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Effects of Peroxisome Proliferator-Activated Receptor Ligands on the Hypothalamic-Pituitary-Gonadal Axis of the male Goldfish

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**Effects of Peroxisome Proliferator-Activated Receptor Ligands on
the Hypothalamic-Pituitary-Gonadal Axis of the Male Goldfish**

Colin Cameron

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
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List of Abbreviations

2EHXA	-	2-ethylhexanoic acid
AC	-	Adenylyl cyclase
ATP	-	Adenosine triphosphate
BEZ	-	Bezafibrate
cAMP	-	Cyclic Adenosine Monophosphate
CLO	-	Clofibrate
DAG	-	Diacylglycerol
DBP	-	Dibutyl phthalate
DEHP	-	Di(2-ethylhexyl) phthalate
ERK 1/2	-	Extracellular Signal-regulated Kinase 1/2
FEN	-	Fenofibrate
GEM	-	Gemfibrozil
GH	-	Growth Hormone
hCG	-	Human Chorionic Gonadotropin
IP3	-	Inositol trisphosphate
LH	-	Luteinizing Hormone
LH-R	-	Luteinizing hormone receptor
MAPK	-	Mitogen-activated Protein Kinase
MEHP	-	Mono(2-ethylhexyl) phthalate
MEK	-	Mitogen-activated protein kinase kinase
PBR	-	Peripheral Benzodiazepine Receptor
PFAAs	-	Perfluorinated Alkyl Acids
PFOA	-	Perfluorooctanoic acid
PFOS	-	Perfluorooctanesulfonic acid
PHs	-	Phthalates
PIP2	-	Phosphatidylinositol 4,5-bisphosphate
PKA	-	Protein kinase A
PKC	-	Protein kinase C
PLC	-	Phospholipase C
PPAR α	-	Peroxisome Proliferator-Activated Receptor α
PPAR β	-	Peroxisome Proliferator-Activated Receptor β
PPAR γ	-	Peroxisome Proliferator-Activated Receptor γ
Raf	-	Proto-oncogene serine/threonine-protein kinase
ROS	-	Rosiglitazone
StAR	-	Steroidogenic Acute Regulatory Protein

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Abstract

Fibrate pharmaceuticals are peroxisome proliferator-activated receptor α (PPAR α) ligands that are detected in sewage treatment plant effluents, surface water and groundwater. In a previous study, male goldfish (*Carassius auratus*) exposed to the fibrate gemfibrozil (GEM) in the water exhibited decreased plasma testosterone concentrations. The following studies investigate the effects of GEM and other PPAR ligands on components of the hypothalamic-pituitary-gonadal axis of the male goldfish; the pituitary and testes. *In vitro* cultures of dispersed goldfish pituitary cells and testis fragments were employed to study these two tissues in isolation. The secretion of neither luteinizing hormone nor growth hormone from pituitary cells was affected in a manner that could explain decreases in circulating testosterone. Testosterone synthesis in testis fragments stimulated with human chorionic gonadotropin (hCG) was suppressed following treatment with GEM. This inhibition appeared to be the result of impaired mitochondrial cholesterol transport. However, changes in the expression of two proteins important for mitochondrial cholesterol transport, steroidogenic acute regulatory protein and peripheral benzodiazepine receptor, were not affected by GEM treatment. Investigations on the involvement of extracellular-regulated signal kinase 1/2 (ERK 1/2) in the steroidogenic pathway revealed that phosphorylated ERK 1/2 (P-ERK 1/2) is required for hCG-stimulated testosterone synthesis and that GEM treatment decreased levels of P-ERK 1/2 in the mitochondria of this tissue. The main conclusions from these studies include the following: (1) The effect of waterborne GEM on the HPG axis of the goldfish is a

direct effect on the testis, (2) GEM impairs mitochondrial cholesterol transport in the goldfish testis, (3) Phospho-ERK 1/2 is required for hCG-stimulated steroidogenesis in the goldfish testis, and (4) the proposed mechanism by which GEM impairs mitochondrial cholesterol transport is through a reduction in mitochondrial phospho-ERK 1/2.

