1996). The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. *Biochimica et Biophysica Acta* **1302**, 93-109.

Scown, T. M., van Aerle, R., Johnston, B. D., Cumberland, S., Lead, J. R., Owen, R. and Tyler, C. R. (2009). High doses of intravenously administered Titanium Dioxide nanoparticles accumulate in the kidneys of rainbow trout but with no observable impairment of renal function. *Toxicological Sciences* **109**, 372-380.

Seiliez, I., Panserat, S., Skiba-Cassy, S., Fricot, A., Vachot, C., Kaushik, S. and Tesseraud, S. (2008). Feeding status regulates the polyubiquitination step of the ubiquitin-proteasome-dependent proteolysis in rainbow trout (Oncorhynchus mykiss) muscle. *Journal of Nutrition* **138**, 487-491.

Sever, N., Song, B.-L., Yabe, D., Goldstein, J. L., Brown, M. S. and DeBose-Boyd, R. A. (2003a). Insig-dependent Ubiquitination and Degradation of Mammalian 3-Hydroxy-3-methylglutaryl-CoA Reductase Stimulated by Sterols and Geranylgeraniol. *Journal of Biological Chemistry* **278**, 52479-52490.

Sever, N., Yang, T., Brown, M. S., Goldstein, J. L. and DeBose-Boyd, R. A. (2003b). Accelerated Degradation of HMG CoA Reductase Mediated by Binding of Insig-1 to Its Sterol-Sensing Domain. *Molecular Cell* **11**, 25–33.

Shao, W. and Espenshade, P. J. (2012). Expanding Roles for SREBP in Metabolism. *CELL METABOLISM* **16**, 414-419.

Sharpe, R. L., Drolet, M. and MacLatchy, D. L. (2006). Investigation of de novo cholesterol synthetic capacity in the gonads of goldfish (Carassius auratus) exposed to the phytosterol beta-sitosterol. *Reproductive Biology and Endocrinology* **4**.

Shek, A. and Ferrill, M. J. (2001). Statin-fibrate combination therapy. *The Annals of Pharmacotherapy* **35**, 908-917.

Shimano, H. (2009). SREBPs: physiology and pathophysiology of the SREBP family. *FEBS Journal* **276**, 616-621.

Shimomura, I., Shimano, H., Horton, J. D., Goldstein, J. L. and Brown, M. S. (1997). Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *Journal of Clinical Investigation* **99**, 838-845.

Shitara, Y., Hirano, M., Sato, H. and Sugiyama, Y. (2004). Gemfibrozil and Its Glucuronide Inhibit the Organic Anion Transporting Polypeptide 2 (OATP2/OATP1B1:SLC21A6)-Mediated Hepatic Uptake and CYP2C8-Mediated Metabolism of Cerivastatin: Analysis of the Mechanism of the Clinically Relevant Drug-Drug Interaction between Cerivastatin and Gemfibrozil. *Journal of Pharmacology and Experimental Therapeutics* **311**, 228-236.

Shitara, Y. and Sugiyama, Y. (2006). Pharmacokinetic and pharmacodynamic alterations of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors: Drug-drug interactions and interindividual differences in transporter and metabolic enzyme functions. *Pharmacology & Therapeutics* **112**, 71-105.

Shobab, L. A., Hsiung, G.-Y. R. and Feldman, H. H. (2005). Cholesterol in Alzheimer's disease. *The Lancet Neurology* **4**, 841-852.

Simons, K. and Ikonen, E. (2000). How cells handle cholesterol. *SCIENCE* **290**, 1721-1726.

Siperstein, M. D. (1984). Role of cholesterogenesis and isoprenoid synthesis in DNA replication and cell growth. *Journal of Lipid Research* **25**, 1462-1468.

Siperstein, M. D. (1984). Role of cholesterogenesis and isoprenoid synthesis in DNA replication and cell growth. *Journal of Lipid Research* **25**, 1462-8.

