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The Characterization of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (HMGCoAR) in Rainbow Trout (*Oncorhynchus mykiss)* and the Effect of Statin Drugs on HMGCoAR

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Chelsie M. Estey

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Abstract

The presence of pharmaceuticals in the aquatic environment is a growing area of concern. The objective of this thesis was to examine the effects of statin drugs, a class of pharmaceuticals prescribed to lower endogenous cholesterol production by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR), in the rainbow trout *Oncorhynchus mykiss*. The study also aimed to provide some insight into mechanisms governing the control of HMGCoAR in fish.

Two statin drugs were used in this study, cerivastatin (CVT) and atorvastatin (AVT). Cerivastatin inhibited hepatic microsomal and brain homogenate HMGCoAR activities when incubated *in vitro* and following an *in vivo* intra-peritoneal injection. Atorvastatin reduced HMGCoAR activity *in vitro* following incubation with liver microsomes and brain homogenates.

Fasting trout for 14 days resulted in a significant decrease in plasma cholesterol and glucose levels compared with the fed-controls. A significant decrease was observed in brain homogenates prepared from fish fasted for 14 days and re-fed for 7 days.

Phosphorylation is an important regulator of mammalian HMGCoAR activities. In trout a significant decrease in HMGCoAR activity was observed when liver microsomes were incubated in a buffer that should stimulate AMPK.

Two HMGCoAR subtypes were found in rainbow trout. HMGCoAR-1 mRNA is present in higher quantities than HMGCoAR-2 however HMGCoAR-1 is located in a limited number of tissues. HMGCoAR-2 mRNA appeared in all tissues assessed.

The results of this thesis indicate that HMGCoAR shares some similar control mechanisms with mammals. These results also demonstrate that statin drugs in the aquatic environment have the potential to disrupt HMGCoAR in fish.

Abstract

The presence of pharmaceuticals in the aquatic environment is a growing area of concern. The objective of this thesis was to examine the effects of statin drugs, a class of pharmaceuticals prescribed to lower endogenous cholesterol production by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR), in the rainbow trout *Oncorhynchus mykiss*. In addition, the study also aimed to provide some insight into mechanisms governing the control of HMGCoAR in fish. Statin drugs are detected in wastewater treatment plant (WWTP) influent, effluent, and surface waters post-WWTP.

Two statin drugs were used in this study, cerivastatin (CVT) and atorvastatin (AVT). Cerivastatin inhibited hepatic microsomal and brain homogenate HMGCoAR activities when incubated *in vitro* and following an *in vivo* intra-peritoneal injection. Atorvastatin reduced HMGCoAR activity *in vitro* following incubation with liver microsomes and brain homogenates. The IC_{50} value for the CVT-exposed liver microsomes was 26 and 17 nM for 250 and 75 g trout, respectively, and for brain homogenates 17 and 23 nM, respectively. The IC_{50} value for the AVT-exposed liver microsomes prepared from 75 g fish was 23 nM and from brain homogenates 17 nM.

Fasting trout for 14 days resulted in a significant decrease in plasma cholesterol and glucose levels compared with the fed-controls, while in the re-fed fish glucose levels were significantly higher than the fed-control and cholesterol levels remained significantly reduced. A minor decrease in HMGCoAR activities in liver microsomes occurred in the fasted fish as well as a significant decrease in brain homogenates prepared from fish fasted for 14 days and re-fed for 7 days.

Phosphorylation is an important regulator of mammalian HMGCoAR activities, and my study indicates that this is also the case in rainbow trout. A non-significant decrease in HMGCoAR activity was observed when liver microsomes were incubated in a buffer that should stimulate protein kinase A (PKA) and PKC; incubating in an AMPK-stimulating buffer led to a significant decrease in HMGCoAR activity.

Two HMGCoAR subtypes were found in rainbow trout. Based on real-time PCR analysis, mRNA of the first subtype is present in higher quantities than the second subtype however HMGCoAR-1 is located to a limited number of tissues, primarily liver, brain, intestine, and gill. HMGCoAR-2 mRNA is more ubiquitous appearing in all tissues assessed. My results support a more important role for HMGCoAR-1 in controlling cholesterol biosynthesis in fish.

The results of this thesis indicate that HMGCoAR shares similar control mechanisms with mammals. These results also demonstrate that statin drugs in the aquatic environment have the potential to disrupt HMGCoAR in a non-target species.

Résumé

La présence des produits pharmaceutiques dans l'environnement est un sujet d'actualité. L'objectif de cette thèse était de déterminer l'impact des statines, une classe de médicaments qui réduit la production endogène de cholestérol, chez la truite arc-en-ciel *Oncorhynchus mykiss*. Ce projet avait aussi comme objectif d'étudier les mécanismes gouvernant le contrôle de l'enzyme HMGCoAR dans les poissons. Les statines ont été détectées dans les eaux municipales usées ainsi que dans les cours d'eau.

Les deux statines cerivastatin et atorvastatin ont été utilisées dans cette étude. Cerivastatin réduit l'activité de HMGCoAR dans les microsomes du foie et dans un homogénat du cerveau suite à une exposition *in vitro* et à une injection IP *in vivo*. Atorvastatin réduit l'activité de HMGCoAR dans les microsomes du foie et dans un homogénat du cerveau suite à une exposition *in vitro*. Les concentrations nécessaires pour inhiber 50% de l'activité enzymatique (IC_{50}) pour les truites de 250 g et 75 g exposées au CVT sont respectivement de 26 et 17 nM dans les microsomes hépatiques et de 17 et 23 nM dans l'homogénat du cerveau. La valeur IC_{50} dans les microsomes hépatiques provenant de truites de 75g exposées à l'AVT est de 23 nM et de 17 nM dans l'homogénat du cerveau.

Les niveaux de cholestérol et de glucose plasmiques ont significativement diminué chez les poissons soumis à un jeûne de 14 jours comparativement aux poissons nourris servant de témoins. Par contre, en comparant les poissons n'ayant pas jeûné avec ceux qui avaient jeûné et par la suite mangé, les niveaux de glucose étaient plus élevés et le cholestérol était plus bas chez ces derniers. Le jeûne a également provoqué une faible diminution de l'activité de HMGCoAR dans les microsomes du foie ainsi qu'une diminution significative dans les homogénats de cerveaux provenant de poissons qui avaient jeûné 14 jours et par la suite mangé pendant 7 jours.

Nous savons que la phosphorylation joue un rôle important dans la régulation de l'activité enzymatique de HMGCoAR chez les mammifères et les résultats de cette étude suggèrent le même phénomène chez les poissons. Une faible diminution de l'activité de HMGCoAR a été observée dans les microsomes du foie incubés en présence de solution tampon qui aurait dû stimuler les kinases A et C (PKA et PKC). Une diminution significative de l'activité de HMGCoAR a été observée dans les microsomes du foie incubés en présence d'une solution tampon qui stimule l'AMPK.

Il existe deux types de HMGCoAR chez la truite arc-en-ciel. Les analyses par réactions en chaîne par polymérase en temps réel (real-time PCR) indiquent que le premier type est présent en plus grande quantité que le deuxième. Toutefois, la distribution de l'ARN messager du type 1 est spécifique au foie, cerveau, intestin et branchies alors que le type 2 est retrouvé dans tous les tissus examinés. Mes résultats indiquent que l'enzyme HMGCoAR-1 joue un rôle important dans le contrôle de la biosynthèse du cholestérol chez les poissons.

Les résultats de cette étude indiquent que les mécanismes de contrôle de l'enzyme HMGCoAR chez la truite arc-en-ciel sont semblables à ceux des mammifères. Ces résultats démontrent également que les statines retrouvées dans l'environnement aquatique peuvent modifier l'activité enzymatique de HMGCoAR dans certaines espèces de poissons.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviation	Description
ACC	acetyl-CoA carboxylase-1
AMPK	AMP-activated protein kinase
AVT	atorvastatin
CEPA	Canadian environmental protection act
CoQ_{10}	ubiquinone
CVT	cerivastatin
ERA	environmental risk assessment
GEM	gemfibrozil
HMGCoAR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HMGCoAS	3-hydroxy-3-methylglutaryl coenzyme A synthase
HDL	high density lipoproteins
LDL	low density lipoproteins
PKA	protein kinase A
PKC	protein kinase C
PPCPs	pharmaceuticals and personal care products
WWTP	wastewater treatment plant

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degree will certainly open many doors in the future. It has served as a stepping stone into a life-long journey of learning. It has taught me to question things and to relish in trying to find the answer. Regardless of where life leads I know that my many memories of time spent in the Moon lab learning and laughing with wonderful people will remain with me.

Chapter 1 General Introduction

I. Rationale

The pharmaceuticals used in this study, cerivastatin and atorvastatin, are prescribed to lower the endogenous production of cholesterol in humans. Their mode of action is to competitively inhibit the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR), the rate-limiting enzyme in endogenous cholesterol biosynthesis (Kennelly and Rodwell, 1985). Atorvastatin is detected in wastewater treatment plant (WWTP) effluents and in surface waters in Ontario (Metcalfe *et al.*, 2003). It is also amongst the world's most prescribed medicines, and prescription rates have steadily increased for the past several years (Rx List, 2005). There is significant published literature on the ubiquitous nature of pharmaceuticals in the environment and these studies demonstrate that some of these drugs alter the physiology and biochemistry of non-target aquatic organisms (Halling-Sørensen *et al.*, 1998; Daughton and Ternes, 1999; Heberer, 2002; Kolpin *et al.*, 2002; Lange and Dietrich, 2002; Miao, *et al.*, 2002; Seiler, 2002; Sanderson *et al.*, 2004; Mimeault *et al.*, 2005; Jjemba, 2006).

Presently there are no studies that examine the effects of statin drugs in the aquatic environment on fish, although one study did examine the effects of statins in the higher aquatic plant *Lemna gibba* (Brain *et al.*, 2006). As this class of pharmaceuticals is measured in WWTP effluent and surface waters, it is important to examine their effects on non-target organisms given that prescription rates are increasing and more will enter the aquatic environment as human use increases.

The present study will use rainbow trout, *Oncorhynchus mykiss*, to investigate the effects of atorvastatin and cerivastatin on HMGCoAR activities and control.

II. Pharmaceuticals in the environment

Pharmaceuticals and personal care products (PPCPs) in the environment are an emerging area of concern. The increased interest in this area comes with an ever-expanding literature pointing to the ubiquitous nature of pharmaceutical products in the aquatic environment (Halling-Sørensen *et al.*, 1998; Daughton and Ternes, 1999; Kolpin *et al.*, 2001; Heberer, 2002; Lange and Dietrich, 2002; Miao, *et al.*, 2002; Seiler, 2002; Sanderson *et al.*, 2004; Mimeault *et al.*, 2005; Jjemba, 2006). Pharmaceuticals are designed to elicit a specific biological response in humans and to persist long enough to have their desired effect. In addition, pharmaceuticals are designed to act on specific cellular effectors, and many if not most such effectors, are highly conserved at least across vertebrates but also amongst some invertebrate and plant species. These features, among others, suggest these products may potentially act on non-target organisms as they do in mammals but may also have unintended effects especially given their persistent or pseudo-persistent (continuously added to the environment) nature. Aquatic organisms are particularly vulnerable targets as they may be exposed to PPCPs from WWTP effluent for the entire duration of their life or at critical points within their life history (Fent *et al.*, 2006).

The prescription rates of pharmaceutical products continue to rise as populations' age. In the European Union approximately 3000 different products are used in human medicine (including contraceptives, anti-inflammatory drugs, lipid regulators, etc.); in addition a large number of pharmaceuticals are used in veterinary medicine (including antibiotics and anti-inflammatory drugs) (Fent *et al.*, 2006).

Human pharmaceuticals, for the most part, enter the environment through ingestion and excretion ultimately entering municipal WWTPs where they can be degraded, adsorbed to

sewage sludge, or become diluted into the surrounding water (Carlsson *et al.*, 2006). Their presence may also be augmented as people dispose of their unused drugs down the drain or in their garbage (Jones *et al.*, 2001). Pharmaceuticals can be excreted either as the parent compound or as metabolites, which may also be active (Seiler, 2002). Pharmaceuticals applied to the terrestrial environment (e.g. from agricultural practices, manure from animals treated with drugs, application of sewage sludge to lands, etc.) can enter the aquatic environment by run-off and seepage into the groundwater (Fig. 1.1).

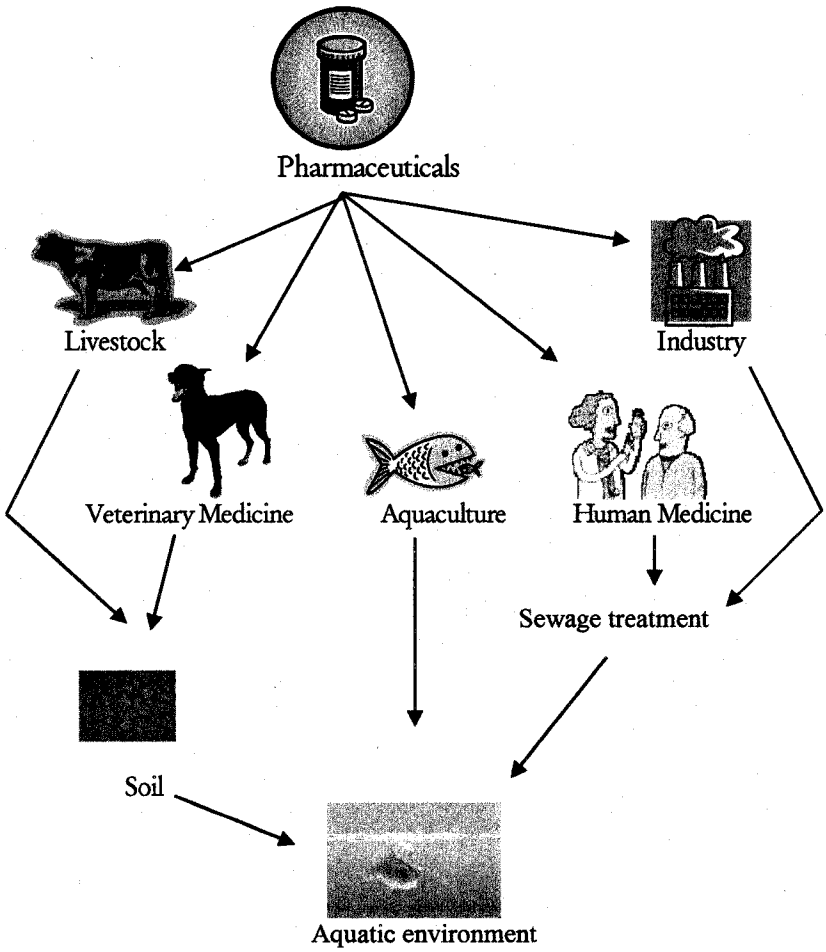


Figure 1.1. A flowchart outlining some common routes of release of pharmaceuticals into the aquatic environment.

Thus pharmaceuticals are continually being added to the environment and aquatic organisms may be chronically exposed to an increasing list of PPCPs especially as the North American and European populations' age.

The concern is the environmental persistence, toxicity, potency, and biological activity of these products. One such example of the negative impact of biologically active compounds in the environment is estrogen and estrogen-like compounds. Natural and synthetic estrogens are measured in municipal WWTP effluents, the primary source of estrogens in the environment (Brion *et al.*, 2004). Natural estrogens originate primarily from human females that produce 10-30 μg daily (Aldercruetz *et al.*, 1994). Estrogens can also come from the use of conjugated estrogens in the treatment of cancer, osteoporosis, menopause (estrogen replacement therapy), and hypogonadism (Arcand-Hoy *et al.*, 1998). Synthetic estrogens including the oral contraceptive 17α-ethinylestradiol also enter the environment through WWTP effluent (Williams *et al.*, 1999). Zebrafish exposed to environmentally relevant concentrations of 17α-ethinylestradiol show delayed sexual maturation, skewed sex ratios in favor of females, induction of vitellogenin and production of ovatestes in males (Hill and Janz, 2003). The consequence of exposure to such compounds in the environment is potentially an alteration in the fitness of fish populations.

Pharmaceuticals other than estrogens are now appearing in the environment post-WWTP. Many examples of diverse drug classes are detected in municipal wastewaters at levels in the ng/L to μg/L range, including anti-inflammatory agents (e.g. ibuprofen, aspirin, diclofenic), stimulants (e.g. caffeine), analgesics (e.g. acetaminophen), lipid and cholesterol regulators (e.g. atorvastatin, gemfibrozil) and antibiotics (e.g. oxytetracycline) (Daughton and Ternes, 1999; Kolpin *et al.*, 2001; Heberer, 2002; Metcalfe *et al.*, 2003; Carlsson *et al.*, 2006;

Gagné *et al.*, 2006; Jjemba, 2006). The sheer number of different compounds entering the environment is of concern. In major urban centers these drugs are coming from municipal WWTPs into the aquatic environment continually and can persist for days or more (Gagné *et al.*, 2006).

A. Effects of human pharmaceuticals in the aquatic environment

The effects of exposure to PPCPs are not a laboratory phenomenon. The presence of a pharmaceutical in WWTP effluent in the USA was first reported by Garrison *et al.* (1976) when clofibric acid was detected at levels of 0.8-2.0 μg/L. Several studies report that caged male fish held in WWTP effluents respond by producing vitellogenin, a well-known biomarker of estrogen exposure (Sumpter and Jobling, 1995; Harries *et al.*, 1997; Sumpter, 1998). Wild populations of roach (*Rutilis rutilis*) were sampled upstream and downstream of WWTPs throughout the British Isles and histological examination identified many of the male fish as "intersex", e.g. having both male and female gonadal traits (Jobling *et al.*, 1998). The proportion of intersex males ranged from 4% at a control site to 100% at two sites located downstream from a WWTP (Jobling *et al.*, 1998). Another study by Nash *et al.* (2004) found that long-term exposure to ethinylestradiol resulted in reproductive failure in fish. A study by Mimeault *et al.* (2005) used the fibrate drug gemfibrozil (GEM), a lipid-regulating drug that is detected in wastewater, surface water, and drinking water in North America and Europe (Ternes, 1998; Metcalfe *et al.*, 2004). Mimeault *et al.* (2005) found that GEM was taken-up from the surrounding water at environmental levels (1.5 μg/L) and concentrated in the blood of goldfish (*Carassius auratus*) up to 500-fold. In this same study fish were exposed to aqueous concentrations including an environmentally-relevant concentration of GEM for 96 h and 14

days and plasma testosterone levels were reduced by over 50% (Mimeault *et al.*, 2005). This study demonstrates that environmental levels of an environmentally ubiquitous drug, GEM, has the potential to bioconcentrate in fish plasma and alter steroid hormone levels, potentially disrupting reproductive ability. Another study examined the cytotoxic and oxidative effects of PPCPs detected in municipal WWTP effluents in Ontario and found that many drugs were detected in the effluent, including caffeine, ibuprofen, cotinine, gemfibrozil, and bezafibrate (Gagné *et al.*, 2006). Nearly all of the compounds tested accelerated oxidation of NADPH and induced lipid peroxidation when incubated with rainbow trout microsomes *in vitro* indicating that these drugs have oxidizing properties (Gagné *et al.*, 2006).

As can be seen from the growing literature on the effects of pharmaceuticals in the aquatic environment, this is an evolving area of research. It is important that we gain a better understanding of the effects of the various classes of drugs on non-target organisms. If there are consequences of the presence of these compounds in the environment, treatment of municipal wastewater may need to be altered and remediation programs may need to be implemented. Ultimately legislation may need to be enacted to address these issues.

B. Legislation regarding human pharmaceuticals

Despite literature pointing to the presence of significant quantities of pharmaceuticals being released into the aquatic environment, specific regulations for ecological risk assessment are largely lacking (Fent *et al.*, 2006). It is important to be able to predict the potential of a drug to cause detrimental effects to the environment and its inhabitants, therefore environmental risk assessments (ERA) are conducted to examine this probability. It is only recently that regulatory bodies have released guidelines on how pharmaceuticals should be assessed (Fent *et al.*, 2006).

The first ERA that was a requirement for registration of pharmaceuticals was developed in 1995 by a European Union Directive and the corresponding Note for Guidance for Veterinary Pharmaceuticals (EMEA, 1998; Fent *et al.*, 2006). More recently a draft guideline was released indicating that authorization for a human medicinal product must be accompanied by an ERA (EMEA, 2005). The Canadian Environmental Protection Act (CEPA) 1999 requires that all new substances registered in Canada be examined for their potential risks to the Canadian environment and human health (EII, 2006). The Environmental Assessment Regulations cover all new substances contained in products regulated under the Food and Drugs Act, including pharmaceuticals and veterinary drugs. The United States Food and Drug Administration published guidelines for the assessment of human drugs, stating that applicants in the US are required to provide an ERA report when the anticipated introduction of the active ingredient of the pharmaceutical product entering the aquatic environment achieved a concentration $\geq$ 1 μg/L (FDA-CDER, 1998).

III. Statin drugs and human health

This thesis will examine the effects of a class of drugs designed to lower human plasma cholesterol levels. These drugs called "statins" are amongst the most prescribed pharmaceuticals in the Western world as complications of obesity continue to escalate. In 1997, combined prevalence of overweight and obese men and women in Canada was >50% and >30%, respectively (Allison and Saunders, 2000). Overweight and obesity are associated with secondary conditions including elevated plasma cholesterol, hypertension, and coronary heart disease (Allison and Saunders, 2000). Elevated plasma cholesterol predisposes humans to develop atherosclerotic lesions that lead to the formation of coronary artery atherosclerosis,

increasing the risk of myocardial infarction (Jones and Lefer, 2001). Statins are prescribed to overcome elevated levels of cholesterol and to reduce the potential risk of heart attacks. In Canada it is estimated that 36% of all deaths result from cardiovascular disease (Baker and Tarnopolsky, 2001) and coronary heart disease is the leading cause of death in Western countries (Castelli *et al.*, 1986, Grundy, 1990; Moghadasian, 1999).