Résumé

Les fibrates sont des médicaments qui sont des ligands pour le récepteur au facteur de prolifération des peroxysomes α (PPAR α) et qui sont détectés dans les effluents d'usines de traitement des eaux usées, et dans les eaux de surface et souterraines. Dans une étude précédente, la fibrate gemfibrozil (GEM) causa une diminution de la concentration de testostérone dans le plasma des cyprins dorés (*Carassius auratus*) mâles. Les études suivantes investiguèrent les effets de GEM et autres ligands de PPAR sur les gonades et l'hypophyse, composantes de l'axe hypothalamo-hypophyso-gonadal (HHG) des cyprins dorés mâles. Des cultures *in vitro* de cellules hypophysaires dispersées et des fragments de gonades furent utilisés pour étudier ces tissus en isolation. La sécrétion d'hormone lutéinisante (LH) et d'hormone de croissance (GH) des cellules hypophysaires n'ont pas été changées d'une manière qui expliquerait la diminution de testostérone circulante. La synthèse de testostérone dans les fragments de gonades stimulée par l'hormone gonadotrope chorionique humaine (hCG) fut supprimée suivant un traitement avec GEM. Cette inhibition paraît être le résultat du transport mitochondrial de cholestérol affaibli. Par contre, l'expression de deux protéines importantes pour le transport mitochondrial de cholestérol, la protéine régulatrice stéroïdogénique aigüe et récepteur périphérique de benzodiazépine, ne fut pas affectée par les traitements de GEM. Les investigations dans la participation de la kinase de signal de régulation extracellulaire 1/2 (ERK 1/2) dans la voie stéroïdogénique révélèrent que la ERK 1/2 phosphorylée (P-ERK 1/2) est nécessaire pour la synthèse de testostérone stimulée

par hCG et un traitement de GEM décroît les niveaux de P-ERK 1/2 dans les mitochondries de ce tissu. Les principales conclusions de ces études sont les suivantes: (1) l'effet de GEM d'origine hydrique sur l'axe HHG des cyprins dorés est un effet direct sur les gonades, (2) GEM affaiblit le transport mitochondrial de cholestérol dans les gonades des cyprins dorés mâles, (3) P-ERK 1/2 est nécessaire pour la stéroïdogénèse stimulée par l'hCG, et (4) le mécanisme proposé par lequel GEM affaiblit le transport mitochondrial de cholestérol et par une réduction de P-ERK 1/2 mitochondrial.

Chapter 1: General Introduction

1.1 Statement of the problem

Many human pharmaceuticals are now detected in the environment worldwide but particularly in surface water. Acute toxicity of these compounds at the measured concentrations (ng/L to µg/L) is unlikely. The sub-lethal effects of these compounds have not been fully studied in any species but nonetheless evidence is accumulating to support the notion that these pharmaceuticals present a threat to aquatic species. Pharmaceuticals entering surface water from sewage treatment facilities are considered pseudopersistent, as they often enter surface water at a rate that is higher than or equal to their rate of degradation. Endocrine disruption is an interference in the normal functioning of the endocrine system by exogenous compounds resulting in detrimental effects to an individual's physiology. Many pharmaceuticals are potential endocrine disruptors as these compounds are designed to be highly active at the receptor or enzyme level, directly modulating endocrine systems, or indirectly affecting metabolic pathways that are regulated by endocrine systems.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors involved in metabolic regulation. Their endogenous ligands include fatty acids, prostaglandins, leukotrienes, and eicosanoids. Many synthetic compounds are capable of binding to and activating these receptors, including fibrate and thiazolidinedione pharmaceuticals, phthalate ester plasticizers, and perfluorinated

compounds (Desvergne and Wahli, 1999; Corton et al., 2000; Corton and Lapinskas, 2005; DeWitt et al., 2009). The fibrate drugs, synthetic ligands for peroxisome proliferator-activated receptor α (PPAR α), were designed as therapies to treat human hyperlipidemia and hypercholesterolemia. Fibrates are frequently detected in surface water and there is evidence that they may affect the reproductive systems of mammals and fish.

In goldfish (*Carassius auratus*) exposed to waterborne gemfibrozil (GEM) at environmental concentrations, the plasma concentration of GEM was highly elevated (bioconcentration >100x) and testosterone concentrations were significantly depressed (Mimeault et al., 2005). This thesis examines the hypotheses that GEM affects the reproductive axis of the goldfish, a model Cyprinid species, by directly impairing endocrine function of the pituitary gland and testes. This Introduction will review the relevant research in this field with support from observations in mammalian models where gaps exist in the current state of knowledge of these systems in fish.