Sirtori, C. R. (1993). Tissue selectivity of hydroxymethylglutaryl coenzyme A (HMG CoA) reductase inhibitors. *Pharmacology and Therapeutics* **60**, 431-459.

Skiba-Cassy, S., Lansard, M., Panserat, S. and Françoise, M. (2009). Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **297**, R1421-R1429.

Skolness, S. Y., Durhan, E. J., Jensen, K. M., Kahl, M. D., Makynen, E. A., Villeneuve, D. L. and Ankley, G. T. (2012). Effects of gemfibrozil on lipid metabolism, steroidogenesis, and reproduction in the fathead minnow (Pimephales promelas). *Environmental Toxicology and Chemistry* **31**, 2615-2624.

Song, B. L., Javitt, N. B. and DeBose-Boyd, R. A. (2005). Insig-mediated degradation of HMG CoA reductase stimulated by lanosterol, an intermediate in the synthesis of cholesterol. *CELL METABOLISM* **1**, 179-189.

SoRelle, R. (2001). Baycol withdrawn from market. *Circulation* **104**, E9015-E9016.

Spencer, C. M. and Barradell, L. B. (1996). Gemfibrozil. A reappraisal of its pharmacological properties and place in the management of dyslipidaemia. *Drugs* **51**, 982-1018.

Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E. and Fruchart, J.-C. (1998). Mechanism of action of fibrates on lipid and lipoprotein metabolism. *Circulation* **98**, 2088-2093.

Stancu, C. and Sima, A. (2001). Statins: mechanism of action and effects. *Journal of Cellular and Molecular Medicine* **5**, 378-387.

Stegman, J. J., Goldstone, J. V. and Hahn, M. E. (2010). Perspectives on Zebrafish as a model in environmental toxicology. In *Fish Physiology*, vol. 29 eds. S. F. Perry M. Ekker A. P. Farrel and C. J. Brauner), pp. 367-439. Amsterdam: Academic Press.

Stocco, D. M. (2001). StAR protein and the regulation of steroid hormone biosynthesis. *Annual Review of Physiology* **63**, 193-213.

Stocco, D. M. and Clark, B. J. (1996). Regulation of the Acute Production of Steroids in Steroidogenic Cells. *Endocrine Reviews* **17**, 221-244.

Sun, L.-P., Seemann, J., Goldstein, J. L. and Brown, M. S. (2007). Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: Insig renders sorting signal in Scap inaccessible to COPII proteins. *Proceedings of the National Academy of Sciences* **104**, 6519-6526.

Sun, W., Lee, T.-S., Zhu, M., Gu, C., Wang, Y., Zhu, Y. and Shyy, J. Y.-J. (2006). Statins activate AMP-Activated protein kinase In vitro and In vivo. *Circulation* **114**, 2655-2662.

Ternes, T. A. (1998). Occurance of drugs in German sewage treatment plants and rivers. *Water research* **32**, 3245-3260.

Thorgaard, G. H., Bailey, G. S., Williams, D., Buhler, D. R., Kaattari, S. L., Ristow, S. S., Hansen, J. D., Winton, J. R., Bartholomew, J. L., Nagler, J. J. et al. (2002). Status and opportunities for genomics research with rainbow trout. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **133**, 609-646.

Thorpe, J. L., Doitsidou, M., Ho, S.-Y., Raz, E. and Farber, S. A. (2004). Germ cell migration in Zebrafish is dependent on HMGCoA Reductase activity and prenylation. *Developmental Cell* **6**, 295–302.

Topics, D. (2011). Top 200 brand drugs by retail dollars in 2010. In http://drugtopics.modernmedicine.com/drugtopics/data/articlestandard//drugtopics/252011/727252/article.pdf.

Trudeau, V. L., Mecalfe, C. D., Mimeault, C. and Moon, T. W. (2005). Pharmaceutical in the environment: Drugged fish? In *Biochemistry and Molecular Biology of Fishes*, vol. 6 eds. T. P. Mommsen and T. W. Moon). Amsterdam: Elsevier. pp. 475-493.