Statin drugs affect the endogenous production of cholesterol that accounts for more than two-thirds of cholesterol present in the human body (Liao, 2005); the remaining cholesterol is derived from the diet. Changes in endogenous cholesterol synthesis occurs as the result of the reversible inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase or HMGCoAR, the enzyme responsible for catalyzing the conversion of HMGCoA to mevalonate, the rate limiting step in the cholesterol synthesis pathway (Fig. 1.2). The affinity of HMGCoAR for statins is several orders of magnitude higher than the natural substrate, HMGCoA (Moghadasian, 1999). Mevalonate production is the committed step in cholesterol synthesis because it is characterized by an irreversible reduction of HMGCoA by HMGCoAR and is thus an important target to control endogenous cholesterol synthesis (Moghadasian, 1999). Inhibition of HMGCoAR results in reduced cholesterol synthesis and impaired feedback inhibition of cholesterol on both HMGCoAR and HMGCoA synthase (HMGCoAS) (Fig. 1.2) (Baker and Tarnopolsky, 2001). These drugs are also capable of increasing the number of hepatic low density lipoprotein (LDL) receptors on the cell-surface to enhance uptake and catabolism of LDL, thus lowering serum cholesterol levels and increasing the ratio of high density (HDL) to low density (LDL) lipoproteins in the blood (Liao, 2005).

Presently five statin drugs are marketed in North America including atorvastatin (Lipitor®), fluvastatin (Lescol®), lovastatin (Mevacor®), pravastatin (Pravachol®), and

simvastatin (Zocor®) (Baker and Tarnopolsky, 2001). These statins can be divided into two groups: those administered as the pro-drug (lactone form) (e.g. lovastatin and simvastatin) that are processed in the body to produce the active metabolites, and as the active form (acid form) (e.g. pravastatin, fluvastatin, atorvastatin, and cerivastatin) that are taken in the active form (Moghadasian, 1999; Ishigami *et al.*, 2001). Statins are susceptible to an interconversion between the active (hydroxy acid) and inactive (lactone) form that is pH dependent (Baker and Tarnopolsky, 2001; Grabarkiewicz *et al.*, 2006). Hepatic hydrolysis at an alkaline pH activates the lactone pro-drug (Baker and Tarnopolsky, 2001). Each statin has a structural component resembling HMGCoA but differs from this natural substrate in that they are more hydrophobic (Istan, 2003). HMGCoAR undergoes a conformational change that exposes a hydrophobic binding site that allows the hydrophobic groups of the statin molecules to bind (Istan, 2003).

Of the statins marketed, Lipitor® or atorvastatin manufactured by Pfizer, Inc, is one of the world's most prescribed medicines. With coronary heart disease recognized as a principal cause of mortality in developed countries, the use of statin drugs including Lipitor® will continue to increase (Liao, 2005). In addition numerous reports indicate that there are pleiotropic effects of statins, including usefulness in the treatment of Alzheimer's, in reducing inflammation, and in enhancing antioxidant properties (Jones and Lefer, 2001; Liao, 2005). These benefits may go beyond cholesterol-lowering effects and may contribute to further increases in prescription rates for this class of pharmaceuticals. Thus more of these drugs will enter the aquatic environment. In 2003 Metcalfe and colleagues reported the concentration of various statins in WWTP influent, effluent and surface water (Metcalfe *et al.*, 2003; Miao and Metcalfe, 2003). Atorvastatin was found at levels of 76 ng/L in the influent, 37 ng/L in the treated effluent, and 1 ng/L in the surface water (Miao and Metcalfe, 2003). Their study detected

atorvastatin, lovastatin, pravastatin, and simvastatin in the untreated influent and in the treated effluent; however only atorvastatin was detected in surface waters (Miao and Metcalfe, 2003).

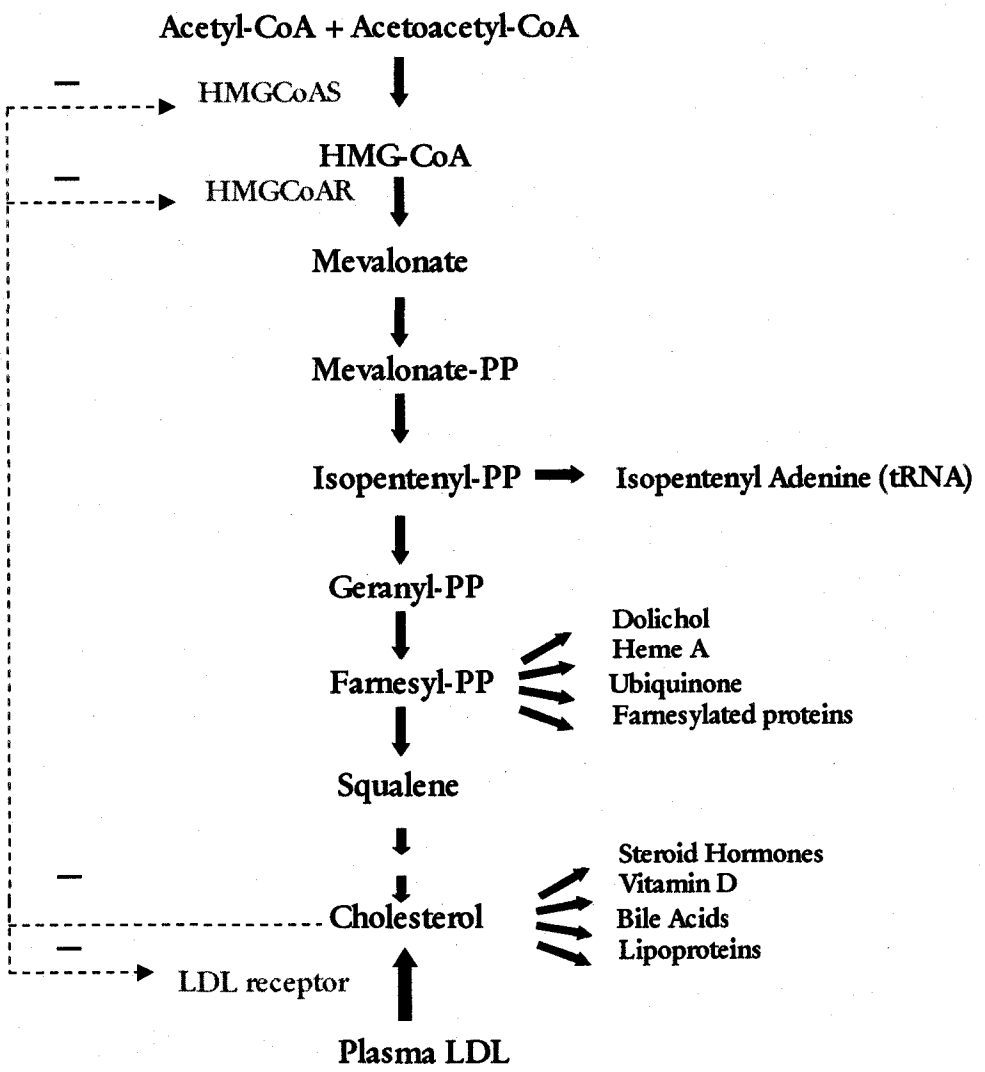


Figure 1.2. The mevalonate pathway in animal cells. The major product of this pathway is cholesterol, which is obtained from both endogenous and exogenous sources. Statin drugs competitively inhibit HMGCoAR activities. Also noted are the paths of inhibition throughout this scheme. The mevalonate pathway also produces non-sterol isoprenoids, as seen on the right (adapted from Goldstein and Brown, 1990).

This project will focus on cerivastatin (CVT) or Baycol® manufactured by Bayer Inc. and atorvastatin (AVT) or Lipitor® manufactured by Pfizer, Inc. CVT was one of the first statins marketed but was removed from the market in 2001. AVT is of particular interest as this statin is found in surface waters in Canada (Miao and Metcalfe, 2003) and is the leading statin prescribed today. Assuming HMGCoAR inhibitors lower endogenously produced cholesterol in non-target organisms as in mammals, effects on cholesterol dynamics in these organisms may be expected. Cholesterol is involved in many important processes including modulating membrane fluidity and as a precursor to all steroid hormones (Stryer, 1995) (Fig. 1.3).

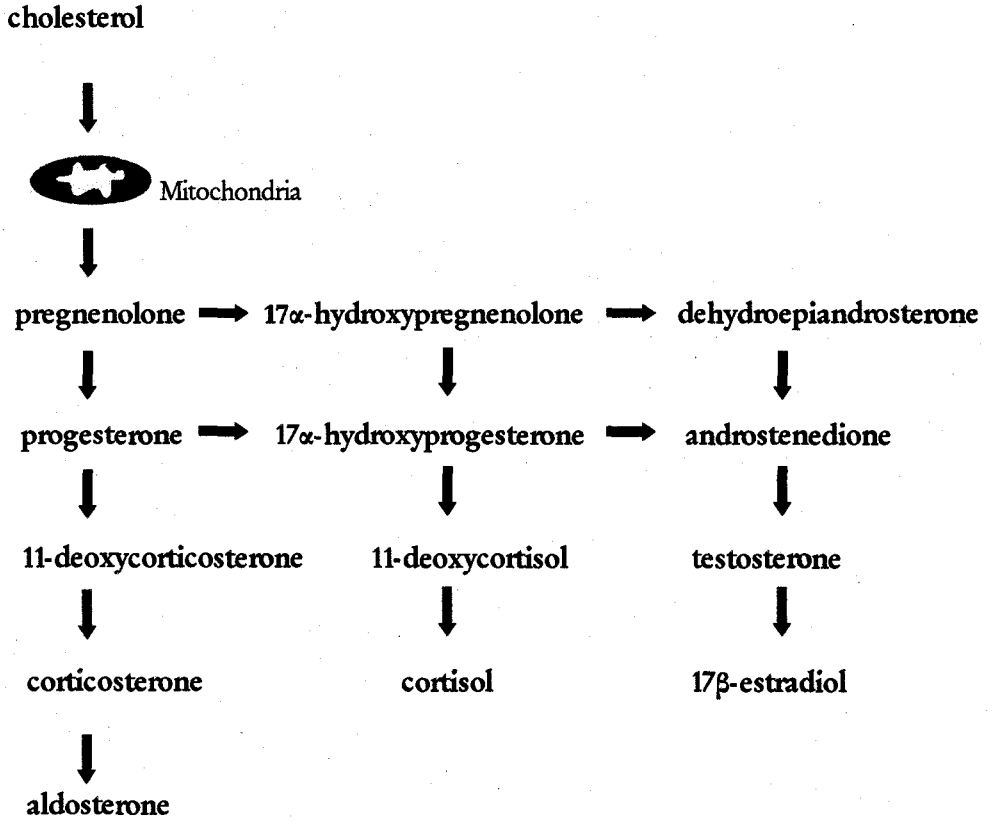


Figure 1.3. The major pathways of steroid biosynthesis. Listed are the biosynthetic pathways leading from cholesterol to the major steroids products of the adrenal, ovary, and testis (adapted from Stocco, 2001).

IV. Cholesterol in vertebrates

Very little is known regarding cholesterol dynamics in fish, in particular as it relates to steroidogenesis. In fact it is unclear precisely the role of blood cholesterol (from lipoproteins) and tissue cholesterol biosynthesis in the production of steroids in mammals (Azhar *et al.*, 2003). Steroid hormones are synthesized in the adrenals, gonads, placenta and central nervous system of mammals (Stocco, 2000). Without sufficient amounts of cholesterol, steroidogenesis may be impaired, meaning that sufficient levels of glucocorticoids such as cortisol/corticosterone, sex hormones such as estrogen and testosterone, and mineralocorticoids such as aldosterone in mammals, may be compromised. Glucocorticoids are involved in the stress response, they promote gluconeogenesis, suppress inflammation and play a role in electrolyte and water excretion (Mommsen *et al.*, 1999). Estradiol is involved in many steps mediating reproduction, including vitellogenesis in oviparous species and embryo implantation in viviparous species (Trudeau *et al.*, 2005). Testosterone is involved in spermatogenesis, development, and it initiates and maintains male secondary sexual characteristics (Cavaco *et al.*, 1998). In mammals, aldosterone is involved in regulating ion balance by promoting the re-absorption of sodium, chloride and bicarbonate ions in the kidneys (Becker *et al.*, 2003). Thus steroid hormones are critical to survival and thus sufficient amounts of cholesterol are necessary to ensure these processes occur at an appropriate rate.

Fish are continually facing environmental stressors including temperature changes, infection, and pollutants, and as such, they need the capacity to respond to these stressors to ensure survival. Without an adequate supply of cholesterol the fish may be unable to mount an appropriate stress response. Insufficient amounts of cholesterol for steroidogenesis could also have severe implications on reproduction and subsequent fitness of fish populations. Deb and

Bhattacharya (1986) tracked the incorporation of intramuscularly injected (4-^{14}C)-cholesterol into ovarian tissue and subsequently sex steroid hormones in freshwater perch (*Anabas testudineus*). They found that circulating cholesterol is the primary source of substrate for ovarian steroidogenesis prior to, but not during or after, spawning periods. In male fish, androgens are involved in spermiation and the acquisition of secondary sexual characteristics; without stimulation by androgens breeding behaviors and breeding success may be affected (Cavaco *et al.*, 1998). Estrogens play an important role in development and reproduction. For example, estrogens control the production of vitellogenin from which a major component of fish oocytes is derived (Hill and Janz, 2003).

The importance of cholesterol synthesis to whole body cholesterol homeostasis can be examined using statins that lower serum cholesterol by inhibiting HMGCoAR (Ness and Chambers, 2000). Inhibition of this enzyme results in lowered cholesterol synthesis but may also affect the synthesis of other products downstream of mevalonate, including ubiquinone (CoQ_{10}) and dolichol (Fig. 1.2). Ubiquinone is a component of the mitochondrial electron transport chain and thus important in energy production, and in its reduced form, it has antioxidant properties that serve to protect membranes against damage from free radicals (Altekin *et al.*, 2002). Altekin *et al.* (2002) demonstrated that treatment with simvastatin decreased plasma ubiquinone levels in humans and Folkers *et al.* (1990) reported that blood levels of ubiquinone were reduced by nearly 75% in humans treated with lovastatin. Dolichol increases cell membrane fluidity and permeability, participates in *n*-glycosylation of proteins required for their transport and function, and is involved in the synthesis of phosphatidylinositol anchors for membrane proteins (Krag, 1998). Although statins are designed to inhibit

cholesterol biosynthesis by blocking the activity of HMGCoAR, there are other products in the mevalonate pathway that may also be affected.

Given continuous increases in prescription rates for statin drugs and the fact that in 2003 AVT was already detected in surface waters, examining the effect of statin drugs on non-target organisms, particularly fish, is timely. This study will provide insight into whether or not statin drugs act in fish as they are designed to act in mammals, by inhibiting HMGCoAR. With this knowledge we could implement monitoring programs to examine the effects of these drugs in natural aquatic systems as environmental levels of statins will presumably continue to rise. With advanced warning on how these drugs may affect non-target species, we can take action to avoid harmful effects in wild populations.

V. Hypothesis and Prediction

There is an expanding literature pointing to the ubiquitous nature of PPCPs in the aquatic environment (Daughton and Ternes, 1999; Halling-Sørensen *et al.*, 1998; Kolpin *et al.*, 2001; Miao, *et al.*, 2002; Sanderson *et al.*, 2004; Fent *et al.*, 2006). In terms of human use of pharmaceuticals, atorvastatin, a cholesterol-reducing drug, is one of the world's best selling medicines (RxList, 2005). In a study done by Miao and Metcalfe (2003) atorvastatin was detected in both sewage influent and effluent, and it was the only statin measured in surface water. Given their ever-increasing popularity and the emergence of literature on the pleiotropic effects of statins, it is important to examine the potential affects of statin drugs on aquatic species.

These studies are undertaken to examine the effect of statin drugs on HMGCoAR in the rainbow trout *Oncorhynchus mykiss*. It is hypothesized that exposure to cerivastatin and

atorvastatin will decrease HMGCoAR activity in tissues where cholesterol synthesis is active. HMGCoAR activities will be assessed *in vitro* in liver microsomes and brain homogenates, two tissues where HMGCoAR transcript levels are among the highest.

In mammals, fasting was found to decrease HMGCoAR enzyme activities (Bucher *et al.*, 1960; Linn, 1967; White and Rudney, 1970; Slakey *et al.*, 1972). Slakey *et al.* (1972) found that fasting resulted in decreased enzyme activities for five of seven enzymes examined in the cholesterol pathway, including HMGCoAR which fell to a greater extent than the other enzymes. I predict that with-holding food will result in a decrease in HMGCoAR enzyme activity in trout. Previous studies in mammals report that HMGCoAR is regulated by reversible phosphorylation/dephosphorylation (Hunter and Rodwell, 1980; Feingold *et al.*, 1983; Kennelly *et al.*, 1983; Kennelly and Rodwell, 1985). This regulatory mechanism will be examined using the trout enzyme and it is predicted that when incubated under conditions that stimulate phosphorylation, HMGCoAR activities will decrease.

Studies using mammals found that fasting activated AMP-activated protein kinase (AMPK) in some tissues (Witters *et al.*, 1994; Culmsee *et al.*, 2001; Proszkowiec-Weglarz *et al.*, 2006). When AMPK is activated it increases cellular energy supply by turning on ATP-generating pathways and decreasing energy demand by turning off ATP-utilizing pathways. This leads to inhibition of the synthesis of hepatic fatty acid and cholesterol due to the phosphorylation of acetyl-CoA carboxylase-1 (ACC), HMGCoAR, and hormone-sensitive lipase (Clarke and Hardie, 1992; Gillespie and Hardie, 1992; Proszkowiec-Weglarz *et al.*, 2006). Although the precise mechanism of phosphorylation is not understood, it is known that AMPK is activated during fasting in higher vertebrates, which if HMGCoAR is controlled by phosphorylation in fish, may decrease HMGCoAR activities.

Previous studies reported that two HMGCoAR subtypes exist in the zebrafish (*Danio rerio*) (Thorpe *et al.*, 2004). Using cloning and sequence analyses, these transcripts will be investigated in the rainbow trout.

Chapter 2 Characterization of Rainbow Trout HMGCoAR

I. Introduction

3-Hydroxy-3-methylglutaryl coenzyme A reductase or HMGCoAR is located to the endoplasmic reticulum of mammalian cells and catalyzes the reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) to mevalonate, the rate-limiting step in the pathway for the endogenous synthesis of cholesterol and isoprenoids (Marrero *et al.*, 1986; see Fig. 1.2). The enzyme is believed to be composed of two portions, a membrane anchoring region consisting of an 8-membrane spanning domain and a catalytic domain that projects into the cytoplasm (Luskey and Stevens, 1985; Kumagai *et al.*, 1995; Corsini *et al.*, 1995). HMGCoAR activity is found in most mammalian tissues although liver and intestine activities are amongst the highest (Kennelly and Rodwell, 1985; Ness and Chambers, 2000). These two tissues together are responsible for as much as 75% of mammalian cholesterol biosynthesis (Turley *et al.*, 1981; Kennelly and Rodwell, 1985). Cholesterol is an important membrane sterol, a precursor to bile acids and steroid hormones, and a component of hedgehog protein, a signaling molecule involved in embryogenesis (Ness and Chambers, 2000). In addition to cholesterol production, the mevalonate pathway also produces ubiquinone (CoQ_{10}) and heme A involved in the mitochondrial electron transport chain, dolichol needed for glycoprotein synthesis, and intracellular messengers including steroid hormones in animals, cytokines in plants, farnesylated mating factors in fungi, and juvenile hormones in insects (Fig. 1.2) (Goldstein and Brown, 1990; Ness *et al.*, 1994; Hargreaves *et al.*, 2005). Given these multiple roles, it is not surprising that HMGCoAR is highly regulated including control over the amount of enzyme (transcription, translation, protein degradation) and by reversible phosphorylation of the existing enzyme (Goldstein and Brown, 1990). The intricacy of this regulation was first discovered through the

use of HMGCoAR inhibitors from fungi, including compactin and lovastatin (Endo, 1988; Goldstein and Brown, 1990). This enzyme is also known to be the target of statin drugs to reduce endogenous cholesterol synthesis (Maher and Thompson, 1990; Moghadasian, 1999; Baker and Tarnopolsky, 2001).

There is evidence for both transcriptional control by cholesterol (in hamsters and mice) and translation control (in rats) of HMGCoAR (Ness and Chambers, 2000). When hamsters were fed a diet containing 2% cholesterol for 14 days, a 6-fold reduction in hepatic HMGCoAR mRNA level was observed (Goldstein *et al.*, 1986); this significant reduction was not seen using rats under similar experimental conditions (Ness *et al.*, 1991). These data indicate that transcriptional control could play an important role in feedback regulation in cholesterol-sensitive animals like hamsters and mice but not in rats that are more resistant to dietary cholesterol manipulation (Goldstein *et al.*, 1986; Ness *et al.*, 1991; Shimomura *et al.*, 1997; Ness and Chambers, 2000). In other experiments, rats fed a diet containing 2% cholesterol over a 48 h period showed a 6-fold decrease in specific HMGCoAR protein synthesis but not general hepatic protein synthesis (Chambers and Ness, 1998; Ness and Chambers, 2000). This decrease accounted for the reduction seen in both HMGCoAR protein levels and enzyme activity (Chambers and Ness, 1998). Both sterols (e.g. cholesterol) which regulate at the level of transcription, and non-sterols (e.g. farnesol) are reported to be involved in the rate of degradation of HMGCoAR at least in mammalian cell lines (Goldstein and Brown, 1990; Gil *et al.*, 1985; Kumagai *et al.*, 1995; Meigs *et al.*, 1996; Meigs and Simoni, 1997). It is also found using liver tissue that hormones including thyroid hormones improve mRNA transcript stability, and glucocorticoids destabilize while estrogen stabilizes HMGCoAR mRNA (Ness and Chambers, 2000; Baker and Tarnopolsky, 2001).