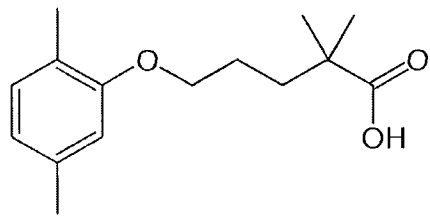
1.2 Peroxisome proliferators in the environment

Peroxisome proliferators (PPs) are defined as compounds that stimulate increases in cellular peroxisome size and density through binding to and activating peroxisome proliferator-activated receptors (PPARs). The degree of cellular peroxisome proliferation is species-specific and the effects of PPs are not limited to peroxisome proliferation. The term PP is more widely used to describe any ligand

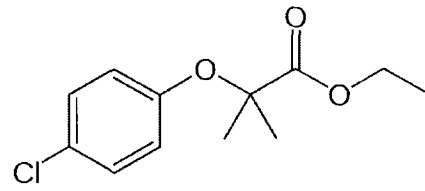
having biological effects through PPARs. Peroxisome proliferators detected in the environment include phthalate ester plasticizers (Oehlmann et al., 2009), perfluorinated compounds (Jensen and Leffers, 2008), and pharmaceuticals including nonsteroidal anti-inflammatories and fibrates (Kolpin et al., 2002; Metcalfe et al., 2003ab), the subject of this investigation (Fig. 1.1).

Fibrate pharmaceuticals are detected in sewage treatment plant (STP) effluents, surface water, groundwater, and even drinking water supplies worldwide. The major route of entry of pharmaceuticals into the aquatic environment is through STPs via excretion or direct disposal of pharmaceuticals into the wastewater stream (Heberer, 2002; Fent et al., 2006; Rahman et al., 2009; Snyder and Benotti, 2010). From STPs, pharmaceuticals may be either released in effluent directly into surface water or deposited into sewage sludge. Sewage sludge containing pharmaceuticals applied as fertilizer may then result in pharmaceuticals being transported in surface run-off, making its way into the aquatic environment (Xia et al., 2005). Groundwater supplies may become contaminated through exchange with surface water (Heberer, 2002).

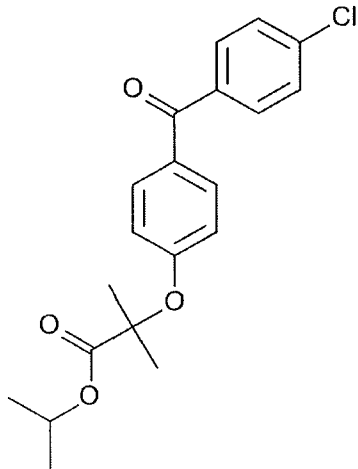
Fibrates are detected in both STP effluents and surface water in various locations within Canada. In the effluents of 18 STPs throughout Canada, the maximum measured concentration of bezafibrate (BEZ) was 0.6 µg/L and for GEM 1.3 µg/L (Metcalfe et al., 2003a). In STP effluents in Peterborough, Ontario, mean concentrations for BEZ, clofibrac acid (CLOa), and GEM were 0.259, 0.044, and 1.493 µg/L, respectively (Metcalfe et al., 2003b). Another group reported a maximum



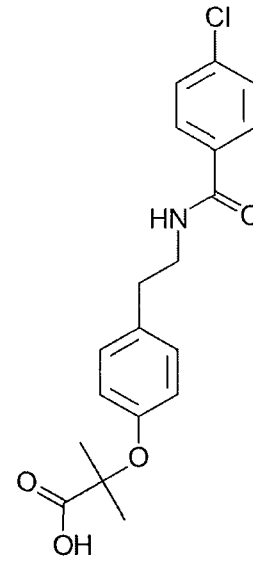
gemfibrozil



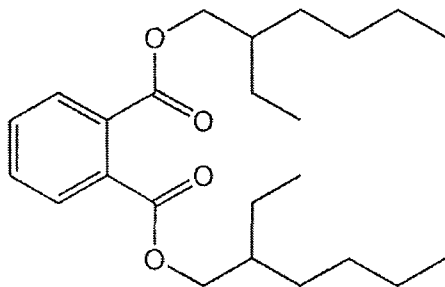
clofibrate



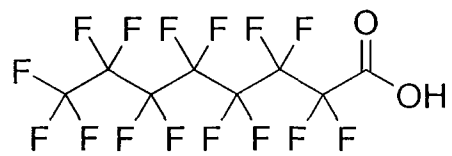
fenofibrate



bezafibrate



di(2-ethylhexyl) phthalate



perfluorooctanoic acid

Figure 1.1. Chemical structure of selected peroxisome proliferators.