Tseng, H.-P., Hseu, T.-H., Buhler, D. R., Wang, W.-D. and Hu, C.-H. (2005). Constitutive and xenobiotics-induced expression of a novel CYP3A gene from zebrafish larva. *Toxicology and Applied Pharmacology* **205**, 247-258.

van Raalte, D. H., Li, M., Pritchard, P. H. and Wasan, K. M. (2004). Peroxisome proliferator-activated receptor (PPAR)-alpha: A pharmacological target with a promising future. *Pharmaceutical Research (Dordrecht)* **21**, 1531-1538.

Velasco-Santamaria, Y. M., Korsgaard, B., Madsen, S. S. and Bjerregaard, P. (2011). Bezafibrate, a lipid-lowering pharmaceutical, as a potential endocrine disruptor in male zebrafish (*Danio rerio*). *Aquatic Toxicology* **105**, 107-118.

Voss, B., Jankowsky, H. D. and Weddigen, P. (1986). Temperature dependence of lipogenesis in isolated hepatocytes from rainbow trout (Salmo gairdneri). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **83**, 13-22.

Walley, T., Folino-Gallo, P., Stephens, P., Van Ganse, E. and EuroMedStat, g. (2005). Trends in prescribing and utilization of statins and other lipid lowering drugs across Europe 1997–2003. *British Journal of Clinical Pharmacology* **60**, 543-551.

Wang, W. and Wong, C.-W. (2010). Statins enhance peroxisome proliferator-activated receptor gamma coactivator-1 alpha activity to regulate energy metabolism. *Journal of Molecular Medicine (Berlin)* **88**, 309-317.

Wang, X., Sato, R., Brown, M. S., Hua, X. and Goldstein, J. L. (1994). SREBP-1 , a Membrane-Bound Transcription Factor Released by Sterol-Regulated Proteolysis. *Cell* **77**, 53-62.

Waterham, H. R. and Wanders, R. J. A. (2000). Biochemical and genetic aspects of 7-dehydrocholesterol reductase and Smith-Lemli-Opitz syndrome. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1529**, 340-356.

Wells, R. M. G. and Pankhurst, N. W. (1999). Evaluation of simple instruments for the measurement of blood glucose and lactate, and plasma protein as stress indicators in fish. *Journal of the World Aquaculture Society* **30**, 276-284.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiological Reviews* **77**, 591-625.

Wensel, T. M., Waldrop, B. A. and Wensel, B. (2010). Pitavastatin: A New HMG-CoA Reductase Inhibitor. *Annals of Pharmacotherapy* **44**, 507-514.

Westerfield, M. (2000). The zebrafish book. A guide for the laboratory use of zebrafish (Dnio rerio). Eugene: University of Oregon Press.

Xu, L. and Simoni, R. D. (2003). The inhibition of degradation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase by sterol regulatory element binding protein cleavage-activating protein requires four phenylalanine residues in span 6 of HMG-CoA reductase transmembrane domain. *Archives of Biochemistry and Biophysics* **414**, 232-243.

Yabe, D., Brown, M. S. and Goldstein, J. L. (2002). Insig-2, a Second Endoplasmic Reticulum Protein That Binds SCAP and Blocks Export of Sterol Regulatory Element-Binding Proteins. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 12753-12758.

Yang, T., Espenshade, P. J., Wright, M. E., Yabe, D., Gong, Y., Aebersold, R., Goldstein, J. L. and Brown, M. S. (2002). Crucial Step in Cholesterol Homeostasis: Sterols Promote Binding of SCAP to INSIG-1, a Membrane Protein that Facilitates Retention of SREBPs in ER. *Cell* **110**, 489–500.

Yokoyama, C., Wang, X., Briggs, M. R., Admon, A., Wu, J., Hua, X., Goldstein, J. L. and Brown, M. S. (1993). SREBP-1, a Basic-Helix-Loop-Helix-Leucine Zipper Protein That Controls Transcription of the Low Density Lipoprotein Receptor Gene. *Cell* **75**, 187-197.