HMGCoAR activity has been extensively studied in vertebrates (Hunter and Rodwell, 1980; Gregory *et al.*, 1972; Marrero *et al.*, 1986; Field *et al.*, 1987), including a few studies in fish (Hunter and Rodwell, 1980; Teichert and Wodtke, 1987). Early evidence found that reversible phosphorylation-dephosphorylation was a key mechanism regulating existing HMGCoAR activities. Mg·ATP decreased HMGCoAR activities in many tissues including brain, liver, and intestine (Beg *et al.*, 1973; Shah, 1981; Ingebritsen *et al.*, 1978; Nordstrom *et al.*, 1977; Gebhard *et al.*, 1985) and in a variety of animal species including pigs, dogs, rodents, chickens, frogs, catfish, insects, and humans (Kennelly and Rodwell, 1985). Catfish (*Ameiurus melas*) HMGCoAR is inactivated by Mg·ATP and reactivated using a broadly specific phosphoprotein phosphatase (Hunter and Rodwell, 1980). Several studies found that sodium fluoride (NaF), a potent inhibitor of phosphatase activities, blocked the activation of HMGCoAR (Nordstrom *et al.*, 1977; Oku *et al.*, 1984). A purified cytosolic phosphorylase *a* phosphatase was found to reactivate a Mg·ATP-inactivated HMGCoAR (Ingebritsen *et* al., 1978; Kennelly and Rodwell, 1985) and a microsomal HMGCoAR kinase inactivated HMGCoAR (Beg *et al.*, 1980; Ferrer and Hegardt, 1984) which could then be activated by dephosphorylation using phosphatases (Gil *et al.*, 1980; Ferrer and Hegardt, 1984). These studies clearly support control of mammalian and potentially fish HMGCoAR activities by reversible phosphorylation-dephosphorylation.

The strong diurnal rhythm in the activity of hepatic HMGCoAR observed in mammals (Shapiro and Rodwell, 1971; Edwards and Gould, 1972) was thought to be controlled by the state of phosphorylation but more recent studies showed that the changes in enzyme activity were associated with changes in the level of HMGCoAR protein (Ness and Chambers, 1996). Peak enzyme activities occurred after consumption of food (Ness and Chambers, 2000) which

may be linked to stimulation by insulin (leads to increased reductase activity by transcriptional effects) an effect which is countered by glucagon which opposes insulin's transcriptional control of HMGCoAR (Ness and Chambers, 2000; Baker and Tarnopolsky, 2001). Other studies have demonstrated that fasting decreases HMGCoAR activities which subsequently recover with re-feeding. These data suggest that cholesterol synthesis is controlled by the levels of HMGCoAR activity during most of the period of fasting and re-feeding (Slakey *et al.*, 1972; White and Rudney, 1972).

Studies have reported that fasting activates AMP-activated protein kinase (AMPK) activities in specific tissues (Witters *et al.*, 1994; Culmsee *et al.*, 2001; Proszkowiec-Weglarz *et al.*, 2006). AMPK responds primarily to intracellular AMP/ATP ratios (Friesen and Rodwell, 1997) although numerous hormones also affect activities (Hardie, 2006) leading to the inactivation of HMGCoAR and acetyl-CoA carboxylase-1 (ACC) (Carling *et al.*, 1989; Parker *et al.*, 1989; Gillespie and Hardie, 1992; Proszkowiec-Weglarz *et al.*, 2006). Whether the fish enzyme is controlled by specific protein kinases (e.g. PKA, PKC, and AMPK) is unclear. However, a study by Teichert and Wodtke (1987) showed that carp (*Cyprinus carpio*) HMGCoAR activities were affected by the presence of NaF which inhibits the activity of protein phosphatases, and by NaCl.

Many studies have looked at HMGCoAR in mammals however few studies have investigated HMGCoAR in fish and the mechanisms by which this enzyme is controlled. Given the significance of HMGCoAR in the mevalonate pathway, the regulation of this enzyme in fish is important. This study characterizes HMGCoAR activities in the rainbow trout *Oncorhynchus*

mykiss and whether changes in food intake and phosphorylation may affect these activities. These studies will lead to further research to determine the impact of statin drugs on HMGCoAR activity.

II. Materials and Methods

A. Materials

3-Hydroxy-3-methylglutaryl coenzyme A, glucose-6-phosphate, NADP and glucose assay chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Glucose-6-phosphate dehydrogenase (yeast grade I) was purchased from Roche (Laval, QC, Canada). 3-Hydroxy-3-methyl(3-^{14}C)glutaryl coenzyme A was purchased from Amersham Biosciences (Baie d'Urfe, QC, Canada). Plastic-backed thin layer chromatography (TLC) plates (silica gel 60, 20 x 20 cm) were obtained from VWR (Ville Mont-Royal, QC, Canada) and were later purchased from Whatman (Maidstone, Kent, England). Ninety-six well flat bottom plastic microtiter plates were obtained from Corning Inc. (Corning, NY, USA). Cholesterol kits were purchased from Wako Diagnostics (Richmond, VA, USA). TRIzol, DNase I, and primers were purchased from Invitrogen Life Technologies (Burlington, ON, Canada). All other chemicals were purchased from local suppliers.

B. Experimental Design

Fish

Rainbow trout, *Oncorhynchus mykiss* (primarily females) were purchased locally from Linwood Acres Trout Farm (Campbellcroft, ON). Fish were acclimated to 13°C for at least 1 week in 1.2 m^3 tanks supplied with water from the City of Ottawa that was oxygenated and dechloraminated at the University of Ottawa Aquatic Care Facility. Trout were used exclusively from two weight classes, 75 g and 250 g. Fish were fed commercial trout feed *ad libitum* and held under a photoperiod regimen that mimicked the natural light cycle in the City of Ottawa; these fish were used for the HMGCoAR enzyme characterization study. All experiments were

conducted under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhere to the guidelines set forth by the Canadian Council for Animal Care for the use of animals in teaching and research.

Fasting/Re-feeding study

A second group of 75 g trout held under the same conditions as above were subsequently divided into three treatment groups: control, fasted for 14 days, and fasted and re-fed for 7 days (called re-fed). The control and re-fed groups were fed 1% of body weight for the duration of the experiment.

At the end of the treatment period fish were anaesthetized with benzocaine [1 ml benzocaine (10 mg/L)/2 L of water] and weighed. Blood was removed from the caudal vessel using a 23-gauge needle attached to a heparinized 1 ml syringe. Following exsanguinations, fish were euthanized by trans-spinal sectioning. Whole brain and liver were removed and portions were snap frozen in liquid nitrogen and kept at -80°C until analyzed. Plasma was separated from red blood cells by a 10 min centrifugation at 10,000 x g (Beckman-Coulter microfuge R centrifuge) and aliquots were stored frozen at -80°C until analyzed. The portions of the tissue to be used for preparation of homogenates and microsomes (see below) were kept on ice until processed. Tissues from groups of two fish were pooled to provide adequate sample for analysis. The frozen tissue samples were later used for isolation of total RNA and the plasma was used for cholesterol and glucose analyses.

C. Tissue preparation

Preparation of the liver microsomes

Livers were rinsed with cold 0.25 M sucrose, weighed, minced using a razor blade and then placed in a tight fitting hand-held homogenizer. The tissue was homogenized using 5 strokes in 50% (w/v) isolation buffer (0.25 M sucrose, 5 mM EDTA, and 50 mM NaCl, pH 7.5), then diluted a further 25% (w/v) and homogenized with 2 additional strokes. The homogenate was poured into 1.5 ml plastic centrifuge tubes and centrifuged at 10,000 x g for 15 min at 4°C (Beckman-Coulter microfuge R centrifuge); the supernatant was then transferred to a new 1.5 ml centrifuge tube and again centrifuged at 10,000 x g for 15 min at 4°C. The resulting supernatant was then centrifuged at 100,000 x g for 60 min at 4°C using a Beckman-Coulter Optima TL Ultracentrifuge. The resulting microsome pellet was resuspended by gentle homogenization in buffer B (30 mM imidazole, 5 mM β-mercaptoethanol, pH 7.5) and aliquots stored at -80°C until assayed within a three week period.

Preparation of brain homogenate

The entire brain was rinsed with cold 0.25 M sucrose as above. The hindbrain was removed and discarded as preliminary assays found it contained low enzyme activity. The brain was homogenized in isolation buffer as above, the homogenate centrifuged in 1.5 ml plastic tubes at 10,000 x g for 15 min at 4°C, transferred to new 1.5 ml centrifuge tubes and again centrifuged at 10,000 x g for 15 min at 4°C. The resultant supernatant was aliquoted and stored at -80°C until assayed within a three week period.

D. Estimates of HMGCoAR activities

This assay was modified from a previous fish study by Teichert and Wodke (1987). Liver microsomes and brain homogenates were thawed on ice and diluted in buffer B to 0.6 mg and 0.5 mg protein per 50 µl, respectively. Fifty µl buffer C (100 mM KH_2PO_4, 500 mM NaCl,

60 mM EDTA, and 5 mM β-mercaptoethanol, pH 7.5) was added to the microsomes. The mixture was incubated for 10 min prior to addition of 50 µl cofactor-substrate solution consisting of (final concentrations) 4.5 µmol glucose-6-phosphate, 3 units glucose-6-phosphate dehydrogenase, 450 nM NADP, and 20 nmol ^{14}C labeled HMGCoA/non-radioactive HMGCoA each dissolved in buffer A (50 mM KH_2PO_4, 250 mM NaCl, 30 mM EDTA, and 5 mM β-mercaptoethanol, pH 7.5). This mixture was allowed to incubate for 30 min at 25°C. Reactions were stopped by adding 15 µl 10 M HCl and the samples were kept at 25°C for 1 h to ensure complete lactonization of mevalonic acid (Teichert and Wodtke, 1987). Protein was sedimented by centrifugation at 3000 rpm for 4 min. Plastic-backed silica gel TLC plates were marked and cut into equal sections of 6.6 cm. Thirty µl of the protein free mixture was spotted onto the bottom of each plate (R_f = 0.0). Plates were developed in tanks in a solvent of benzene and acetone (1:1 v/v). Once the solvent migrated to the top of the plate (R_f = ~10), the plates are removed from the chambers and dried in a fumehood. The dried plates were cut into strips corresponding to the different zones of migration of each component of the reaction: e.g. HMGCoA/HMG, R_f ~0.0; mevalonolactone, R_f ~0.4. Each zone was scrapped into vials containing 5 ml high flash point Safety-Solve scintillation cocktail (Research Products International, Mount Prospect, IL, USA). The radioactivity was assessed using a Beckman-Coulter LS6500 scintillation counter; 3 min was sufficient to collect significant counts. Background was assessed by adding 15 µl 10 M HCl to either the microsome or homogenate preparations prior to the addition of the cofactor-substrate solution and processing this mixture as above. The activity of HMGCoAR was expressed as pmol/min/mg of protein based upon estimates of the specific activity of the ^{14}C-HMGCoA added to each mixture.

Phosphorylation-dephosphorylation experiments

Liver microsomes were prepared and used to examine the effect of phosphorylation and dephosphorylation conditions on HMGCoAR activity. Microsome protein (0.6 mg) was incubated for 2 h in a total volume of 100 µl with one of four buffers that promote phosphorylation (protocol modified from Storey, 1994):

1) control incubation: 2 mM EDTA, 2 mM EGTA, 25 mM NaF
2) PKA stimulation: 5 mM Mg·ATP, 25 mM NaF, 1mM cAMP
3) PKC stimulation: 5 mM Mg·ATP, 25 mM NaF, 1.3 mM $CaCl_2$, 7 µg/ml phorbol myristate acetate
4) AMPK stimulation: 5 mM Mg·ATP, 25 mM NaF, 1 mM AMP

Following incubation at 25°C, preparations were assayed for HMGCoAR activities as noted above.

Dephosphorylation used liver microsomes (0.6 mg protein) and a 2 h incubation period in a total volume of 100 µl using one of three buffers as modified from Teichert and Wodtke (1987) and Storey (1994):

1) control: 0.25 M sucrose, 5 mM EDTA
2) phosphorylation buffer: 0.25 M sucrose, 5 mM EDTA, 50 mM NaF
3) dephosphorylation buffer: 0.25 M sucrose, 5 mM EDTA, and 50 mM NaCl

Following incubation at 25°C, preparations were assayed for HMGCoAR activities as above.

E. Biochemical assays

Protein determination

Bovine serum albumin (BSA) standards ranging from 0 to 1 mg/ml were prepared in distilled and autoclaved water. Ten µl sample or standard were loaded onto a 96 well microtiter plate and 200 µl of the prepared BioRad dye reagent concentrate (Hercules, CA, USA) was added to each well. The plate was stirred briefly and incubated at room temperature for 5 min. Absorbance values were assessed with a Spectramax Plus 384 spectrophotometer (Molecular

Devices, Sunnyvale, CA, USA) at 595 nm. Sample concentrations were determined from the BSA standard curve.

Cholesterol measurements

Total plasma cholesterol levels were determined using a Wako Cholesterol E kit. In brief, 3 µl of sample (plasma, standard, or blank) was added to individual wells of a 96 well microtiter plate. Three-hundred µl color reagent was added, plates mixed well and allowed to incubate at 37°C for 5 min. Absorbance is then measured at 600 nm using the microplate spectrophotometer (as above). Cholesterol values are calculated based on a standard curve provided in the kit.

Glucose measurements

Plasma glucose values are determined by loading 10 µl glucose standards or plasma samples onto a 96 well microtiter plate. Two-hundred µl glucose reaction mixture (60 mM Trizma base, 40 mM Tris-HCl, 1 mM $MgSO_4$, 2 mM NAD^+, 1 mM ATP, 0.1 units/ml G6PDH, *Leuconostoc*) was added to each well. The plate was incubated for 5 min at room temperature and absorbance assessed using the microplate spectrophotometer at 340 nm. Ten µl hexokinase was added to each well, and the mixture incubated at room temperature for 30 min and a final reading taken at 340 nm. The difference between the two readings was assessed and compared to a glucose standard curve to determine glucose concentrations.

F. Analysis of trout HMGCoAR mRNA

RNA isolation

Frozen tissue was ground in liquid N_2 using a RNase-free mortar and pestle. The crushed tissue (50-100 mg) was transferred to a 1.5 ml RNAse-free plastic centrifuge tube. RNA

isolations were carried out using TRIzol (Invitrogen Life Technologies, Burlington, ON, Canada) according to the manufacturer's protocol. Tissue samples were homogenized in 1.0 ml TRIzol reagent and incubated at room temperature for 5 min. Then 0.2 ml chloroform was added to each sample tube, the samples were shaken by hand for 15 sec and then incubated at room temperature for an additional 3 min. Samples were then centrifuged at 4°C for 15 min at 12,000 x g. The upper aqueous phase of each sample was removed and transferred to a new 1.5 ml RNase-free centrifuge tube. The RNA was precipitated through the addition of 0.5 ml isopropyl alcohol. Samples were incubated at room temperature for 10 min and centrifuged for 10 min at 12,000 x g at 4°C. Following centrifugation the supernatant was removed and the RNA pellet rinsed once with 1 ml 75% ethanol. Samples were vortexed and centrifuged at 4°C for 5 min at 7,500 x g. The supernatant was again removed and the pellet was air-dried in the fumehood for 5-10 min. RNA pellets were dissolved in 50 µl DEPC water and incubated at 55-60°C for 10 min. Samples were stored at -80°C.

DNase treatment of RNA samples

Samples were DNase-treated using the Deoxyribonuclease I kit (Invitrogen Life Technologies, Burlington, ON, Canada) to remove any contaminating DNA according to the manufacturer's protocol. In summary, 1 µg RNA was mixed with 1 µl 10X DNase I reaction buffer, 1 µl DNase I and DEPC water added on ice to make a total volume of 10 µl in a PCR tube. The tubes were removed from the ice and incubated at room temperature for 15 min followed by the addition of 1 µl 25 mM EDTA solution. The tubes were then incubated at 65°C for 10 min.

cDNA synthesis

cDNA synthesis was performed using the 1st Strand cDNA Synthesis Kit and M-MLV RT (Invitrogen Life Technologies, Burlington, ON, Canada) according to the manufacturer's protocol. In summary, 1 µl oligo (dT), 5 µl DNase-treated total RNA, 1 µl 10 mM dNTP mix, and 5 µl sterile distilled water were added to a RNase-free centrifuge tube. The contents were heated to 65°C for 5 min and then quickly chilled on ice. The tube was briefly centrifuged followed by the addition of 4 µl 5X first-strand buffer, 2 µl 0.1M DTT, and 1 µl RNaseOUT. The contents of the tube were gently mixed and incubated at 37°C for 2 min. One-µl M-MLV RT was added and mixed by pipetting the mixture up and down, the tube was then incubated for 50 min at 37°C. The reaction was inactivated by heating at 70°C for 15 min. All cDNA was stored at -20°C.

PCR Reaction

The PCR reaction was carried out using the Invitrogen™ Life Sciences *Taq* DNA Polymerase kit. Each reaction contained 16.4 µl water (autoclaved and distilled), 2.5 µl 10X PCR buffer, 1 µl forward primer, 1 µl reverse primer, 2 µl dNTP (2.5 mM), 1.0 µl $MgCl_2$ (50 mM), 1.0 µl cDNA template and 0.125 µl *Taq*. To avoid pipetting errors, a master mix was made up with all ingredients that were common to all PCR tubes.

Real-time PCR

Isolation of tissue RNA. Total cellular RNA was isolated from frozen tissues of the rainbow trout using TRIzol reagent as above with the following exception. Four µl linear acrylamide (2 mg/µl) (Ambion, Austin, TX) was added prior to the addition of isopropanol; samples were then placed on dry ice for 30 min. This modification assisted in the precipitation of RNA from tissues of small size, thus increasing the yield of RNA (Gaillard and Strauss,

1990). RNA concentrations and quality were verified using spectrophotometry (OD at 260 nm) and RNA gel electrophoresis, respectively.

Real-time PCR analysis of HMGCoAR subtypes 1 and 2 tissue distribution. HMGCoAR subtypes 1 and 2 and β-actin (reference control) were assessed using real-time PCR analysis and DNA amplification using SYBR green (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. The degenerate primers were designed from a multiple alignment (for HMGCoAR-1: *Dicentrarchus labrax*, accession #AY424801; *Gallus gallus,* accession #AB109635; *Rattus norvegicus*, accession #BC064654) (for HMGCoAR-2: *Homo sapiens*; accession #AAH33692; *Rattus norvegicus*; accession #NP_037266; *Dicentrarchus labrax*, accession #AAR02862; *Danio rerio*, accession #s NP_001073446 and; NP_001014314) using CODEHOP (http://bioinformatics.weizmann.ac.il/blocks/codehop. html) and the gene specific primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were synthesized (Invitrogen) to yield 180 bp

HMGCoAR-1 real-time PCR 5'-CTCTGGCCAGGTTACAGGAG-3'(F)

5'-TCTACCAGGGCTTCAGTGCT-3'(R)

HMGCoAR-2 real-time PCR 5'-TCCCTGAGCTCCAGGTTCTA-3'(F)

5'-CATGGCTGAGCCCACTAGAT-3'(R)

HMGCoAR2 degenerate primer

5'-GGCGACGCCATGGGNATGAAYATG-3'(F)

5'-GCAGGCGCCCTGCACNCCNARCAT-3'(R)

β-actin 5'-CGTCCCAGGCATCAGGGAGT-3' (F)

5'-TCTCCATGTCGTCCCAGTTG -3' (R).

HMGCoAR consensus sequence

5'-CTCTGGCCAGGTTACAGGAG-3'(F)

5'-TCTACCAGGGCTTCAGTGCT-3'(R)

The Q-PCR products were cloned to ensure that the amplification was identical to the original sequence. Forty cycles of a two-step PCR protocol were used: 1x 2 min at 94°C, 40x (30 sec at 94°C, 30 sec at 58°C, 1 min at 72°C), and 1x 10 min at 72°C. A no template control for each master mix and a no RT (reverse transcriptase) control were included in each analysis. A threshold value is set above baseline that reflects the average of emittance during the initial PCR cycles. The Ct value (cycle number) is the point at which the emittance passes above the baseline (threshold) and thus mirrors the accumulation of PCR product in a specific well of the PCR reaction. The relative mRNA expression level of the gene of interest was determined by comparing the different Ct values between the gene of interest and that of the internal control (β-actin). Thus the lower the Ct value difference, the higher mRNA expression level of the gene of interest compared with the internal control and vice versa. The slopes of the standard curves for HMGCoAR-1, HMGCoAR-2, and β-actin were –3.221, -3.085 and –3.015, respectively, giving a corresponding primer efficiency of 104.4%, 110.9% and 114.6%, respectively.

Q-PCR data were reported as 2^(-ΔCt) with β-actin as the reference gene. ΔCt is the difference between the Ct values of the HMGCoAR subtypes and β-actin gene.

G. Statistical analysis

Experimental results are presented as means ± standard error of the mean (SEM). A value of $p < 0.05$ was considered significant. All statistical analyses were conducted using SigmaStat™ 3.1 software (SPSS Corp., Chicago, IL, USA). Statistical significance was tested with one-way ANOVA; where significant differences were detected an appropriate post-hoc test was used (Dunn's where samples sizes were not equal; Tukey in all remaining experiments).

III. Results

A. Tissue distribution for HMGCoAR subtypes 1 and 2 in rainbow trout

Semi-quantitative PCR

A tissue distribution of HMGCoAR mRNA was completed using semi-quantitative PCR analysis and 18S as the control gene. The tissues with the most intense bands were brain, gill, intestine, liver, and stomach (Fig. 2.1).

Real-time PCR

A second tissue distribution was undertaken when it was found that the zebrafish (*Danio rerio*) contained two HMGCoAR subtypes (Thorpe *et al.*, 1994). This experiment utilized real-time PCR a more quantitative technique than the previously used semi-quantitative PCR. β-Actin was present in all samples and variation between tissues was minimal, thus it was used to compare relative HMGCoAR transcript levels between tissues for the real-time PCR analysis. mRNA expression of HMGCoAR subtype 1 (HMGCoAR-1) (Fig. 2.2A) was seen in liver (the highest expression), brain (olfactory lobe/cerebrum, optic lobe, cerebellum, hypothalamus, and pituitary), intestine, and gill. HMGCoAR-2 was more ubiquitous in nature but was present at lower levels than HMGCoAR-1. This second subtype was found in all tissues examined (liver, gill, heart, intestine, kidney, white muscle, and 5 brain parts; Fig. 2.2B).