concentration of GEM of 0.436 µg/L in STP effluents discharging into the Thames River of southern Ontario (Lishman et al., 2006). In Quebec, fibrates detected in municipal wastewater effluents include BEZ at concentrations up to 72 ng/L and GEM at concentrations up to 84 ng/L (Gagné et al., 2006). In surface waters adjacent to STP effluent discharge sites in Ontario, various fibrates were detected including CLOa (maximum 0.175 µg/L), GEM (maximum 0.112 µg/L), and BEZ (maximum 0.2 µg/L) (Metcalf et al., 2003b). Lower concentrations in surface water would be predicted based on partial removal of these pharmaceuticals in STPs as well as dilution with surface water. However, it is apparent that significant amounts of these pharmaceuticals are entering the aquatic ecosystem.

1.3 Peroxisome proliferator-activated receptors

Three different PPAR isoforms, PPAR α , PPAR β (or δ) and PPAR γ , encoded by different genes, have been identified (Berger and Moller, 2002). These receptors belong to the nuclear receptor superfamily in which an early gene duplication event generated 6 nuclear receptor subfamilies: Thyroid Hormone Receptor-like, Retinoid X Receptor-like, Estrogen Receptor-like, Nerve Growth Factor IB-like, Steroidogenic Factor-like, and Germ Cell Nuclear Factor-like. Subsequent gene duplication events resulted in vertebrate-specific genes within each of these nuclear receptor subfamilies (i.e.: PPAR α , PPAR β , PPAR γ within the Thyroid Hormone Receptor-like subfamily) (Laudet, 1997; Escriva et al., 2000; Escriva et al., 2002; Michalik et al., 2002). Within the PPARs, there is a higher similarity between PPAR α and PPAR β as

compared to PPAR γ (Laudet, 1997). The PPARs share structural similarities with the nuclear receptor superfamily members including a ligand-independent transcriptional activation (AF-1) domain (A/B region), a DNA-binding domain (C region), a hinge domain (D region), and domains for ligand binding, receptor dimerization, and ligand-dependent transactivation (AF-2) (E region) (Corton et al., 2000; Feige et al., 2006). The major endogenous PPAR ligands are polyunsaturated fatty acids, some saturated and mono-unsaturated fatty acids, and eicosanoids. Synthetic ligands include fibrates, thiazolidinediones, non-steroidal anti-inflammatory drugs, and phthalates (Desvergne and Wahli, 1999; Corton et al., 2000; Corton and Lapinskas, 2005). As nuclear receptors, PPARs function as transcription factors regulating the transcriptional activity of target genes. Upon ligand binding, PPARs heterodimerize with the retinoid X receptor (RXR), bind to PPAR response elements (PPREs) on genomic DNA, and activate the transcription of target genes (Berger and Moller, 2002). Response element binding by PPARs is dependent upon the presence of direct repeat (DR)-1 elements spaced by a single nucleotide (consensus sequence: 5'-AGGTCANAGGTCA-3')(Palmer et al., 1995; Berger and Moller, 2002). In contrast to these effects mediated through PPREs, PPARs may also indirectly affect transcription by either inhibiting the DNA interactions of other transcription factors ("tethering") or by competing for cofactors required by other transcription factors ("squenching") (Feige et al., 2006). Additionally, PPARs may also be involved in non-nuclear cell signalling including intracellular phosphorylation cascades (Gardner et al., 2005; Nakai et al., 2008; Lombardi et al., 2008) and

changes in intracellular calcium ion concentrations (Ropero et al., 2009; Suh et al., 2008).