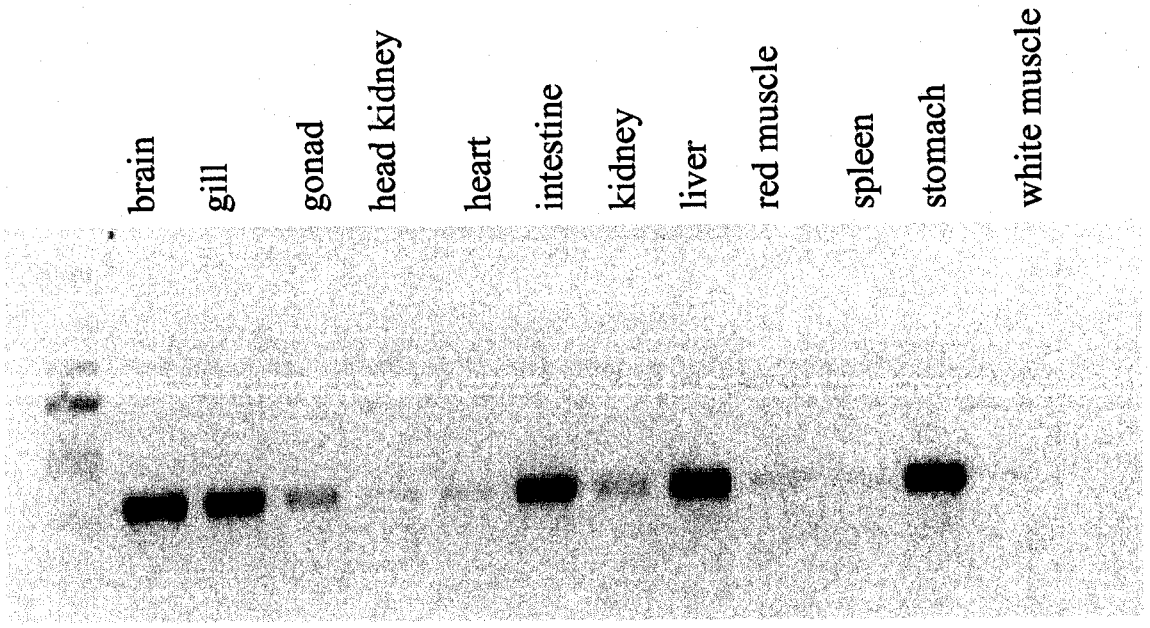


Figure 2.1: A tissue distribution for HMGCoAR in rainbow trout using semi-quantitative PCR analysis. The most intense bands are brain, gill, intestine, liver, and stomach, and ladder is located on the left.

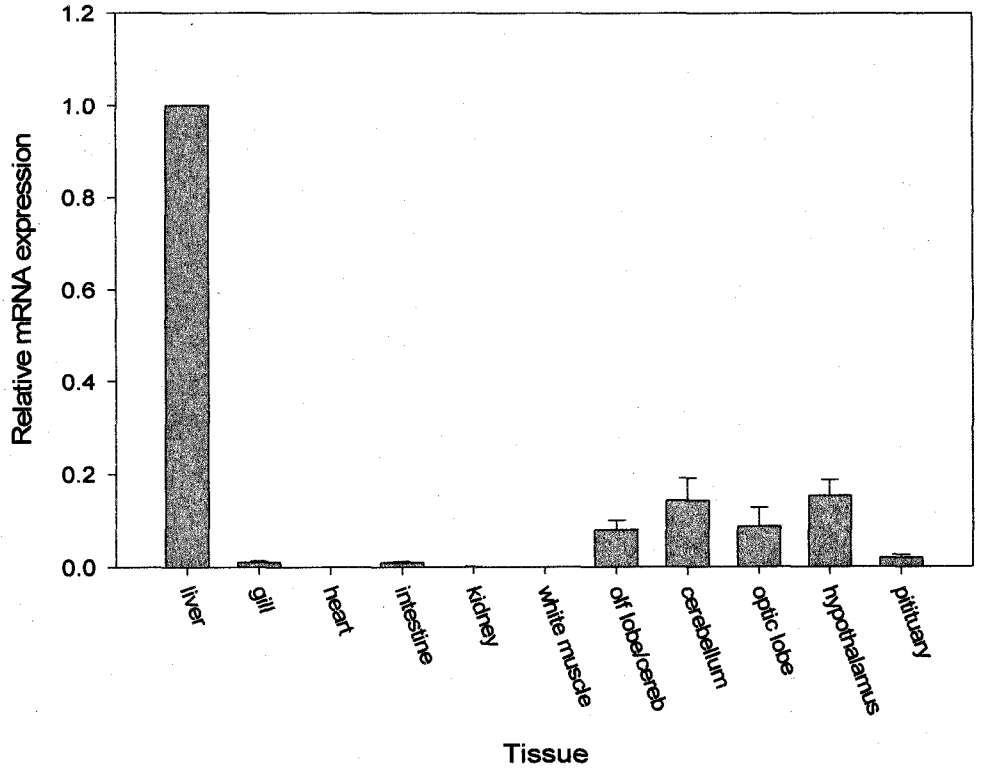


Figure 2.2A. Tissue distribution for HMGCoAR subtype 1 using real-time PCR analysis; data generated by Xi Chen.

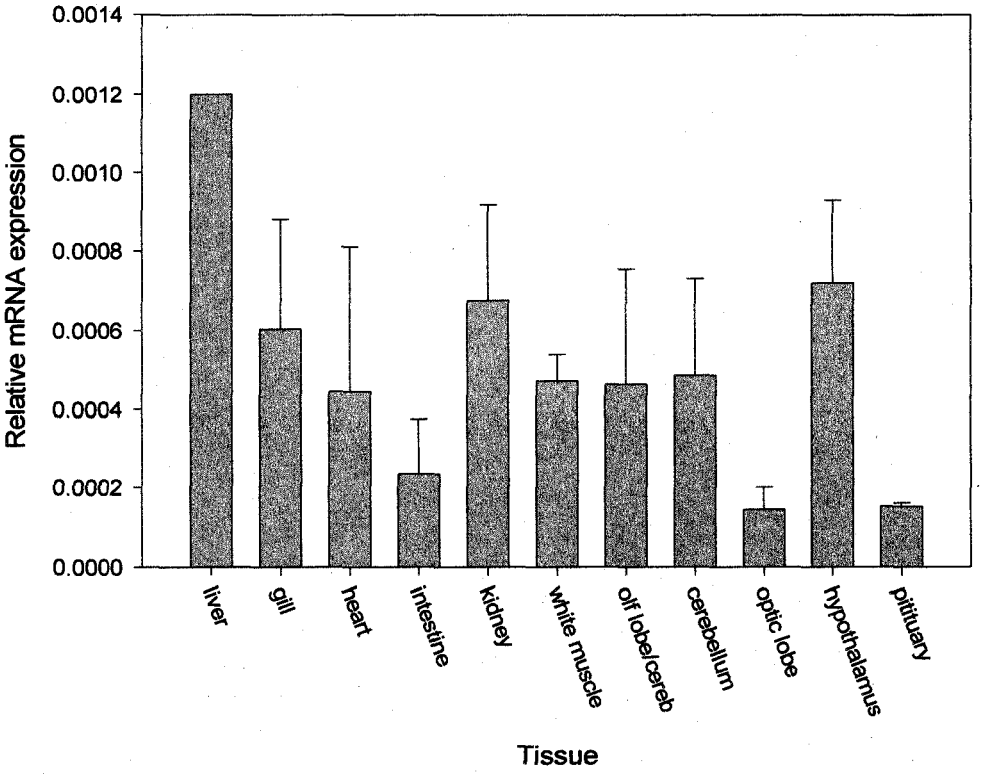


Figure 2.2B. Tissue distribution for HMGCoAR subtype 2 using real-time PCR. Note that the relative expression of HMGCoAR-2 is much lower than the expression levels of HMGCoAR-1; profiles of the subtypes and β-actin were completed on the same Q-PCR plate allowing direct comparisons. Real-time PCR analysis completed by Xi Chen.

B. Optimization of the HMGCoAR assay

The assay for HMGCoAR was optimized for rainbow trout using a modification of the protocol reported by Teichert and Wodtke (1987). HMGCoAR activity increased linearly with protein content (Fig. 2.3) and as assay temperature increased (Fig. 2.4). All subsequent assays used 0.6 mg liver microsome protein and 0.5 mg brain homogenate protein and incubations were at 25°C.

C. Liver and brain HMGCoAR activities

Non-significant changes occurred in HMGCoAR activities throughout the year in both liver microsomes and brain homogenates (Fig 2.5A and 2.5B). A general trend to decrease HMGCoAR activities during the summer months exists for both liver and brain activities, but these differences are not significant.

D. Enzyme activities and metabolites from fasted and re-fed fish

Non-significant changes occurred in HMGCoAR activities in liver microsomes from the 14 day fasted and the 7 day re-fed groups compared with controls (Fig. 2.6A). Brain homogenate HMGCoAR activities decreased in the fasted group but this decrease achieved significance only in the 7 day re-fed group compared with the control group (Fig. 2.6B).

Plasma glucose levels in the fasted group were significantly lower compared with the control group while values were higher than the control in the re-fed group; the fasted and re-fed groups were significantly different from each other (Fig. 2.7A).

Plasma cholesterol levels were significantly reduced when compared to the control group in both the 14 day fasted and 7 day re-fed groups (Fig. 2.7B).

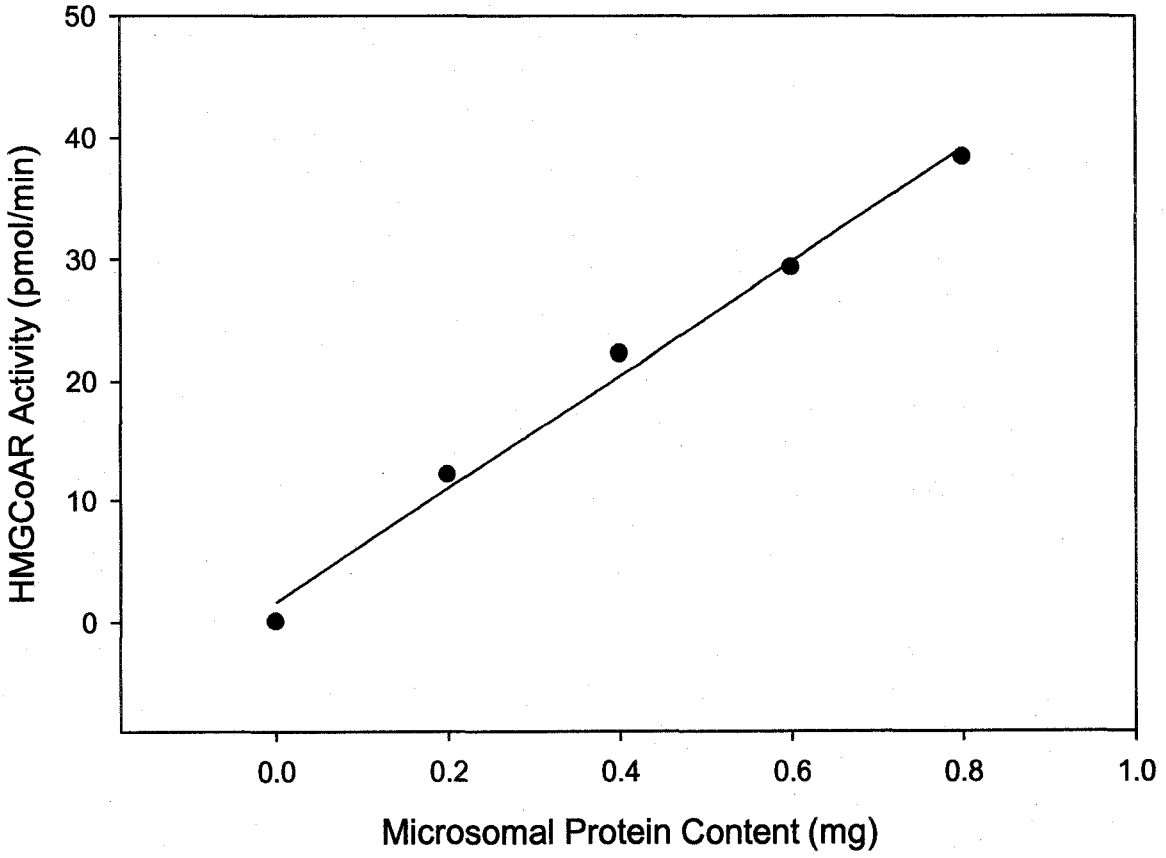


Figure 2.3. HMGCoAR activities as a function of liver microsome protein added to the assay; temperature was 25°C, $R^2 = 0.9909$.

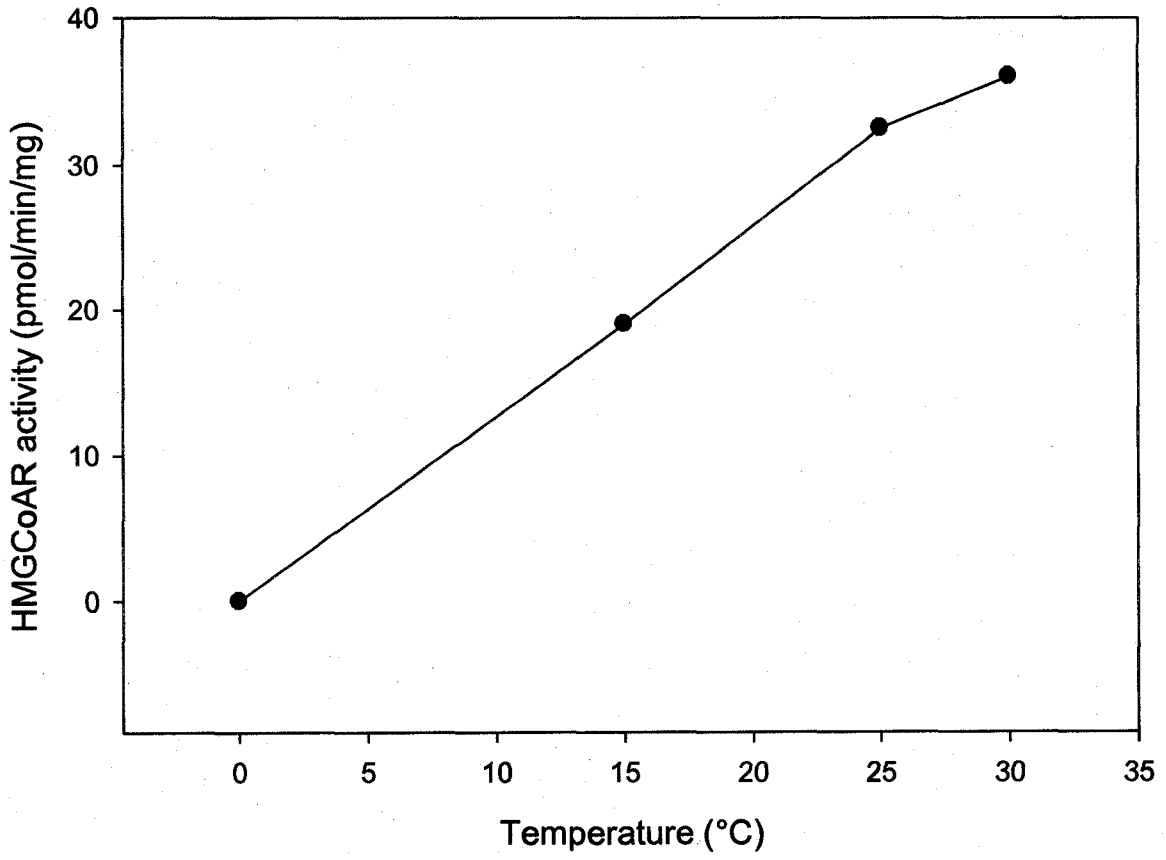


Figure 2.4. HMGCoAR activity of liver microsomes as a function of incubation temperature; 0.6 mg protein was added to each assay.

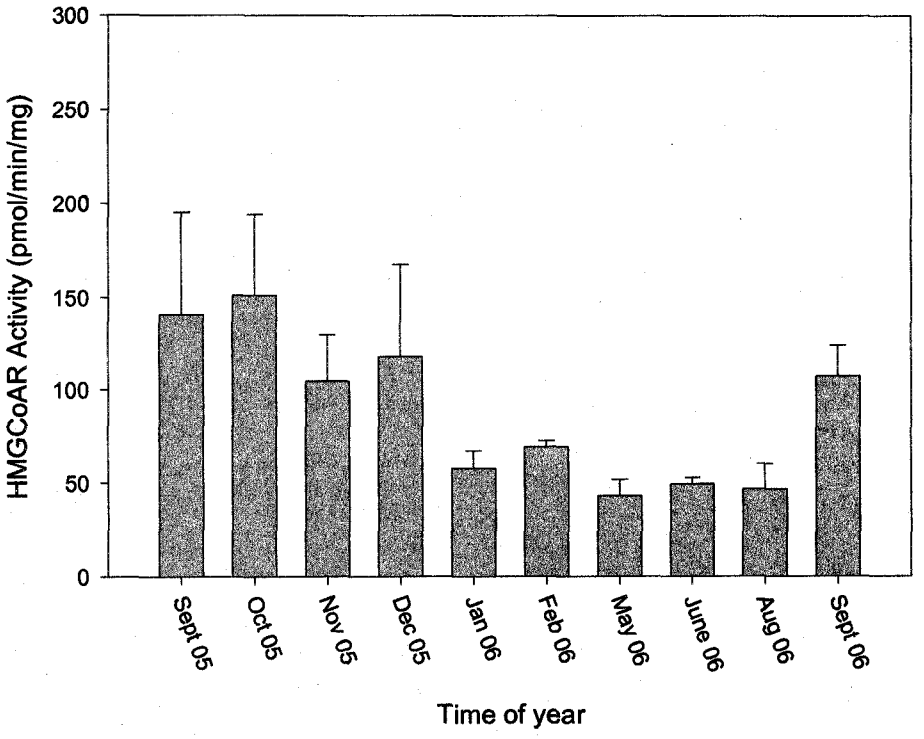


Figure 2.5A. HMGCoAR values from liver microsomes over a one year period. Liver microsomes were taken from 75 g rainbow trout. Data represent mean + SEM (n = 3-4).

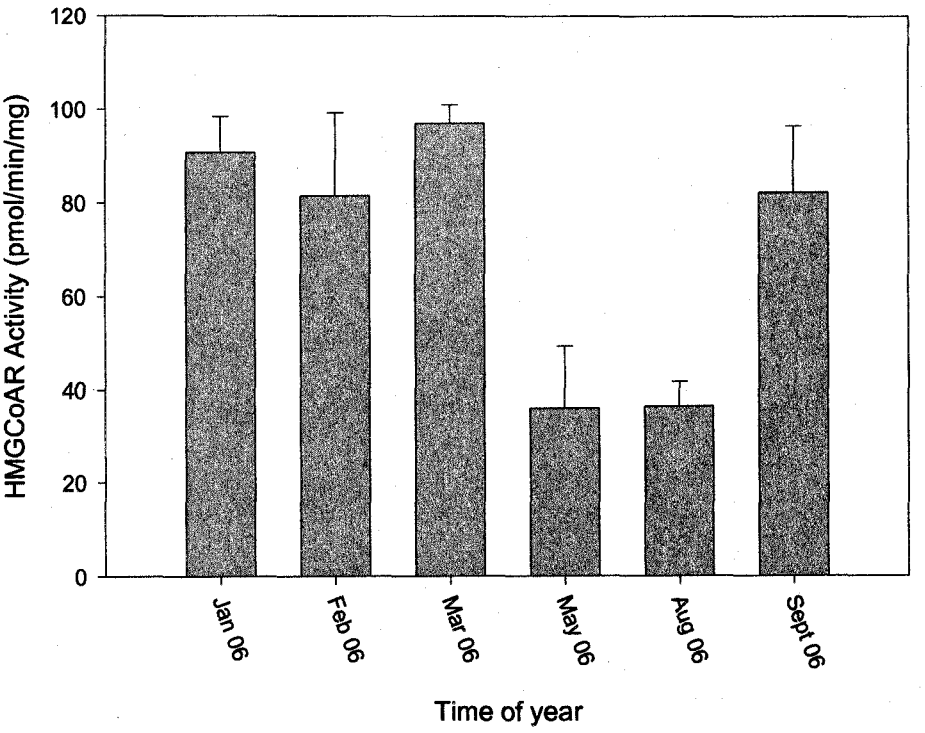
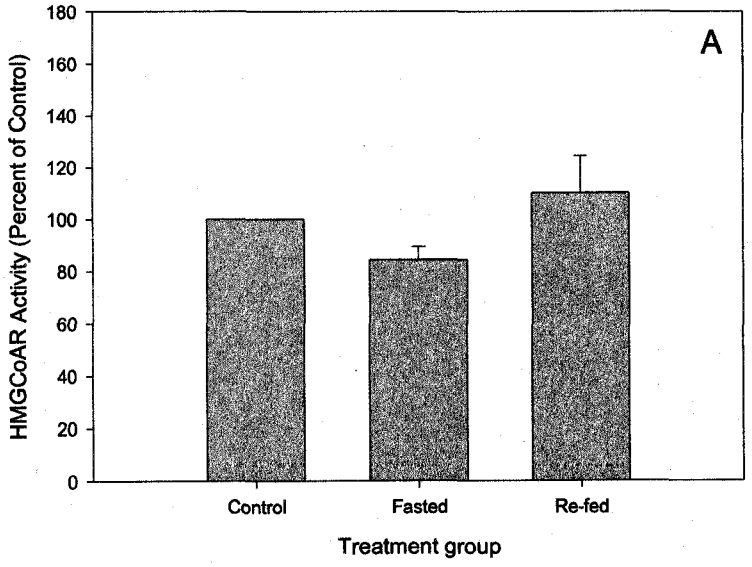


Figure 2.5B. HMGCoAR values from brain homogenates over a one year period. Brain homogenates were taken from 75 g rainbow trout. Data represent mean + SEM (n = 2-4).



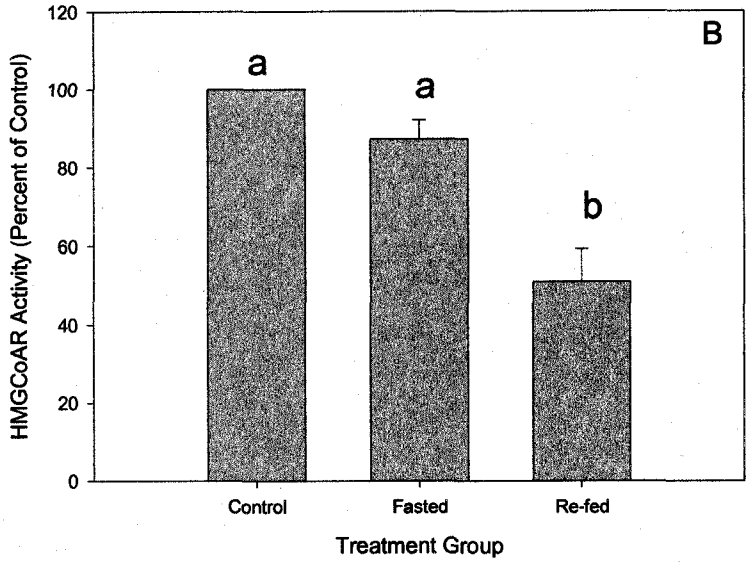


Figure 2.6. HMGCoAR activity (% of control) from trout liver microsomes (A) and brain homogenates (B). Rainbow trout were fasted for 14 days, a separate group was fasted then re-fed for 7 days, and a control group was fed 1% body weight for the duration of the experiment. (A) Data represent mean + SEM (n = 6 for control and re-fed group, n = 5 for fasted group). (B) Data represent mean + SEM (n = 4 for control group, n = 6 for fasting and re-fed groups). Letters that differ are significantly different from each other by a one-way ANOVA ($p<0.05$ followed by Tukey test).

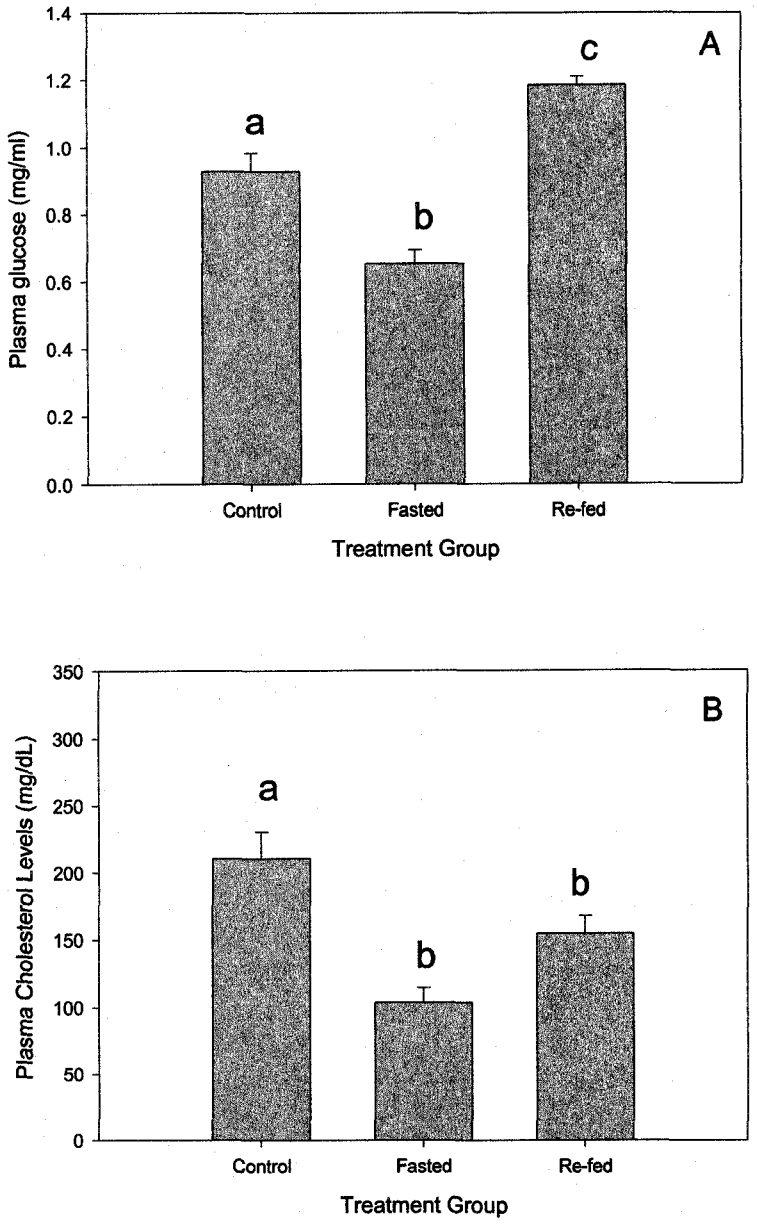


Figure 2.7. Plasma glucose (A) and cholesterol (B) values in rainbow trout. Rainbow trout were fasted for 14 days, a separate group was fasted then re-fed for 7 days, and a control group was fed 1% body weight for the duration of the experiment. Data represent mean + SEM (n = 6). Letters that differ are significantly different from each other by a one-way ANOVA ($p<0.05$ followed by Tukey test).

E. Phosphorylation-dephosphorylation control of HMGCoAR activities

Liver microsomes were incubated with three buffers designed to stimulate PKA, PKC, or AMPK (see Materials and Methods) activities leading to possible phosphorylation of HMGCoAR. All three phosphorylating conditions reduced HMGCoAR activities, although only the condition stimulating AMPK resulted in a significant decrease in activities compared with the control incubations (Fig. 2.8).

HMGCoAR activities were also assessed in buffers containing NaF and NaCl; NaF is a known inhibitor of protein phosphatase activities (Feingold *et* al., 1983; Teichert and Wodtke, 1987). As indicated in Figure 2.9, NaCl increased while NaF decreased HMGCoAR activities in liver microsomes compared with control incubates. However, neither group was statistically significant from the control group, although each were significantly different than each other.

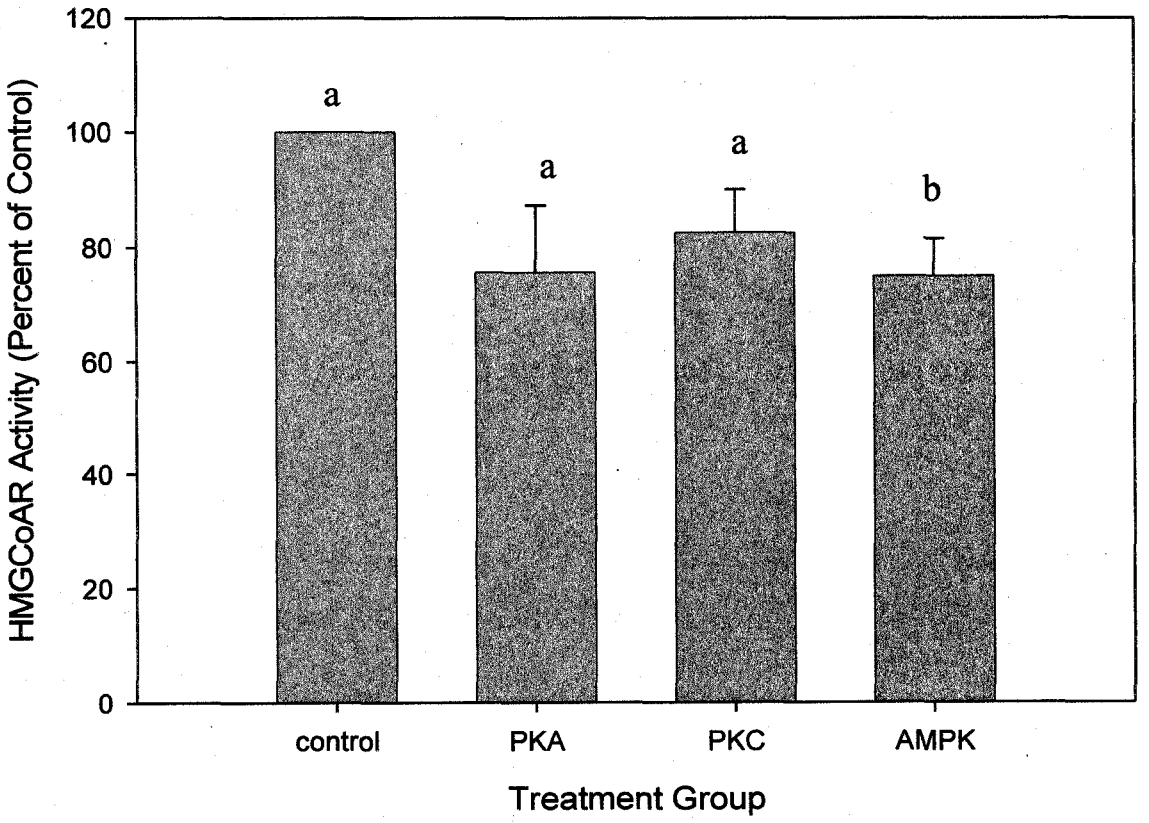


Figure 2.8. HMGCoAR activity (% of control) in microsomes incubated for 2 h under conditions that stimulate activities of PKA, PKC, and AMPK as indicated in the Materials and Methods. Following incubation HMGCoAR activities were assessed. Data represent mean + SEM (n = 6). Letters that differ are significantly different from each other by a one-way ANOVA on ranks ($p<0.05$ followed by Tukey test).

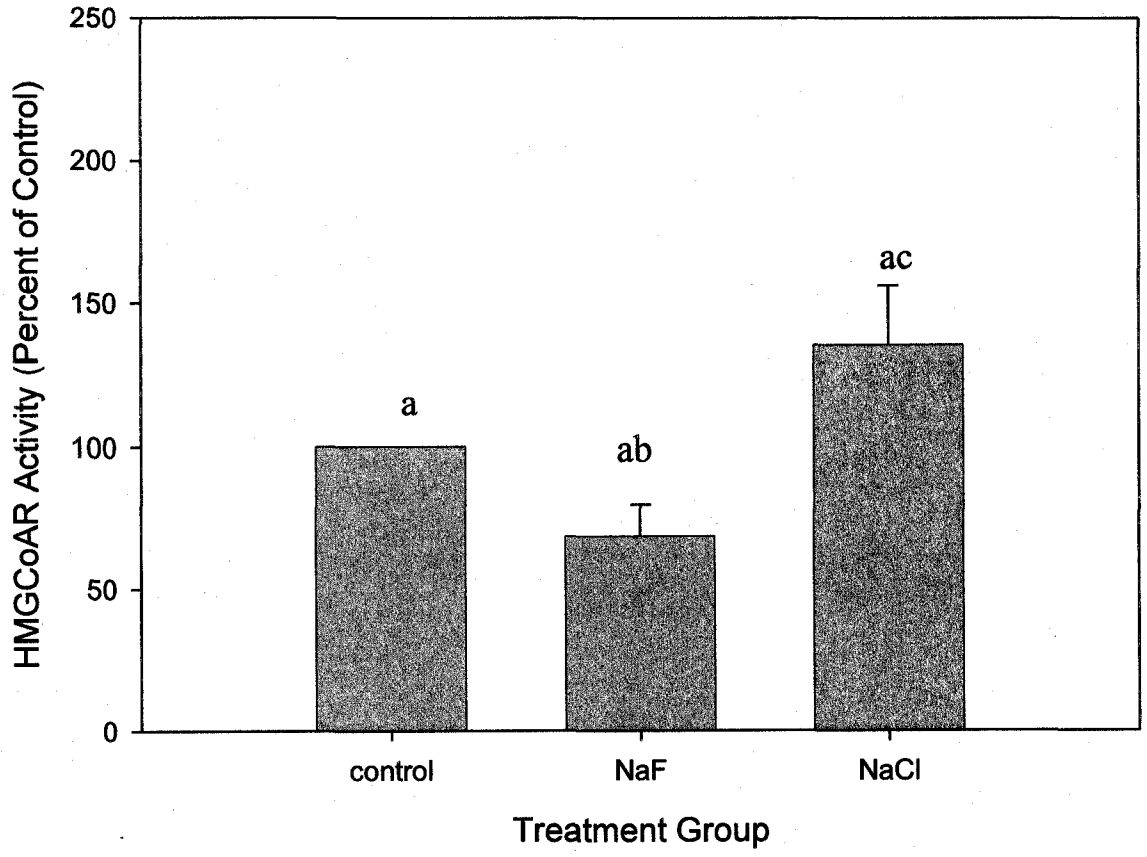


Figure 2.9. HMGCoAR activity in liver microsomes (% of control) incubated with buffers containing 50 mM NaF or 50 mM NaCl. Following incubation for 2 h HMGCoAR activities were assessed. Data represent mean + SEM (n = 4). Letters that differ are significantly different from each other by a one-way ANOVA ($p<0.05$ followed by Tukey test).

IV. Discussion

This study elucidated the distribution pattern of HMGCoAR subtypes in various tissues of the rainbow trout and some of the mechanisms governing the control of HMGCoAR, such as cholesterol mediated regulation and reversible phosphorylation/dephosphorylation.

A. mRNA analysis

HMGCoAR is found in most mammalian tissues although liver generally expresses some of the highest levels of this enzyme (Ness and Chambers, 2000). In some animals, such as the rat, liver expresses both the highest HMGCoAR activity and cholesterol synthesis capacity; however, in other species the adrenals, intestine, skin, or carcass exhibit the highest levels of cholesterol synthesis (Spady and Dietschy, 1983). No information is available concerning the tissue distribution of HMGCoAR mRNA in fish. Using semi-quantitative RT-PCR the most intense bands of trout HMGCoAR mRNA were observed in whole brain, gill, intestine, liver, and stomach. The high expression in both the intestine and liver coincides with similar findings in mammals (Kennelly and Rodwell, 1985). HMGCoAR is also found in the brain of mammals (e.g. rats) (Ness and Chambers, 2000) and rainbow trout. Thorpe *et al.* (2004) reported the presence of two HMGCoAR transcripts in zebrafish. Two HMGCoAR subtypes were also found to exist in rainbow trout and these were cloned and sequenced, and the sequences aligned with sequences found on GenBank (Appendix 1) and a preliminary phylogenetic analysis (Appendix 2) was undertaken by Xi Chen in our laboratory. HMGCoAR-1 was present in much higher quantities than HMGCoAR-2, although in fewer tissues. HMGCoAR-2 was more ubiquitously distributed, but was detected at levels approximately 1000-times lower than those observed for HMGCoAR-1. This is only the second species where two HMGCoAR subtypes are reported. In

zebrafish HMGCoAR-2 is present in 4-cell stage embryos; HMGCoAR-1 expression was not observed prior to day 4 and was then only found in the liver and intestine of the embryos (Thorpe *et al.*, 2004). Gornati *et al.* (2005) completed a phylogenetic analysis comparing sea bass (*Dicentrarchus labrax*) HMGCoAR with those sequences of other vertebrates. Sequence identity of sea bass HMGCoAR with human HMGCoAR was 76% (Gornati *et al.*, 2005). In sea bass the N- and C- terminal portions are the most conserved and when compared to the corresponding portions of other vertebrates identities range from 82 to 86% (Gornati *et al.*, 2005). The region of the HMGCoAR-1 that was cloned in rainbow trout shares 84% similarity with HMGCoAR-2 from rainbow trout, 82% with human HMGCoAR, and 88% with sea bass HMGCoAR, and the region of the trout HMGCoAR-2 gene that was cloned shares 84% similarity with rainbow trout HMGCoAR-1, 81% with human HMGCoAR, and 87% with sea bass HMGCoAR (Appendix 4).

In a companion study where relative HMGCoAR subtype mRNA expression was examined using the same fasting and re-feeding fish as my study (data not included in thesis), it was observed that changes in transcripts occurred primarily for HMGCoAR-1 with little variation observed in HMGCoAR-2 and as seen with the tissue distribution reported here, HMGCoAR-1 was present in much higher quantities compared with HMGCoAR-2 (Xi Chen, personal communication). This supports a primary role for HMGCoAR-1 in the rainbow trout as the main subtype involved in controlling cholesterol production. The fact that it was found at very high levels in the liver, one of the major sites involved in cholesterol synthesis at least in mammals and presumably fish further supports this hypothesis.

B. HMGCoAR activities

Fasting and re-feeding

Little change in liver microsome HMGCoAR activity was observed in the fasted fish and levels returned to normal in the re-fed group. However, a significant decrease was observed in brain homogenates in the re-fed group, which had lower activity than that observed in the fasted group. Plasma cholesterol levels were significantly lower in the fasted group, as were plasma glucose levels. The plasma cholesterol values reported for fish species vary quite substantially both within and between species (Babin and Vernier, 1989). For example Farrell and Munt (1983) report that plasma cholesterol levels are 233 ± 19 mg/dl for brook trout yet are 635 ± 56 mg/dl in Atlantic salmon. Black and Skinner (1986) found that fasting rainbow trout for 8 weeks resulted in cholesterol values of 315 ± 22 mg/dl compared with 416 ± 31 mg/dl for the fed group. In the present study fasted fish had cholesterol values of 103 mg/dl compared with 210 mg/dl for the control group. Studies in mammals have shown that fasting depresses cholesterol synthesis by regulating HMGCoAR, and decreased HMGCoAR activities have been observed following fasting (Bucher *et al.*, 1960; Regen *et al.*, 1966; Linn, 1967; Slakey *et al.*, 1972). The present study indicates that the effects of fasting on fish differ from those seen in mammalian studies where fasting causes a decrease in liver HMGCoAR activities. Blood glucose levels in fishes fluctuate greatly both between species and within species (Navarro and Gutiérrez, 1995). Moon *et al.* (1989) demonstrated that fasting rainbow trout for six weeks resulted in a significant decrease in plasma glucose levels, whereas Vijayan *et al.* (1993) showed that food deprivation in the sea raven (*Hemitripterus americanus*) had no significant effect on plasma glucose, compared with Gutiérrez *et al.* (1991) who found that blood glucose levels decreased after 5 days of fasting in juvenile sea bass. Less active fish have the capacity to maintain glucose levels for longer periods than more active fish, perhaps on account of the reduced demand for

glucose (Navarro and Gutiérrez, 1995). Insulin acts to increase hepatic HMGCoAR activities possibly resulting from changes in transcription of HMGCoAR mRNA (Ness and Chambers, 2000).Other studies demonstrated that rats rendered diabetic had reduced HMGCoAR mRNA levels. It was found that insulin restored the mRNA levels to near normal and this restoration correlated with a decrease in blood glucose levels (Ness *et al.*, 1994a,b). In the present study liver tissue was taken from the fish used in the fasting/re-feeding experiments and it was found that HMGCoAR-1 and HMGCoAR-2 mRNA levels decreased in the fasted fish when compared with control values (Xi Chen, personal communication). Certainly insulin levels fall in fasted rainbow trout (Moon *et al.*, 1989; Navarro and Gutiérrez, 1995) which may as in mammals be at least partially responsible for the changes in HMGCoAR mRNA levels seen in this experiment.

Phosphorylation/dephosphorylation

When purified HMGCoAR kinases became available it was possible to clearly demonstrate that HMGCoAR from rat liver microsomes was inactivated upon phosphorylation (Beg *et* al., 1980; Ferrer and Hegardt, 1984). It was also shown that inactivated HMGCoAR could be reactivated by dephosphorylation using phosphoprotein phosphatases (Gil *et al.*, 1980; Ferrer and Hegardt, 1984). Using conditions that potentially activated phosphorylation by PKA, PKC, and AMPK, *in vitro* trout liver HMGCoAR activities decreased; however only the AMPK incubation conditions result in a significant decrease when compared with the control. Other studies show that AMPK becomes activated upon fasting in various species, including chicken and rat (Munday *et al.*, 1991; Witters *et al.*, 1994; Culmsee *et al.*, 2001; Proszkowiec-Weglarz *et al.*, 2006). Upon activation of AMPK, HMGCoAR is phosphorylated leading to decreased enzyme activity. This study supports a mode of action in the trout as it is in higher vertebrates.

Teichert and Wodtke (1987) isolated carp microsomes in the presence of NaF and NaCl. NaF is an inhibitor of dephosphorylation, it accomplishes this by inhibiting protein phosphatases and NaCl activates the enzyme through dephosphorylation (Teichert and Wodtke, 1987; Proksch *et al.*, 1990). Teichert and Wodtke (1987) found that on average HMGCoAR activities from microsomes isolated in the presence of NaCl were 4-times higher than when microsomes were isolated in the presence of NaF. The results from the present study support these earlier studies showing that HMGCoAR activities in microsomes incubated with NaF and NaCl are significantly different from each other, with the microsomes incubated with NaCl containing buffer exhibiting higher HMCoAR activity than those incubated with NaF containing buffer.

Based on these findings HMGCoAR activities in rainbow trout demonstrate mechanistic properties in common with the mammalian enzyme. However, unlike mammals, it appears that rainbow trout have two subtypes of HMGCoAR similar to zebrafish, where HMGCoAR-1 probably plays the primary role in controlling cholesterol synthesis. Further sequencing of these two subtypes are required to better differentiate the two subtypes from the one form found in mammals; a preliminary phylogenetic analysis (Appendix 2) does support HMGCoAR-1 rather than HMGCoAR-2 being more closely related to mammalian HMGCoAR. This also raises the question as to the role of HMGCoAR-2 and why these fish species have two forms while mammals only have a single form.

Chapter 3 The Effect of Statin Drugs on Trout HMGCoAR

I. Introduction

Recent studies provide evidence that human pharmaceuticals are a component of the effluent discharged from WWTPs (see Chapter 1) but the full extent of this class of compounds as an emerging group of contaminants is not well understood. Pharmaceuticals are designed to be biologically active and most act through specific receptors, enzyme pathways and ion channels. The presence of pharmaceuticals in the environment is of particular concern given the high degree of conservation of functions between species, especially vertebrates, indicating that a drug may act in a non-target organism much the same way it would in the target species (Halling-Sørensen *et al.*, 1998; Daughton and Ternes, 1999). These products are continuously being added to the environment, meaning that aquatic species may be exposed to these compounds throughout their life but also at critical stages of their development.