While PPAR α expression has been detected in most tissues studied, it is most highly expressed in liver where it regulates the expression of several genes controlling fatty acid catabolism including those involved in fatty acid uptake, peroxisomal and mitochondrial β -oxidation, and microsomal ω -oxidation (Desvergne and Wahli, 1999; Berger and Moller, 2002; Lee et al., 2003; Pyper et al., 2010). The fibrates, a group of pharmaceuticals prescribed in the treatment of hyperlipidemia, are synthetic ligands for PPAR α . Their therapeutic effect is based upon increased fatty acid oxidation, and in particular, peroxisomal β -oxidation. Peroxisomes are cellular organelles with a variety of functions including the β -oxidation of very long chain fatty acids. Peroxisome proliferation is an increase in the size and number of cellular peroxisomes, along with an increased rate of peroxisomal β -oxidation, and is reported to occur in the liver (Desvergne and Wahli, 1999). This dramatic increase in the size and number of hepatic peroxisomes is observed in rodents and is dependent upon PPAR α (Lee et al., 1995), although this process does not occur in humans (Desvergne and Wahli, 1999). Increases in peroxisomal fatty acid oxidation may lead to generation of reactive oxygen species and cellular oxidative stress. Hydrogen peroxide is a byproduct of acyl CoA-oxidase activity and, if not neutralized by the enzyme catalase, may result in oxidative damage to the cell (Schrader and Fahimi, 2006).

In contrast to PPAR α , PPAR γ is involved in lipid storage and adipocyte

differentiation, and is expressed in a wide variety of tissues although the highest levels are found in adipose tissue (Desvergne and Wahli, 1999). This receptor is the target of the anti-diabetic thiazolidinedione (TZD) pharmaceuticals which function by improving adipose tissue insulin sensitivity. The tissue expression pattern of PPAR β is typically much broader than that observed for other PPAR isoforms. PPAR β is involved in fatty acid catabolism and anabolism, being functionally similar to both PPAR α and PPAR γ , respectively (Bocher et al., 2002; Dressel et al., 2003; Michalik et al., 2003; Luquet et al., 2005).

In addition to their role in peroxisome proliferation and lipid homeostasis, PPARs have a wider range of biological functions, including effects on reproductive development (Sharpe, 2001), ovarian and testicular steroidogenesis (Gazouli et al., 2002; Schoppee et al., 2002), and cell proliferation and apoptosis (Gonzalez et al., 1998; Heaney, 2003). The role of PPARs in reproductive function are the focus of this thesis. Of particular interest is the potential for gemfibrozil in the aquatic environment to have effects on the reproductive function of aquatic species.

1.4 Gemfibrozil pharmacokinetics in humans and goldfish

Gemfibrozil (GEM) is a fibrate pharmaceutical, prescribed in individuals with elevated plasma triglyceride and cholesterol concentrations, and is a PPAR α agonist. The typical oral dosage of GEM in humans is 600 mg twice per day. Plasma concentrations of GEM reach a peak of 20-50 $\mu\text{g/mL}$ (80-200 μM) within 1-2 h following ingestion and the half-life of GEM in plasma is 1-1.5 h (Miller and Spence,

1998; Kyrklund et al., 2003; Rouini et al., 2006; Rouini et al., 2008). The majority of the GEM absorbed into circulation is either glucuronidated and excreted or eliminated through the feces (Miller and Spence, 1998).

In goldfish injected with GEM (5 ng GEM/g of fish), the mean plasma concentration after 24 h was 10300 µg/mL (41.3 µM), and at 96 h less than 5% of this GEM remained in the plasma (Mimeault et al., 2005). This finding indicates that the residence time of GEM in the goldfish is limited to several days. Goldfish exposed to 1500 µg/L and 10000 µg/L GEM in the water for 96 h had plasma GEM concentrations ranging from 75000-180000 µg/L (300-700 µM) (Mimeault et al., 2005). In a longer-term experiment (14 days), goldfish treated with 1.5 µg/L and 1500 µg/L GEM in the water had plasma GEM concentrations of 170 µg/L (0.68 µM) and 78000 µg/L (311.6 µM), respectively (Mimeault et al., 2005). In both of these experiments with goldfish (96 h and 14 d GEM exposure), plasma testosterone concentrations were significantly decreased in male goldfish within all GEM treatment groups. These plasma GEM concentrations reported for goldfish are also within the range of therapeutic concentrations reported in humans. For in vitro experiments within this thesis, PPAR agonist treatment concentrations were selected to encompass a range that included the plasma concentrations (0.68 µM) observed in goldfish exposed to environmental levels of GEM and the lower spectrum of the pharmacological concentrations observed in humans (100 µM).

1.5 Structure and function of PPARs in fish

All 3 PPARs identified in mammals have been reported in various fish species. Fish PPAR α and PPAR β show high nucleotide sequence similarity when compared to the homologous receptors in mammals, and predicted amino acid sequence identities are in the range of 65-85% for these two receptors. Fish PPAR γ does not appear to be as well conserved, and predicted amino acid sequences generally show identities less than 70% with mammalian receptors. Multiple genes for various PPARs have been described within some fish species (Maglich et al., 2003; Leaver et al., 2007; Robinson-Rechavi et al., 2001). Multiple transcript variants have also been described for Atlantic salmon (*Salmo salar*) PPAR γ (Andersen et al., 2000). In many species, however, only single genes and transcripts have been described for each PPAR, including the goldfish. The DNA- and ligand-binding domains of fish PPARs are highly conserved, with the DNA-binding domain being the most highly conserved region (Boukouvala et al., 2004; Leaver et al., 2005; Kondo et al., 2007; Tsai et al., 2008). Within the ligand-binding domain, four amino acid residues deemed important for ligand binding have been conserved in fish PPAR α , whereas a single substitution has occurred in fish PPAR β , and only two of these residues are conserved in fish PPAR γ (Maglich et al., 2003; Leaver et al., 2005; Kondo et al., 2007; Tsai et al., 2008). These changes may explain differences between mammalian and fish PPAR γ ligand binding. The least conserved region of fish PPARs is the A/B domain (Boukouvala et al., 2004; Leaver et al., 2005; Tsai et al., 2008; Raingeard et al., 2009). This region is important for ligand-independent

transactivation and this suggests differences in receptor activation and cofactor recruitment in fish. The lack of PPAR γ conservation observed between mammalian and fish species may be explained by differences in use of fuels (lipid vs. carbohydrate) and regulation of blood glucose levels in fish (Leaver et al., 2005). This lack of conservation may also be partially due to the fact that comparisons of PPARs have been made between mostly carnivorous fish species and a select few omnivorous mammalian species.

The tissue distribution of PPAR types in fish has been described for some species as generally similar to that of mammals. Such a distribution has been described for all three PPAR types in sea bass (*Dicentrarchus labrax*) (Boukouvala et al., 2004). In the thicklip grey mullet (*Chelon labrosus*), PPAR α was found in all tissues studied but was relatively highly expressed in liver and lower in white muscle (Raingeard et al., 2006). In plaice (*Pleuronectes platessa*) and gilthead sea bream (*Sparus aurata*), the tissue expression profiles for both PPAR α and PPAR β are also similar to those of mammals (Leaver et al., 2005). In other species, the tissue distribution of PPARs differs from that observed in mammals. In species such as the pufferfish (*Fugu rubripes*), a relatively broad expression pattern is observed for PPARs, where all three receptor isoforms are expressed in all tissues studied (brain, gill, gut, heart, liver, ovary) (Maglich et al., 2003). Differences have also been noted for PPAR γ , which was more widely expressed in plaice and sea bream as compared to the situation in mammals (Leaver et al., 2005). In goldfish, all 3 PPARs were broadly expressed in all tissues studied including brain, testes, heart, intestine,

kidney, liver, red muscle, spleen, and white muscle (Mimeault et al., 2006).

PPARs in several fish species bind endogenous and pharmaceutical ligands, and recognize mammalian PPRES, suggesting a receptor function similar to that in mammals. Fish PPAR receptor function has been demonstrated in various studies utilizing *in vitro* ligand-activated transcriptional assays. For the most part, fish PPAR α behaves as expected when compared to mammalian receptors. In a reporter assay using acyl-CoA oxidase PPRES, pufferfish PPAR α was responsive to 5,8,11,14-eicosatetraenoic acid (ETYA), arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and Wy-14,643 (Kondo et al., 2007). Plaice and bream PPAR α are also activated by a wide variety of fatty acids, with high activation being observed with oleic acid, conjugated linoleic acid (CLA), as well as synthetic ligands (Wy-14,643, ETYA) (Leaver et al., 2005). In rainbow trout (*Onchorynchus mykiss*) gill cells transfected with a reporter construct containing the PPRES-1 of the rat liver 3-ketoacyl-CoA thiolase B gene, WY-14,643, GEM and BEZ were able to induce luciferase activity, whereas ROS was not (Liu et al., 2005). Ligands that bind to sea bass PPAR α include linoleic, linolenic, CLA, ETYA, and Wy-14,643, but not EPA or DHA (Boukouvala et al., 2004).