The purpose of a WWTP is to use physical, chemical, and biological means to remove contaminants from water prior to its discharge into the receiving environment. These facilities were not designed to remove specific chemical contaminants, for example pharmaceuticals or their metabolites from the effluent stream (Kolpin *et al.*, 2002). There is little information on the bioaccumulation of pharmaceuticals in biota or through food webs although gemfibrozil, a lipid-lowering drug, is reported to bioconcentrate in the blood of goldfish (*Carassius auratus*) when presented in the water at environmental concentrations (Mimeault *et al.*, 2005). There is also documentation that diclofenac, an anti-inflammatory agent used to treat cattle in Pakistan, accumulates and is detrimental to the survival of vultures in this country (Oaks *et al.*, 2004). The action and fate of pharmaceuticals and their metabolites in the aquatic environment is largely unknown (Fent *et al.*, 2006).

Lipitor® (Atorvastatin, AVT; Pfizer, Inc.) is presently one of the best selling pharmaceuticals in the world (RxList, 2005). This drug belongs to a class of drugs known as "statins" and is prescribed to lower endogenously produced cholesterol by inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase or HMGCoAR, the first committed step in cholesterol synthesis (Hunter and Rodwell, 1980; Kennelly and Rodwell, 1985). Studies are emerging of the alternative benefits of statins including combating Alzheimer's disease and acting as an anti-inflammatory agent (Jones and Lefer, 2001; Liao, 2005). These pleiotropic effects of statins will lead to further increases in prescription rates of these drugs. In 2003 a study examined the concentration of various statins in WWTP influent, effluent and surface waters (Metcalfe *et al.*, 2003; Miao and Metcalfe, 2003). Atorvastatin was found at 76 ng/L in the influent, 37 ng/L in the treated effluent, and at 1 ng/L in the surface water (Miao and Metcalfe, 2003). Atorvastatin was the only statin of those studied detected in surface waters. Given that this product is already present in the aquatic environment and that prescription rates are increasing, it is timely to examine the effects of this class of drug on non-target species, such as fish. A single study found that significant disruptions occurred during zebrafish (*Danio rerio*) development when AVT was used at very high pharmacological doses (Thorpe *et al.*, 2004). Brain *et al.* (2006) demonstrated that AVT is phytotoxic in the higher aquatic plant *Lemna gibba* and found alterations in the pathway products downstream of HMGCoAR. Statin treatment caused plastoquinone and ubiquinone to accumulate; however increasing statin concentration caused a dose dependent decrease in stigmasterol and β-sitosterol (Brain *et al.*, 2006).

This study examines the effects of two statin drugs, cerivastatin (CVT) and atorvastatin (AVT), on HMGCoAR in rainbow trout, *Oncorhynchus mykiss*. The hypothesis to be tested is that these statins will decrease trout HMGCoAR activities as reported in mammals. Both *in vitro*

and *in vivo* tests will be used to examine enzyme activity upon exposure to statins. In addition, other biochemical parameters (e.g. cholesterol and glucose levels), and HMGCoAR transcript levels will be assessed to gain a broader view of how these drugs may influence this non-target species.

II. Materials and Methods

A. Materials

See Chapter 2 for a list of materials used. Atorvastatin (AVT) was donated by Pfizer, Inc. as part of a funded research project awarded to T.W. Moon and cerivastatin (CVT) was obtained from Apin Chemicals (Abingdon, Oxon, UK); both drugs were considered to be 100% pure. All other chemicals were of the highest possible quality and were obtained from local distributors.

B. Experimental Design

Fish

Rainbow trout (*Oncorhynchus mykiss)* were purchased and held at the University of Ottawa Aquatic Care Facility as noted in Chapter 2. All experiments were conducted under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhere to the guidelines set forth by the Canadian Council for Animal Care for the use of animals in teaching and research.

In vitro studies

Liver microsomes and brain homogenates were prepared from 75 and 250 g rainbow trout as noted in Chapter 2. These preparations were incubated *in vitro* with 50 µl of various CVT and AVT concentrations dissolved in buffer C (see Chapter 2), incubated for 15 min prior to addition of the cofactor-substrate, and HMGCoAR activities assessed as in Chapter 2.

In vivo studies - intraperitoneal injections (IP)

Rainbow trout (maximum 12 per tank) were divided into control and treated groups in separate tanks (0.115 m^3); the fish were allowed to adjust to these new conditions for 48 h. At

time zero, trout were lightly anaesthetized using benzocaine [1ml benzocaine (10 mg/l)/2l of water] and given a single 0.5 μl/g fish IP injection of either Cortland's saline solution (containing 0.1 M NaCl, 2 mM KCl, 4.1 mM $MgSO_4$, 5 mM $NaHCO_3$, 0.8 mM glucose, 1.25 mM KH_2PO_4, 2.6 mM $CaCl_2$) (control) or cerivastatin (CVT; 11.2 or 1.4 ng/g fish) dissolved in Cortland's saline. Those fish receiving 11.2 ng CVT per g fish were sampled at 0, 4, 9 and 24 h post-injection while the 1.4 ng CVT group was sampled only at 4 and 24 h post-injection. The high concentration was selected to reflect a human equivalent dose scaled down to fish using an estimated fish total blood volume of approximately 5 ml per 100 g fish (Brill *et al.*, 1998; Skov and Steffensen, 2003). At each time point twelve fish were removed from the tank, anaesthetized in benzocaine and weighed. A blood sample was taken from the caudal vessel using a 23-gauge needle attached to a heparinized 1 ml syringe. Following blood sampling the fish were euthanized by trans-spinal sectioning. The plasma was separated from red blood cells by centrifugation at 10,000 x g for 10 min; the plasma was aliquotted, flash frozen in liquid nitrogen and stored at -80°C until used for analysis. The whole liver and brain were removed for preparation of liver microsomes and brain homogenates as noted above and in Chapter 2. Tissues from groups of two fish were pooled to provide adequate sample for the assay. Liver microsomes and brain homogenates were then assayed for HMGCoAR activity as outlined in Chapter 2 with the exception of increasing the 10 min incubation to 15 min.

C. Biochemical assays

Microsome and homogenate proteins were determined using the BioRad assay and plasma cholesterol and glucose were assayed as noted in Chapter 2.

D. Real-time PCR of rainbow trout liver HMGCoAR mRNA

Real-time PCR of trout HMGCoAR mRNA was undertaken by Xi Chen as noted in Chapter 2.

E. Statistical analysis

Experimental results are presented as means ± standard error of the mean (SEM). A value of $p<0.05$ was considered significant. All statistical analyses were conducted using SigmaStat™ 3.1 software (SPSS Corp., Chicago, IL, U.S.A.). Statistical significance was tested with one-way ANOVA; where significant differences were detected an appropriate post-hoc test was used (Dunn's in cases where samples sizes were not equal; Tukey in all remaining experiments).

III. Results

A. HMGCoAR inhibition by statins *in vitro*

Initial inhibitor studies used trout liver microsomes exposed to cerivastatin (CVT) at 0, 8, 15, and 30 μM (final incubation concentrations). At these concentrations HMGCoAR activities were significantly reduced (Fig. 3.1). Therefore, CVT concentrations were reduced by three-orders of magnitude and liver microsomes prepared from both 75 and 250 g rainbow trout were incubated with concentrations ranging from 0 to 30 nM (Fig. 3.2A). At these concentrations HMGCoAR activity from liver microsomes was significantly reduced compared with the control in 250 g fish at 26 and 30 nM; in 75 g fish, HMGCoAR activities were significantly reduced with 21, 26, and 30 nM CVT.

This study was repeated using brain homogenates prepared from the same fish. All drug concentrations caused a reduction in HMGCoAR activity when compared with the control group. In the homogenates prepared from 250 g fish, HMGCoAR activities were significantly decreased at CVT concentrations of 17, 21, and 30 nM (Fig. 3.2B). The brain homogenates from the 75 g fish were significantly decreased at 21, 26, and 30 nM CVT.

IC_{50} values (concentration resulting in 50% inhibition of HMGCoAR activity) were approximately 26 and 17 nM for the microsomes prepared from the 250 and 75 g fish, respectively. The IC_{50} value for the brain homogenate was 17 and 23 nM from the 250 g and 75 g fish, respectively.

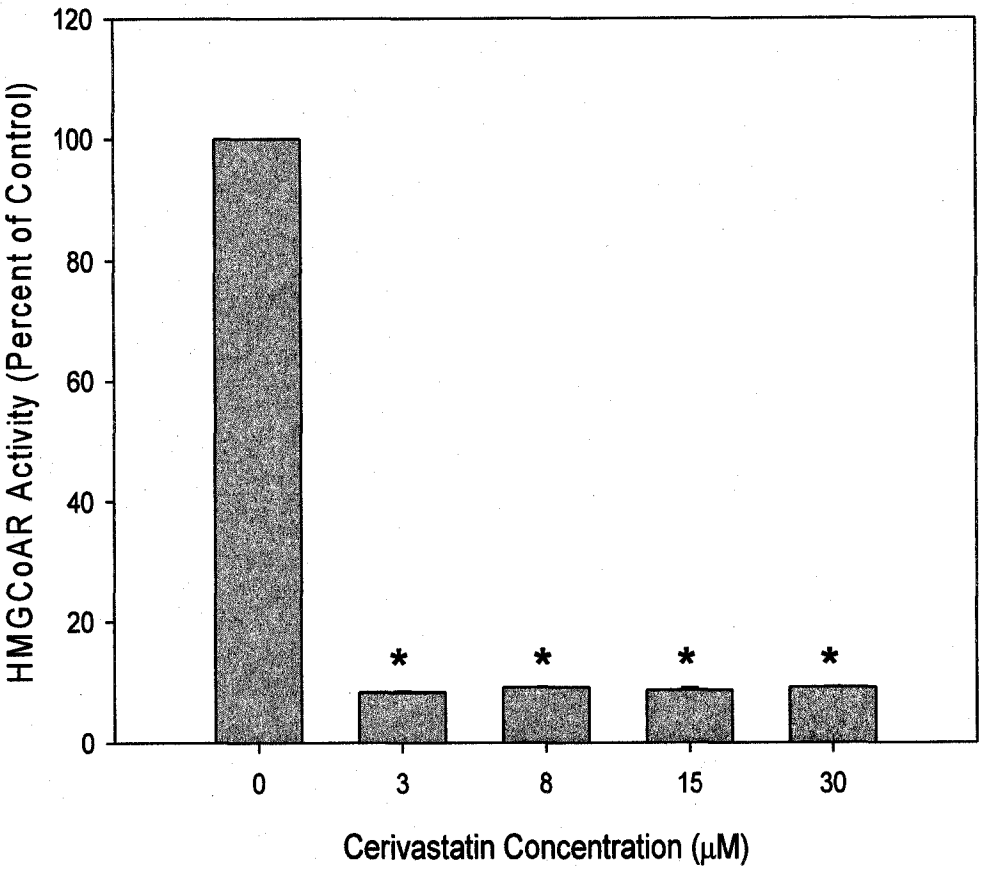


Figure 3.1. Liver microsome HMGCoAR activity with cerivastatin *in vitro*. Rainbow trout liver microsomes (0.6 mg protein) were pre-incubated with cerivastatin for 15 min at 25°C. HMGCoAR activities were assessed as in the Materials and Methods. Data represent mean ± SEM (n = 2). (*) denotes statistically significant from control group ($p<0.05$).

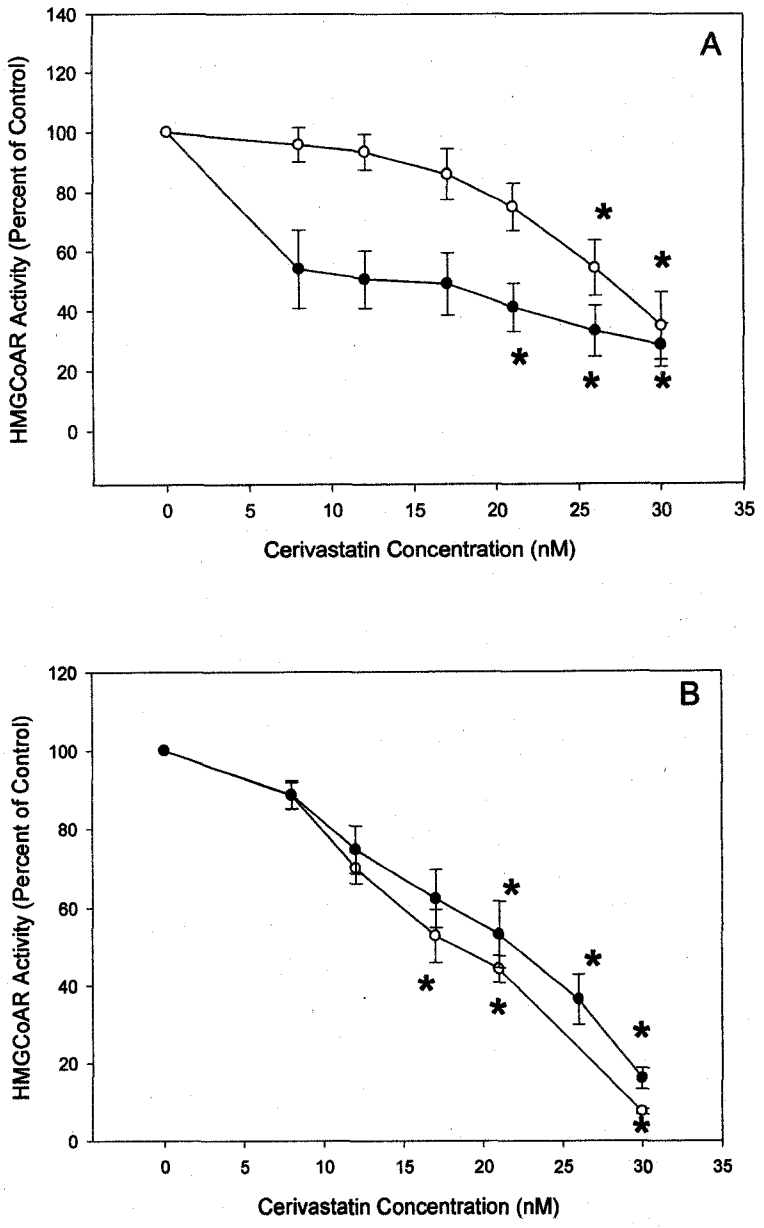


Figure 3.2. Cerivastatin inhibition curves generated using liver microsomes (A) and brain homogenates (B). Liver microsomes (0.6 mg protein) and brain homogenates (0.5 mg protein) were incubated with cerivastatin for 15 min at 25°C. HMGCoAR activities were assessed as in the Materials and Methods. Closed circles 75 g fish and open circles 250 g fish. Data represent mean ± SEM (n = 7). (*) denotes statistically significant from control group ($p<0.05$); one-way ANOVA on ranks followed by Tukey test.

Cerivastatin, allegedly the most potent statin that was commercially available (Stein *et al.*, 1997), affects liver and brain HMGCoAR activities, so the effects of atorvastatin (AVT), the most prescribed statin, were also assessed but only in 75 g rainbow trout *in vitro*. AVT concentrations ranging from 0 to 45 nM were used to assess the effects *in vitro*. At each AVT concentration used, HMGCoAR activities were reduced compared with the control group. These decreases became significant in the liver at 23, 33, and 45 nM AVT (Fig. 3.3A) and in the brain at 17, 23, 33, and 45 nM (Fig. 3.3B). IC_{50} values were approximately 23 nM in the liver microsomes and 17 nM in the brain homogenates. These IC_{50} values are similar to those observed using CVT.

B. HMGCoAR inhibition by statins *in vivo*

After establishing that HMGCoAR activities were diminished with *in vitro* exposure to statins, the *in vivo* effects of statin exposure were assessed. Trout received an IP injection of 11.2 ng CVT per g fish and sampled at 0, 4, 9, and 24 h post-injection. Enzyme activities in liver microsomes were diminished at 0 h post-injection (approximately 10 min post-injection or when the fish had recovered from anaesthesia and was swimming normally) and were significantly reduced at both 4 and 9 h post-injection; activities returned to control values by 24 h post-injection (Fig. 3.4A).

Brain HMGCoAR activities were not significantly affected by CVT injection at any time point assessed (Fig. 3.4B). Neither plasma glucose (Fig. 3.5A) nor cholesterol (Fig. 3.5B) values changed as a function of CVT treatment.

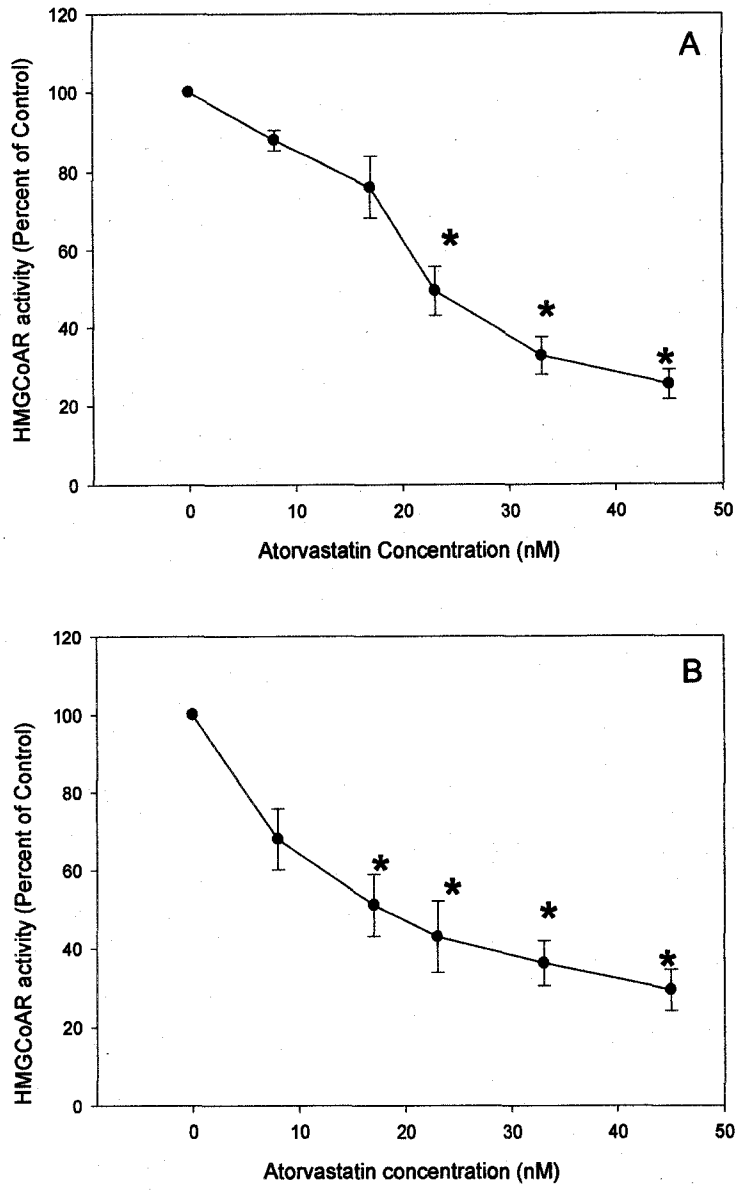


Figure 3.3. HMGCoAR activities in the presence of atorvastatin using trout liver microsomes (A) and brain homogenates (B). Liver microsomes and brain homogenates prepared from 75 g female rainbow trout were incubated with AVT for 15 min at 25 °C and HMGCoAR activity assessed. Data represent mean ± SEM (n = 7). (*) denotes statistically significant from control ($p<0.05$); one-way ANOVA on ranks followed by Tukey test.

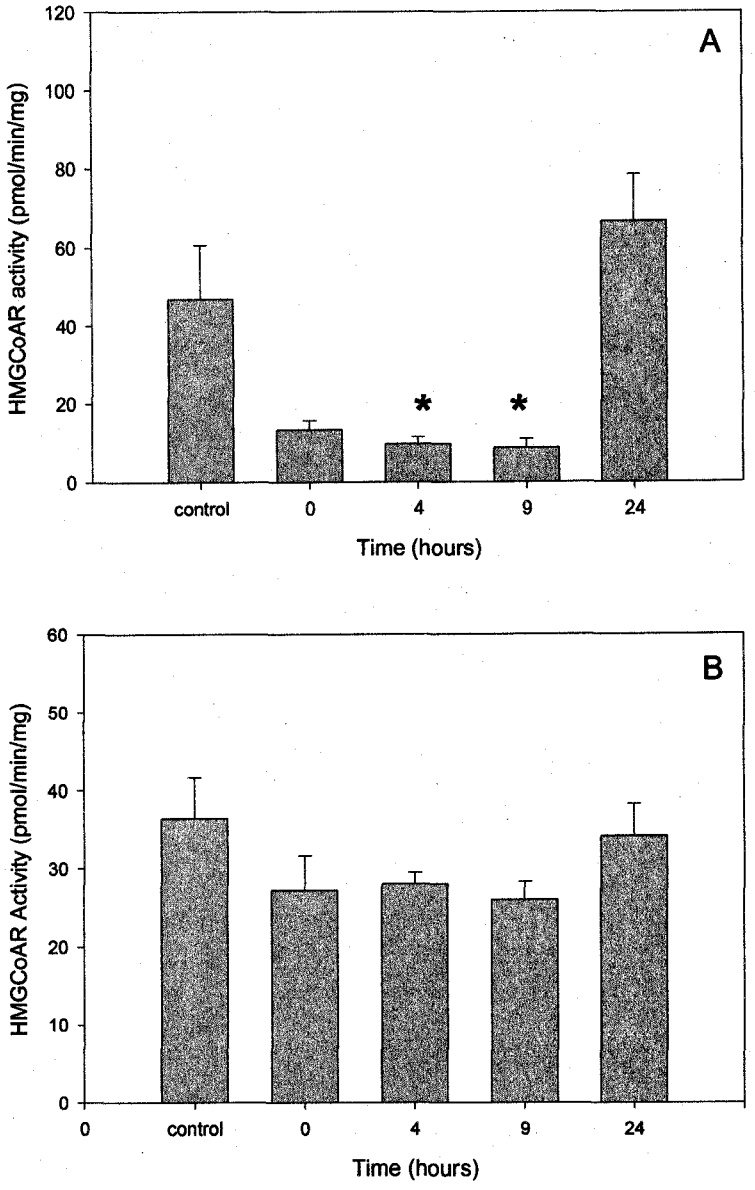


Figure 3.4 . HMGCoAR activities in liver microsomes (A) and brain homogenates (B) following a 11.2 ng CVT per g fish IP injection into 75 g rainbow trout. Data represent mean + SEM (n = 6 for all groups except n = 4 for the control). Control fish were injected with Cortland's saline and fish sampled at time 0 were injected with CVT and sampled upon recovery from anaesthesia (approximately 10 min). (*) denotes statistically significant from control ($p<0.05$); one-way ANOVA on ranks followed by Dunn's method.

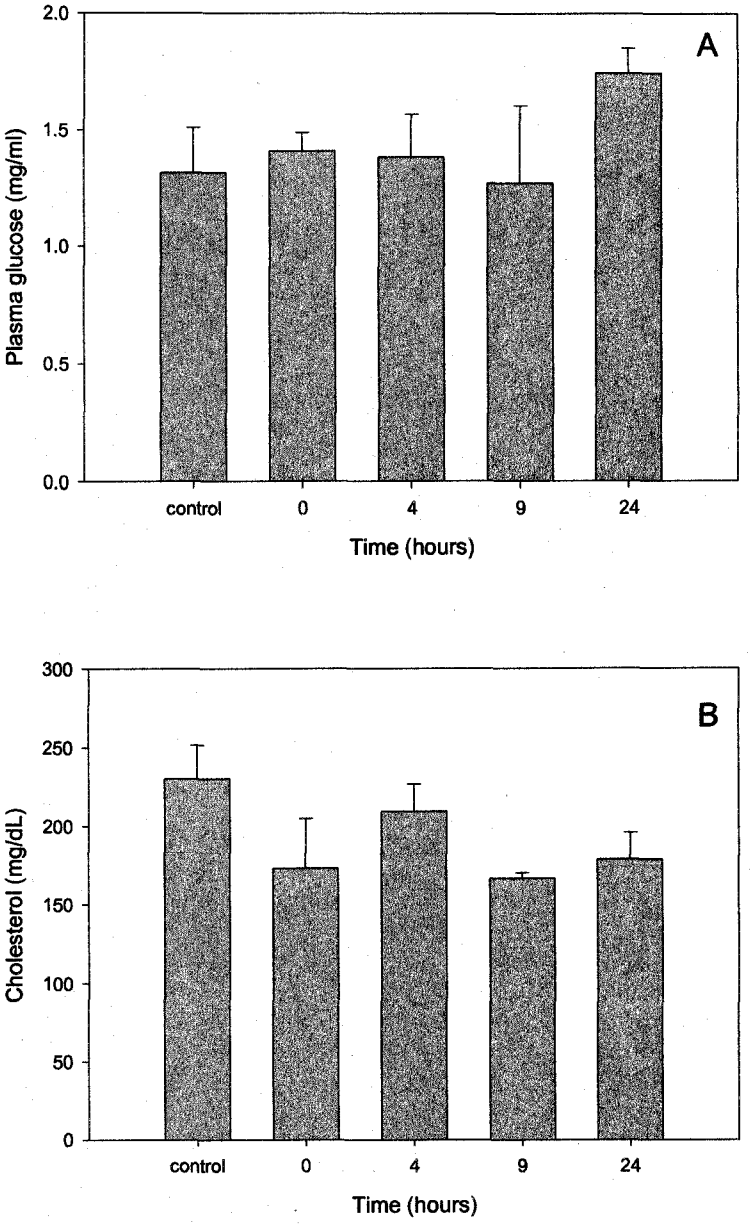


Figure 3.5. Plasma glucose (A) and cholesterol (B) levels in rainbow trout injected with 11.2 ng cerivastatin per g fish as a function of time post injection. Data represent mean + SEM (n = 3 for all groups except n = 4 for the control). Control fish were injected with Cortland's saline and fish sampled at time 0 were injected with cerivastatin and sampled upon recovery from anaesthesia (approximately 10 min). There are no significant differences as a function of time after injection.

A second series of experiments used 1.4 ng CVT per g fish (8X lower than the initial study), and sampling occurred at 4 and 24 h post-injection. In liver microsomes HMGCoAR activities were significantly reduced at 4 h and 24 h post-injection (Fig. 3.6A). The brain homogenate prepared from the injected fish showed a statistically significant decrease in activities at 4 h post-injection; activities returned to control values at 24 h (Fig. 3.6B). Both glucose and cholesterol levels remained unchanged in plasma samples taken at both time points (Fig. 3.7A, B).

C. Relative HMGCoAR mRNA expression

The relative expression of both HMGCoAR-1 and -2 was examined in liver from trout injected with 1.4 ng CVT per g fish. Relative mRNA expression of HMGCoAR-1 increased in liver at 4 h post-injection but significantly decreased at 24 h when compared with the 4 h time point (Fig. 3.8A). There was a steady decrease in relative mRNA expression in the HMGCoAR-2 subtype over the 24 h exposure period (Fig. 3.8B).

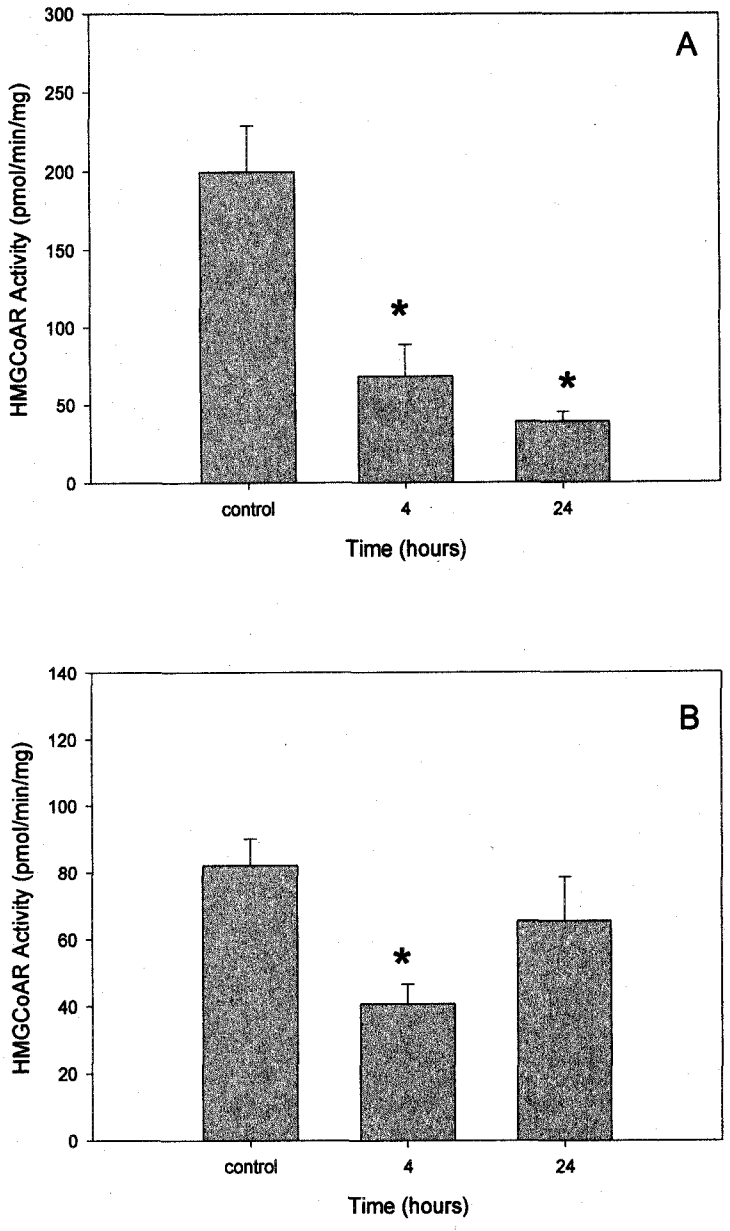


Figure 3.6. HMGCoAR activity in trout liver microsomes (A) and brain homogenates (B) from rainbow trout injected with 1.4 ng CVT per g fish at 4 and 24 h post injection (n = 6; mean + SEM). (*) denotes statistically significant from control ($p<0.05$); one-way ANOVA on ranks followed by Tukey test.

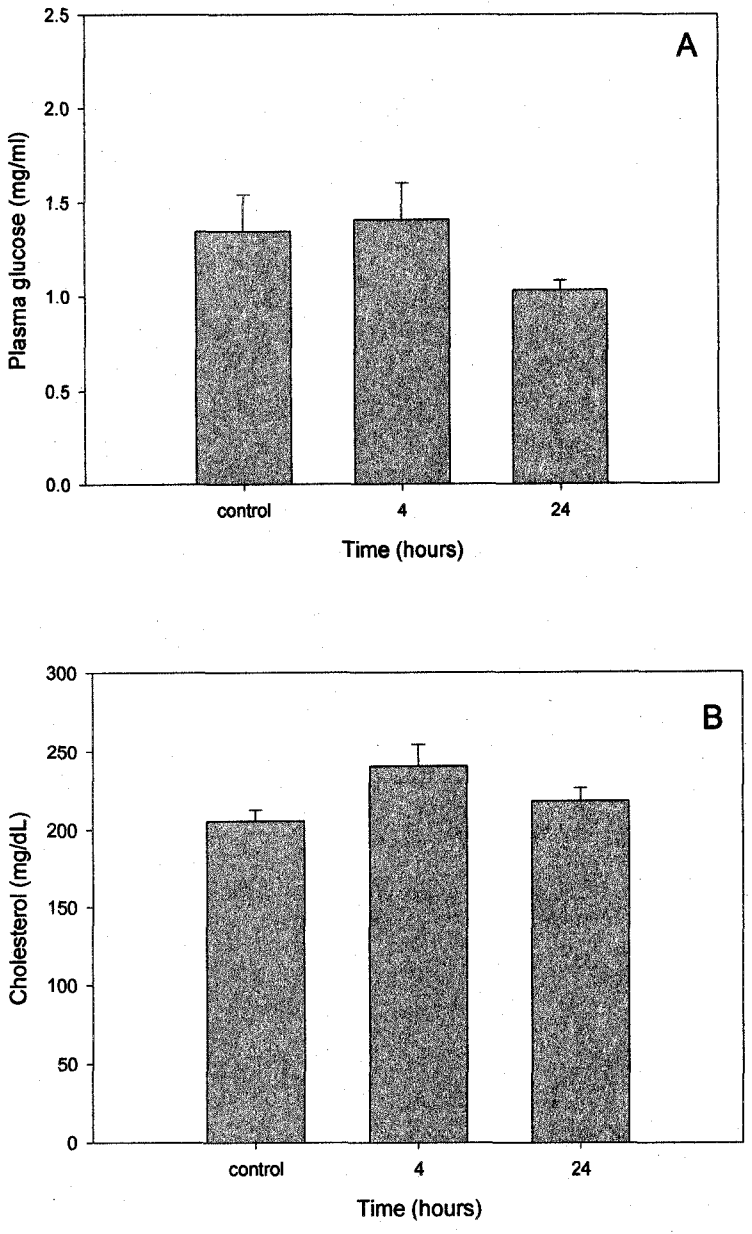


Figure 3.7. Plasma glucose (A) and cholesterol (B) levels in rainbow trout injected with 1.4 ng CVT per g fish at 4 and 24 h post injection (n = 6; mean + SEM).

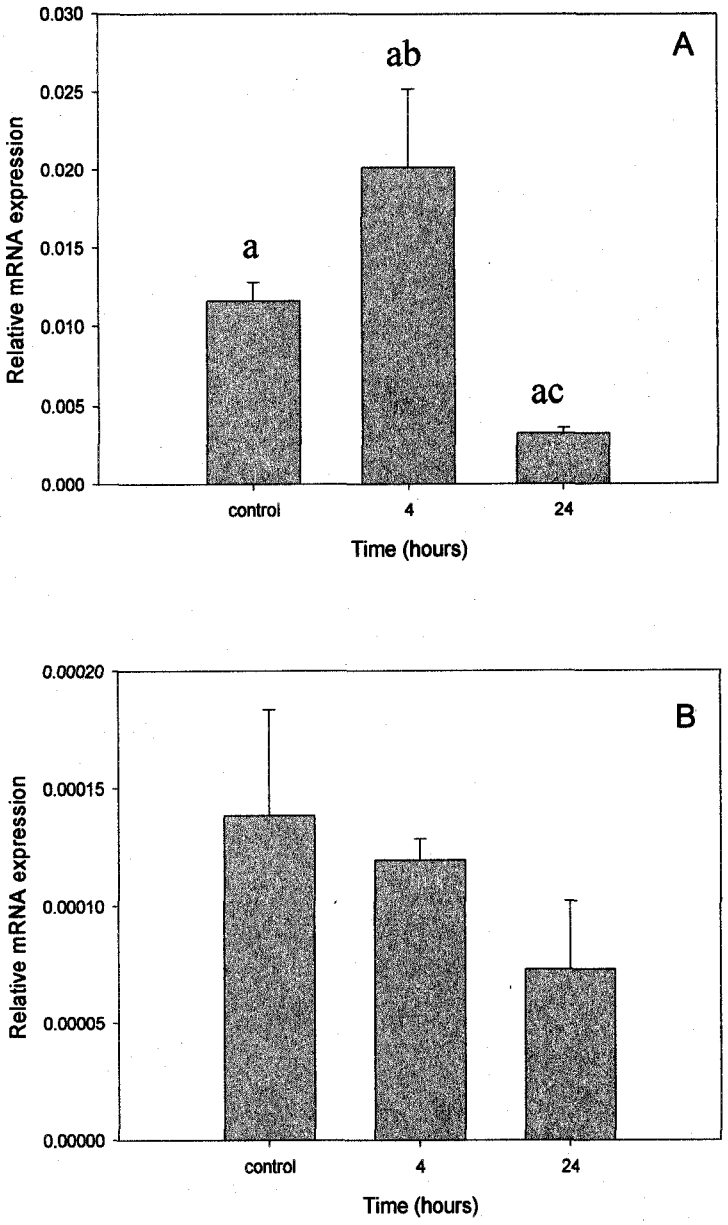


Figure 3.8. HMGCoAR-1 (A) and -2 (B) relative mRNA expression in liver taken from rainbow trout IP injected with 1.4 ng CVT per g fish 4 and 24 h post injection (n = 3; mean + SEM). Real-time PCR analysis completed by Xi Chen.

IV. Discussion

Miao and Metcalfe (2003) reported that statin drugs were present in WWTP influent, effluent and in the surface water post-WWTP. Statin drugs function by inhibiting HMGCoAR, which is a key step in endogenous cholesterol synthesis and in the production other products downstream of mevalonate (e.g. ubiquinone) (Moghadasian, 1999; Ness and Chambers, 2000; Istvan, 2003). The reduction of HMGCoA to mevalonate, which is catalyzed by HMGCoAR, is the rate-limiting step for cholesterol production, and is thus the target of this class of drug. Given the presence of statins and their predicted further increase in the aquatic environment, it is timely to investigate the potential for these drugs to inhibit HMGCoAR in fish as they do in mammals. This study clearly demonstrates using both *in vitro* and *in vivo* methods that rainbow trout liver and brain HMGCoAR activities are inhibited by the two statin drugs used in this study, cerivastatin (CVT) and atorvastatin (AVT).

A. *In vitro* Studies

Endo and Kuroda (1976) reported that an antibiotic isolated from *Phytium ultimum* acted as an inhibitor of cholesterol synthesis in rat liver. Subsequently Endo *et al.* (1976) isolated three fungal metabolites (ML-236A, B, and C) from *Penicillum citrinum* and ML-236B was also isolated by Brown *et al.* (1976) from *Penicillum brevicompactum.* ML-236B or compactin/mevastatin when administered to rats resulted in reductions in plasma cholesterol (Endo *et al.*, 1976). Further studies examined ML-236B and found that this compound inhibited cholesterol synthesis from acetate, acetyl CoA and HMGCoA but did not alter the conversion of mevalonate into sterols, leading the authors to believe that the compound acted on HMGCoAR (Endo *et al.* 1976b). In this study mevastatin at 5 and 50 μM led to a 25 and 70% reduction,

respectively in the incorporation of acetate, acetyl CoA and HMGCoA into sterols, demonstrating an inhibitory effect on HMGCoAR. The affinity of HMGCoAR for statins is in the nM range while the affinity for the natural substrate, HMGCoA, is approximately 30 μM (Endo *et al.*, 1977; Flamberg *et al.*, 1990).

This study provides evidence that both CVT and AVT inhibit HMGCoAR activities in rainbow trout. IC_{50} values (the concentration resulting in 50% inhibition of HMGCoAR activity when compared with a control) for the two statins and trout HMGCoAR are in the nM range, values that are observed for mammalian HMGCoAR (Tables 3.1 and 3.2).

Table 3.1. The IC_{50} (concentration resulting in 50% inhibition of cholesterol synthesis) values reported for a variety of statin drugs in two mammalian cell types. Values are reported as ranges in nM (adapted from Corsini *et al.*, 1995).

Cell Type	Lovastatin	Pravastatin	Simvastatin	Fluvastatin	Cerivastatin	Mevastatin
Rat hepatocytes	3-146 [2, 5, 9-11]	5-500 [1, 4, 9, 11]	3-50 [9, 11, 14]	1.7-52 [5, 10]	NA	4-2000 [1, 6, 7]
Human Hep G2	4-50 [3, 5, 12]	700-2650 [5, 12, 13]	34, 150 [12, 13]	30, 43 [5, 10]	1 [15]	200 [8]

1) Tsujita *et al.*, 1986; 2) Bocan *et al.*, 1992; 3) Beck *et al.*, 1990; 4) Endo *et al.*, 1977; 5) Shaw *et al.*, 1990; 6) Koizumi *et al.*, 1982; 7) Pullinger and Gibbons, 1983; 8) Cohen *et al.*, 1984; 9) Scott, 1990; 10) Parker *et al.*, 1990; 11) Koga *et al.*, 1990; 12) Cohen *et al.*, 1993; 13) Nagata *et al.*, 1990; 14) Ribeiro *et al.*, 1991; 15) Bischoff *et al.*, 1997.

Table 3.2. K_i values (dissociation constant for the inhibitor-enzyme complex) for several HMGCoAR inhibitors (from rat microsome preparations) (adapted from Corsini *et al.*, 1995; *Bischoff *et al.*, 1977).

Substrate	**K_i (nM)**
Lovastatin	0.6, 150*
Pravastatin	2.3
Simvastatin	0.12
Fluvastatin	0.3
Cerivastatin	1.3

B. *In vivo* studies

A few studies are available that examined the effects of CVT administration on various parameters in mammals. Bischoff *et al.* (1997) found inhibition of hepatic cholesterol synthesis in rats using 0.001 mg CVT per kg of body weight, the lowest oral dose tested. The dose which decreased cholesterol synthesis by 50% (pharmacological ED_{50}) was 0.002 mg/kg body weight. Using lovastatin the ED_{50} was 0.3 mg/kg body weight (Bischoff *et al.*, 1997). This same study utilized male and female beagle dogs and an ED_{50} was calculated to be 0.002 mg/kg body weight (from oral administration) and the highest dose used in the study (0.03 mg/kg body weight) decreased the [^{14}C]cholesterol content in the liver by 95%, almost completely inhibiting cholesterol synthesis (Bischoff *et al.*, 1997)

The present IP injection study used CVT doses of 1.4 and 11.2 ng per gram fish, doses that are 100 to 1000 time lower than doses used in the Bischoff *et al.* (1977) study. The higher

dose had no effect on HMGCoAR activities in trout brain homogenates, however, at the lower dose a significant reduction in activity was observed at 4 h post-injection. Ifergan *et al.* (2006) demonstrated that two statins, lovastatin and simvastatin, reduced human blood-brain barrier permeability. However at both doses, hepatic microsome HMGCoAR activities were reduced 4 h post-injection and at the higher dose activities were decreased immediately post-injection. A major difference existed at 24 h where activities remained significantly depressed at the low dose but activities at the high dose had returned to control values. This differential dose effect could be of particular relevance to environmental exposure which typically takes place at low concentrations but over long periods of time. Certainly doses used in these IP experiments are substantially higher than those found in the environment at least for AVT, but no information exists regarding uptake kinetics or turnover of a statin in any fish species. Previous studies by Mimeault *et al.* (2005) reported that gemfibrozil, a lipid-lowering drug does bioconcentrate in goldfish blood; whether a statin drug acts in this manner is unknown. All HMGCoAR inhibitors are absorbed to different degrees across the gut (Moghadasian, 1999). In the case of CVT the plasma half-life is 2-3 h and 30% is excreted in the urine of mammals. This is compared with the half-life of 12-58 h and urinary excretion of 2.3% for AVT (Moghadasian, 1999).

Transcript levels of both HMGCoAR-1 and -2 were affected by the low dose CVT IP injection. At 24 h HMGCoAR-1 relative mRNA levels were significantly reduced compared with the control group, while HMGCoAR-2 exhibited a steady but non-significant decline in relative mRNA expression. These data correlate with the observed changes in HMGCoAR enzyme activities, where there was a steady decrease in activity and the reductions seen at 4 and 24 h were significant when compared with control values. These data support both a direct inhibition by statins on HMGCoAR activities and possible regulation at the mRNA transcription

level in trout. In cultured mammalian cells compactin and lovastatin inhibit the synthesis of mevalonate and lead to an increase in reductase protein within a few hours (Brown *et al.*, 1978; Nakanishi *et al.*, 1988). However, this additional protein is inactive because of the inhibitory actions of the statin drug (Goldstein and Brown, 1990). The observed increase in HMGCoAR protein results from the cumulative effects of induction of transcription which produces increased mRNA levels, mRNA translation taking place at a higher rate, and enzyme degradation occurring more slowly (Nakanishi *et al.*, 1988). Kocarek *et al.* (2002) found that treatment of primary cultured human hepatocytes with HMGCoAR inhibitors produced an increase in HMGCoAR mRNA levels, which is a result of the diminished sterol content.