There are differences in PPAR β and PPAR γ ligand activation between fish and mammals, and also among different fish species. Sea bass PPAR β heterodimerizes with RXR, binds to PPRES, and is activated by linoleic acid, linolenic acid, CLA, and ETYA, but not EPA or DHA (Boukouvala et al., 2004). Pufferfish PPAR β showed only slight and insignificant responses to ARA and EPA

(Kondo et al., 2007). Sea bream PPAR β was only moderately activated by fatty acids and ETYA, and in comparison plaice PPAR β was poorly activated by the same ligands (Leaver et al., 2005). An Atlantic salmon PPAR β variant (ssPPAR β 1A) is activated by monounsaturated fatty acids, 2-bromopalmitate, and GW501516, a mammalian PPAR β ligand. However, another receptor variant (ssPPAR β 2A) is not activated by these compounds but is able to inhibit activity of the other receptor variant (Leaver et al., 2007). Potent activators of fish PPAR γ are yet to be identified. Pufferfish PPAR γ is not activated by fatty acids (AA, EPA, DHA) or the PPAR γ -specific synthetic ligand rosiglitazone (ROS) (Kondo et al., 2007). Plaice and sea bream PPAR γ were not activated by a variety of fatty acids, however PFOA moderately activated the receptor in both species, and ROS was only able to slightly activate sea bream PPAR γ (Leaver et al., 2005). In rainbow trout gill cells transfected with a PPARE reporter construct, ROS treatment was unable to induce luciferase activity (Liu et al., 2005). It could be suggested that the trout gill cells contain active PPARs (likely PPAR α), and either do not express PPAR γ or that this receptor is not responsive to ROS, as is the case with other fish species. Luciferase activity in these cells, however, is induced with ROS in the presence of retinoic acid (Liu, Moon and Trudeau, unpublished results). The absence of effect with any ligand in transcriptional assays could also suggest a deficiency in another aspect of transcriptional complex formation by PPARs such as retinoic acid (the ligand required for RXR heterodimerization with PPARs) or certain PPAR cofactors. Treatment with ROS increased PPAR γ -stimulated PPARE activity in olive flounder

(*Paralichthys olivaceus*) HINAE embryonic cells (Cho et al., 2009), supporting a conservation of PPAR γ ligand binding and transcriptional activities in fishes. Although activation of fish PPAR β and PPAR γ with known ligands in fish is generally inconsistent, all three types of sea bream and plaice PPARs form heterodimers with mouse RXR β and bind to several mammalian PPREs (ACO, CYP4A6z, GSTA1.1, 1.2) (Leaver et al., 2005). It is possible that PPAR β and PPAR γ are functionally conserved in fishes, but that their ligands have not yet been identified.

The physiological roles of PPARs in fish are not well-studied, but several studies provide evidence for a similar function of PPARs in fatty acid metabolism in mammals and fish. Rainbow trout injected with ciprofibrate for three weeks had increased peroxisomal acyl-CoA activity, catalase, and hepatic peroxisomal density (Yang et al., 1990). Fatty acyl-CoA oxidase activity was increased in rainbow trout hepatocytes treated with CLOa or ciprofibrate, but not GEM (Donohue et al., 1993), in rainbow trout receiving intraperitoneal injections of GEM (Scarano et al., 1994), in Atlantic salmon hepatocytes treated with CLOa and BEZ (Ruyter et al., 1997), and in fathead minnow (*Pimephales promelas*) exposed to CLOa (Weston et al., 2009). Japanese medaka (*Oryzias latipes*) exposed to GEM in water increased levels of the hepatic peroxisomal bifunctional enzyme (Scarano et al., 1994). Induction of microsomal lauric acid hydroxylase by peroxisome proliferators, a response well-characterized in rodents, was also reported in bluegill (*Lepomis macrochirus*) liver and in the kidneys of channel catfish (*Ictalurus punctatus*) (Haasch et al., 1998) and sea bass (*Dicentrarchus labrax*) (Sabourault et al., 1999). Fenofibrate-fed rainbow