In mammals sterols repress transcription of HMGCoAR. However, even in the presence of large quantities of sterols HMGCoAR mRNA is transcribed at approximately one-eighth the maximum rate (Nakanishi *et al.*, 1988). When a statin is present HMGCoAR mRNA is translated even in the presence of sterols, but when non-sterols are present in conjunction with sterols (e.g. by administering exogenous mevalonate) translation of HMGCoAR mRNA is reduced (Nakanishi *et al.*, 1988).

Cholesterol levels did not change during either injection study. Inhibition of HMGCoAR should result in decreased cholesterol production, however the exposure period was only 24 h in duration and it is possible that a longer period would be required to diminish cholesterol levels, or that there would be a lag period between reduced enzyme activity and subsequent reductions in pathway products including cholesterol. Previously I did show that a 7 day fast significantly decreased cholesterol levels by approximately 50% (Fig. 2.8), suggesting that plasma cholesterol values can vary in trout. In general plasma cholesterol levels vary substantially between and within fish species (Babin and Vernier, 1989). Wedemeyer and Chatterton (1970)

found that in juvenile rainbow trout plasma cholesterol levels ranged from 161-365 mg/dl. Plasma glucose levels were also unchanged suggesting that CVT does not impact glucose homeostasis. März *et al.* (2003) found that fasting blood glucose tended to be lower in statin users when compared with non-users due to the fact that statins facilitate blood pressure and glucose control (Freeman *et al.*, 2001; Borghi, 2002).

Bischoff *et al.* (1997) observed a reduction in cholesterol levels, but this study was carried out over multiple weeks. Similarly, Shiomi and Ito (1999) injected WHHL rabbits subcutaneously with CVT at a concentration of 0.6 mg/kg/day and after 32 weeks total cholesterol levels had decreased by 39% compared with a control group. The *in vivo* doses used in the present study (0.001-0.01 mg/kg) are within the range of those used by Bischoff *et al.* (1997) (0.001-0.1 mg/kg). It is possible that reductions in cholesterol would have been observed if the present study had been carried out over several weeks rather than several hours.

It is important to note that both CVT and AVT significantly reduced HMGCoAR activities in both *in vitro* and *in vivo* experiments. This is particularly relevant as AVT was detected in surface waters 4 years ago (Miao and Metcalfe, 2003). The results of these experiments serve as a warning of potential effects that the presence of statin drugs in the aquatic environment could have on fish. Although further studies are needed in this area to examine the impact of waterborne exposure on HMGCoAR in fish, these studies provide the framework for experiments showing a potential effect of exposing a non-target organisms, such as fish to statin drugs.

Chapter 4 General Conclusions and Future Studies

The experiments reported in this thesis investigate some basic mechanisms involved in regulating HMGCoAR activities in rainbow trout. The results demonstrate that as in mammals fish HMGCoAR is regulated at both the transcription and enzyme activity levels, and in part by at least reversible phosphorylation/dephosphorylation. Having identified some characteristics of this enzyme in trout, the primary purpose of this project was to examine the effects of statin drugs on HMGCoAR activities.

Based on the results of these studies cerivastatin (CVT) inhibits the enzyme in both *in vitro* and *in vivo* studies while atorvastatin (AVT) inhibits HMGCoAR activities at least *in vitro* (*in vivo* studies were not undertaken). This inhibition takes place at concentrations similar to those observed in mammals but whether these pharmaceuticals would inhibit HMGCoAR and cholesterol synthesis when added into the water at concentrations found in the environment remains to be determined.

To assess the potential affects of statins, additional studies must be completed to enhance the groundwork that has been accomplished in the present study. It would be important to complete a waterborne exposure using environmentally relevant levels of statins (e.g. 1 ng/l AVT; a concentration detected in surface waters by Miao and Metcalfe, 2003) to examine the effects on HMGCoAR transcript and protein levels. A waterborne study would also enable one to examine whether the drug is taken up from the water and if it bioconcentrates in the tissues/plasma of the fish as previously reported for gemfibrozil, a lipid-lowering drug (Mimeault *et al.*, 2005) and fluoxetine, a selective-serotonin re-uptake inhibitor (Brooks *et al.*, 2003). Such studies would require quantification of statin levels in blood and other tissues of the fish; few studies of this kind have been undertaken although Dr. Chris Metcalfe (personal

communication) at Trent University is now able to establish tissue concentrations of at least a few pharmaceuticals. Using such studies body statin turnover could also be assessed to provide a pharmokinetic profile of these drugs.

It would also be informative to complete long-term waterborne exposures to statins to observe changes in plasma cholesterol to link this with changes in HMGCoAR transcript levels and activities. With a longer exposure period changes in cholesterol levels could be established to determine whether fish cholesterol levels are modified in a manner previously observed in mammalian studies. If cholesterol levels change, this may affect those components within the cholesterol synthetic pathway that may be important to health and fitness of the fish.

Examining steroid hormones including testosterone, estradiol, and cortisol, during long-term exposure to statins would demonstrate whether a reduction in cholesterol, as is the intended effect of statins, would in fact translate into serious downstream consequences as many other processes (e.g. steroid hormone production and membrane fluidity) depend on cholesterol.

Another important area worth examining would be the metabolites of these drugs. In some cases the metabolites of statins have inhibitory properties as well and are excreted in higher quantities than the parent compound itself (Moghadasian, 1999; Pfizer, 2005). The metabolites have yet to be measured in the aquatic environment, but presumably they would be found possibly at higher concentrations than the parent compound given the quantities that are excreted in the urine and feces.

Finally, statins are not the only drug received by the environment post-WWTP. Statins can interact with a number of other drugs including fibrate drugs resulting in a condition called rhabdomyolysis, or dissolution of muscle (Omar and Wilson, 2002). Fish are unable to avoid

drug mixtures in their environment so looking at the affects of drug mixtures could be very important.

The present study serves as an initial warning sign that these pharmaceutical products do have the capacity to disrupt HMGCoAR activity in fish. The statins examined in this study act in fish much as they do in mammals and this could be of particular concern to fish being exposed to these products in an aquatic environment throughout the duration of their lives.

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Appendix 1

HMGCoAR multiple sequence alignment completed by Xi Chen using sequences obtained from GenBank (see accession numbers in Appendix 3).

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CLUSTAL W (1.83) multiple sequence alignment

human           -MLSRLFRMHGLFVASHPWEVIVGTVTLTICMMSMNMFTGNNKICGWNYECPKFEEDVLS 59
seabass         -MLTRLFRMHGLLVASHPWEVIVGTVTLTICMMSMNMFTGNDQICGWNFDCPKTEEQILS 59
zebHMG1         -MLTRLFRIHGLFVASHPWEVIVATVTLTICMMSMNMFTGNNQICGWNFDCPKLDEQILS 59
zebHMG2         MMLSRLFRLHGLFVASHPWEVIVGTVAVTMCLMSMNAFAGNDQICGWNHQCPKVQEKIIS 60
RTHMG1          ------------------------------------------------------------
RTHMG2          ------------------------------------------------------------

human           SDIIILTITRCIAILYIYFQFQNLRQLGSKYILGIAGLFTIFSSFVFSTVVIHFLDKELT 119
seabass         SDIIILTITRCIAIVYIYFQFQNLRQLGSKYILGIAGLFTIFSSFVFSTVVIHFLDKELT 119
zebHMG1         SDIIILTITRCIAIVYIYFQFQNLRQLGSKYILGIAGLFTIFSSFVFSTVVIHFLDKELT 119
zebHMG2         GDMAILTITRCIAIIYIYIQFKNLRQLGSKYILGIAGLFTIFSSFVFSTVVVHFLGKELT 120
RTHMG1          ------------------------------------------------------------
RTHMG2          ------------------------------------------------------------

human           GLNEALPFFLLLIDLSRASTLAKFALSSNSQDEVRENIARGMAILGPTFTLDALVECLVI 179
seabass         GLNEALPFFLLLIDLSKACALAKYALSSSSQDEVRDNIARGMAVLGPTFTLDALVECLVI 179
zebHMG1         GLNEALPFFLLLIDLSKACALAKFALSSNSQDEVRENIAKGMAVLGPTFTLDALVECLVI 179
zebHMG2         GLNEALPFFLLLIDLSKACTLAKFALSSNSQEEVRENIAQGMAILGPTFTLDAVVECLVI 180
RTHMG1          ------------------------------------------------------------
RTHMG2          ------------------------------------------------------------

human           GVGTMSGVRQLEIMCCFGCMSVLANYFVFMTFFPACVSLVLELSRESREGRPIWQLSHFA 239
seabass         GVGTMSGVRQLEIMCCFGCMSVLANYFVFMTFFPACVSLVLELSRESQEGHPIWQLSHFS 239
zebHMG1         GVGTMSGVRQLEIMCCFGCMSVLANYFVFMTFFPACVSLVLELSRESREGRPIWQLSHFA 239
zebHMG2         GVGTMSGVRQLEIMCCFGCMSVLANYFVFMTFFPACVSLVLELSRESREGRPIWQLSHFA 240
RTHMG1          ------------------------------------------------------------
RTHMG2          ------------------------------------------------------------

human           RVLEEEE-NKPNPVTQRVKMIMSLGLVLVHAHSRWIADPSPQNSTADTSKVSLGLDENVS 298
seabass         RVMEEEEDNKPNPVTQRVKMIMSLGLVMVHAHSRWIAEPLSINTTVDIPQVGMELDHLSP 299
zebHMG1         RVLEEEEDNKPNPVTQRVKMIMSLGLVMVHAHSRWIADPASSDGTLDLPEVGMSLNENLP 299
zebHMG2         RVLEEEEGNKPNPVTQRVKMIMSLVLALVHAHSRLKTESPAHN--ISSSDVVLPTSNMES 298
RTHMG1          ------------------------------------------------------------
RTHMG2          ------------------------------------------------------------

human           KRIEPSVSLWQFYLSKMISMDIEQVITLSLALLLAVKYIFFEQTETESTLSLKNPITSP- 357
seabass         RRIEPEKPLWHFYLTRMITMDIEQVICLALALLLAIKYIFFEQVEMESTLSLKNPITMSV 359
zebHMG1         KRIEPDMPLWHFYLSRMISMDIEQVITLGLALFLAVKYIFFEQVEMESTLSLKVPTPS-- 357
zebHMG2         DRS------------MSSVDLEQVITLSLALLLAAKYVFFEQAETESSLSIKTTVATPN 345
RTHMG1          ------------------------------------------------------------
RTHMG2          ------------------------------------------------------------

human           -VVTQKKVPDNCCRREPMLVRNNQKCDSVEEETGINRERKVEVIKPLVAETDTPNRATFV 416
seabass         PALTPRRPVETCCRKEPYTPRPLAPTQTVAAPAPTNMEERDEVIRPLSAPPADPQQKSFF 419
zebHMG1         SMLTQKWSPDQCCRKEIPYSTKLD--KPPTPPPVVTKEERDLVIRPLPAPKEPEQKSTFV 415
zebHMG2         CSLINRRGGEERCQRDSAPPKAIKGVNENKS------------------EREAPDRSFS 386
RTHMG1          ------------------------------------------------------------
RTHMG2          ------------------------------------------------------------
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human           VGNSSLLDTSSVLVTQEPEIELPREPRPNEECLQILGNAEKGAKFLSDAEIIQLVNAKHI 476
seabass         VVGG---GEEELRPKTN-QLSIPLTPRGLDECVAILNSPDLGPCFLSDAEVMLLVNSKHI 475
zebHMG1         LGED---ETQENTHVPEPVMSLPAEPRPVEDCLSILKNPDMGARYLSDEEVIVLVNSKHI 472
zebHMG2         IGNP---EDEERD---------KIQCRPVQECLAILKDPKRGAGFLSNAEVMALVESRGI 434
RTHMG1          ------------------------------------------------------------
RTHMG2          ------------------------------------------------------------

human           PAYKLETLMETHERGVSIRRQLLSKKLSEPSSLQYLPYRDYNYS---------------- 520
seabass         PAYKLEAMMERPERGVAIRRQMISAKLPTPSALSSLPYTDYDYSKVIGACCENVIGYMPV 535
zebHMG1         PAYKLEAMMETPERGVMIRRKMLSPKFPEPSTLSCLPYKDYDYSKVMGTCCENVIGYMPV 532
zebHMG2         LGYRLEAVMESPERGVAIRREMLSGKLPDSSALENLPYKHYDYSKVVGSNCENVIGYVPV 494
RTHMG1          ----------------------------------------------CCENVIGYMPI 11
RTHMG2          ------------------------------------------------------------

human           ------------------------------------LLGGGASSRVLADGMTRGPVVRL 543
seabass         PVGVAGPLHLDGKQFQVPMATTEGCLVASTNRGCRAIALGGGASSRILADSMTRGPVVRL 595
zebHMG1         PVGVAGPLLLDGKQFQVPMATTEGCLVASTNRGCRAIALAGGASSRVLADGMTRGPVVRF 592
zebHMG2         PVGVAGPLLLDGKEFHVPMATTEGCLVASTNRGCRAVTLSGGVSSRVLADGMTRGPVVQM 554
RTHMG1          PVGVAGPLHLDGKQFQVPMATTEGCLVASTNRGCRAISLGGGASSRILADGMTRGPVIRC 71
RTHMG2          ------------------------------------------------------------

human           PRACDSAEVKAWLETSEGFAVIKEAFDSTSRFARLQKLHTSIAGRNLYIRFQSRSGDAMG 603
seabass         PSAYQAAEVKAWLESTDGFQAIKEAFDNTSRFARLQKLLVCLAGRNLYIRFHSKTGDAMG 655
zebHMG1         PSACQAAEVKAWLESPEGFKTIKEAFDQTSRFARLEKLLIGLASRNLYIRFQSRTGDAMG 652
zebHMG2         PSACRAAEVKGWLESSEGFTEIKQAFDHTSRFARLDRLQVSLAGRNLYIRFQSQTGDAMG 614
RTHMG1          PSACLAAEVKGWLESTDGFQAIKDAFDHTSRFARLQKLMIGLAGRNLYIRFQSRTGDAMG 131
RTHMG2          ----------------------------------------------------GDAMG 5
                                                                     *****

human           MNMISKGTEKALSKLHEYFPEMQILAVSGNYCTDKKPAAINWIEGRGKSVVCEAVIPAKV 663
seabass         MNMISKGTEQALSRLQQHFPELQVVAVSGNYCTDKKPAAINWIEGRGKSAVCEATIPAKV 715
zebHMG1         MNMISKGTEQALARLKEEFPELQVLAVSGNYCTDKKPAAINWIEGRGKSVVCETTIPAKV 712
zebHMG2         MNMLSKGTEQALGRLQERYPDMRLLAVSGNYCTDKKPAAVNWIHGRGKSTVCEAVIPARV 674
RTHMG1          MNMISKGTEQALARLQEQYPELVVLAVSGNYCTDKKPAAINWIEGRGKSAVCEATIPAKV 191
RTHMG2          MNMLSKGTEQAVSRLQQQFPELQVLAVSGNYCTDKKPSAMNWILGRGKSAVCEVTVPARV 65
                ***:*****:*:.:*:: :*:: ::************:*:*** *****.***..:**:*

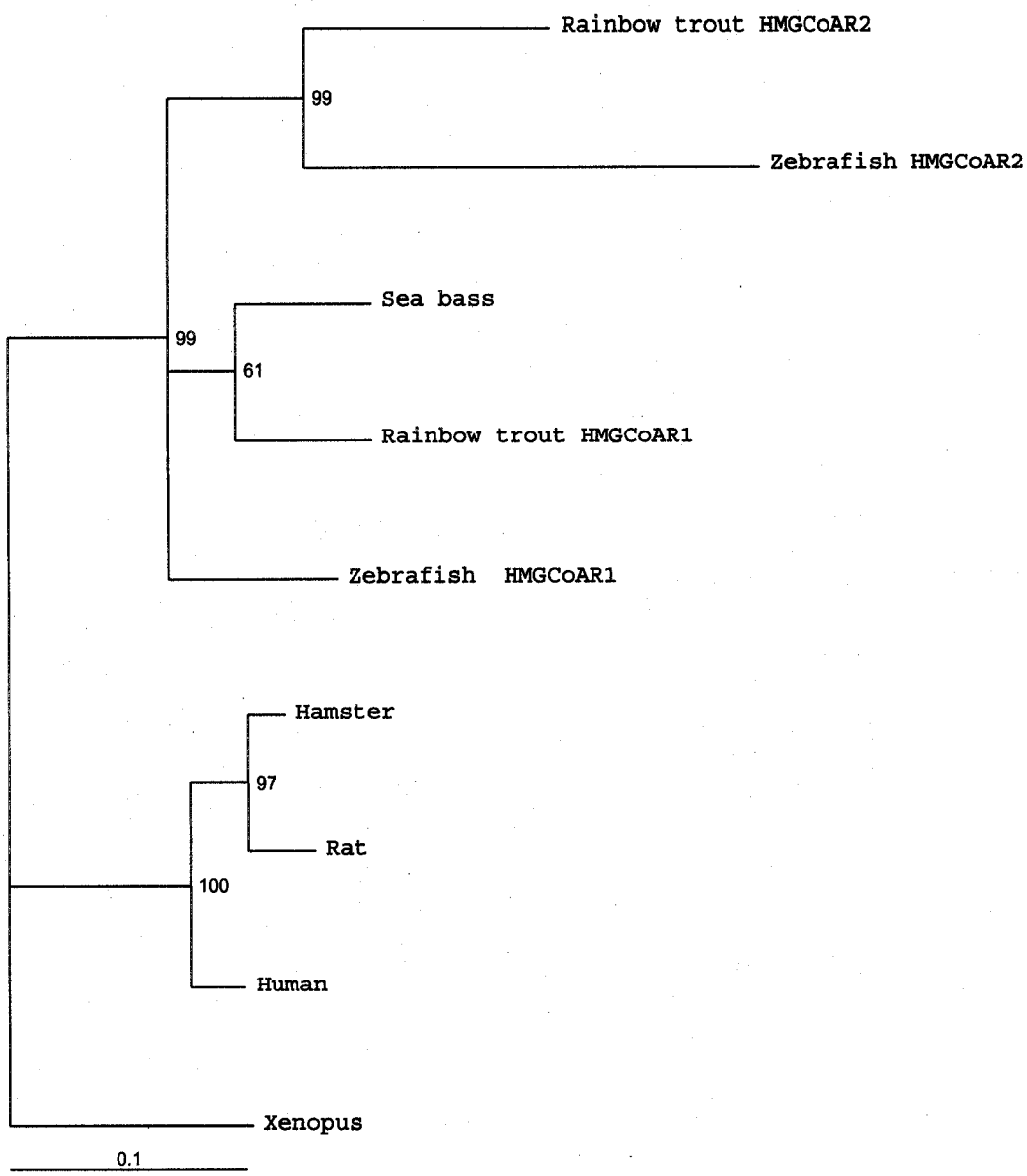
human           VREVLKTTTEAMIEVNINKNLVGSAMAGSIGGYNAHAANIVTAIYIACGQDAAQNVGSSN 723
seabass         VREVLKTTTGALVEVNISKNLVGSAMAGSIGGCNAHAANLVAAIYIACGQDPAQSVGSSN 775
zebHMG1         VKEILKTSTEALVDVNISKNLVGSAMAGSIGGYNAHAANIVAAIYIACGQDPAQTVGSSN 772
zebHMG2         VKEVLKSSTAALVELNISKNLVGSAMAGSIGGFNAHAANIVTAIYIACGQDPAQTVGSSN 734
RTHMG1          VREVLKTSTEALVDVNINKNLVGSAMAGSIGGCN-------------------------- 225
RTHMG2          VREVLKTSTTALVELNINKNLVGSAMAGSIGGFNAHAANIVAAIYIACGQDPAQTVGSSN 125
                *:*:**::* *::::**.************** *

human           CITLMEASGPTNEDLYISCTMPSIEIGTVGGGTNLLPQQACLQMLGVQGACKDNPGENAR 783
seabass         CITLMEASGPTGEDLYISCTMPSIELGTVGGGTNLPPQQACLQMLGVQGASQECPGENAR 835
zebHMG1         CITLMEASGATGEDLYISCTMPSIELGTVGGGTNLPPQQACLKMLGVLGASQECPGENAR 832
zebHMG2         CITLMECVGTSGEDLYVSCTMPSLELGTIGGGTGLPAQHACLQMLGVGGASVQCPGENSR 794
RTHMG1          ------------------------------------------------------------
RTHMG2          CITLMEQVGPEGEDLYISCTMPSIELGTVGGGTNLAPQQSCLQMLGVQGACXW------- 178

human           QLARIVCGTVMAGELSLMAALAAGHLVKSHMIHNRSKINLQDLQGACTKKTA 835
seabass         QLARVVCATVLAGELSLMAALAAGHLVKSHMTHNRSKVNLQETPGTCTNKAS 887
zebHMG1         QLAKVVCATVLAGELSLMSALAAGHLVKSHMTHNRSKVNLQEAPGTCTKQAS 884
zebHMG2         TLARIVCASVLAGELSLMAALAAGHLVQSHMTHNRSKVNLREPEGQPKEAS- 845
RTHMG1          ----------------------------------------------------
RTHMG2          ----------------------------------------------------
```

Appendix 2

A preliminary phylogenetic analysis undertaken by Xi Chen to distinguish between the two HMGCoAR subtypes (HMGCoAR-1 and HMGCoAR-2) identified in this study. Given that they do not group together in the tree, it can be concluded that they represent two separate subtypes of HMGCoAR. Phylogenetic tree was created using PHYLIP (Phylogeny Inference Package).



Appendix 3

Table showing the gene accession number and associated species as listed in GenBank

Species	**Gene Accession Number**
Sea bass	AAR02862
Zebrafish HMG1	XP_684400 replaced by NP_001073446
Zebrafish HMG2	NP_001014314
Human	AAH33692
Golden hamaster	P09610
Rat	NP_037266
Xenopus	P20715

Appendix 4

Identity table comparing the amino acid sequences of both rainbow trout HMGCoAR subtypes with other speices.

	HMGCoAR1	**HMGCoAR2**	**Human**	**Sea bass**
HMGCoAR1		84%	82%	88%
HMGCoAR2	84%		81%	87%

The similarity number is calculated from BLAST using GenBank accession numbers noted in Appendix 3 and amino acid multiple alignments (Clustal W) (Appendix 1).