trout had reduced tissue EPA and DHA and evidence of induction of peroxisomal activities (Du et al., 2004). Indirect evidence for the functional roles of PPARs in fish comes from studies in which dietary lipid composition affected PPAR transcript abundance and the metabolism of fatty acids. Diets high in n-3 highly unsaturated fatty acids resulted in increases in transcript abundance of acyl-CoA oxidase and PPAR β , as well as several markers of oxidative stress in liver of Atlantic salmon (Kjær et al., 2008). High dietary conjugated linoleic acid (CLA) was also found to decrease carcass lipid content, decrease liver PPAR β 2 expression, and increase hepatic beta-oxidation in Atlantic salmon (Leaver et al., 2006).

Other studies in fish have provided data that is not always consistent with PPAR function in mammals. In goldfish exposed to GEM in water, hepatic acyl-CoA oxidase activity, a gene regulated by PPAR α in mammals, was only slightly elevated after 14 days but was reduced after 28 days (Mimeault et al., 2006). These animals, however, did show increased activities of hepatic catalase and other antioxidant enzymes, suggesting that GEM exposure led to oxidative stress. Sea bass injected with clofibrate (CLO) over a two week period did not show induction of any of the mitochondrial, microsomal, or peroxisomal enzymes that are typically associated with peroxisome proliferation (Pretti et al., 1999). Mosquitofish (*Gambusia holbrooki*) treated with waterborne CLO and CLO α had decreased liver catalase activity following acute exposure (96 h), with no changes after chronic exposure (28 d) (Nunes et al., 2004).

Few studies have focused on the potential for environmental exposure to PPs

to affect the reproductive axis of fish. In goldfish, a significant bioconcentration (>100X) of the fibrate drug GEM was observed in the plasma of individuals exposed to environmental GEM concentrations (1.5 µg/L), and this was associated with depressed plasma T concentrations (Mimeault et al., 2005). These changes may have been related to changes in testicular steroidogenic acute regulatory (StAR) protein transcript levels, although this could not completely explain the changes in T (Mimeault et al., 2005). Fathead minnows exposed to high doses of perfluorooctanoic acid exhibited significantly decreased circulating steroids (testosterone [T] in males, T and estradiol [E2] in females), but only modest effects on reproductive performance as measured by time to first oviposition and egg production (Oakes et al., 2004). Clofibric acid treatment decreased sperm counts in fathead minnows and this was associated with a modest (non-significant) decline in plasma testosterone concentrations (Runnalls et al., 2007). Exposure to PPAR ligands may lead to indirect effects on gonadal function by affecting other sites within the hypothalamic-pituitary-gonadal (HPG) axis. Decreases in transcripts for secretogranin-II and GH were observed in pituitary glands of goldfish exposed to GEM in water (Trudeau et al., 2005). Further study is required as these studies suggest that environmental exposure to PPs could lead to decreased reproductive success in fish.

1.6 Reproductive axis and steroidogenesis

Vertebrate reproductive development and function is regulated by the HPG

axis. The HPG axis is highly conserved across vertebrates and is relatively well studied in the fishes. The synthesis and secretion of gonadotropic (luteinizing hormone [LH]; follicle stimulating hormone [FSH]) and somatotropic (growth hormone [GH]) hormones from the pituitary is regulated by both hypothalamic and peripheral factors (Trudeau, 1997; Holloway and Leatherland, 1998). These pituitary hormones are necessary for gonadal development, growth, maturation, and steroid hormone synthesis. Gonadal steroid hormones are important for gonadal function, the development of secondary sexual characteristics, reproductive and parental behaviours, and feedback regulation of pituitary hormone synthesis (Borg, 1994; Nagahama et al., 1994; Trudeau, 1997; Habibi and Huggard, 1998; Holloway and Leatherland, 1998; Amer et al., 2001).

In vertebrates, steroidogenesis occurs in several tissues including gonad, adrenals (or interrenals in fish), placenta and brain. The products of gonadal steroidogenesis are the sex steroids; primarily androgens in males and estrogens in females. Testicular steroidogenesis occurs in specialized cells called Leydig cells and this process requires cholesterol as a precursor molecule (Figure 1.2). Cholesterol for steroid synthesis can be derived from dietary intake or by de novo synthesis at sites outside or within the gonad. The first step in the steroidogenic pathway is the transfer of cholesterol to the inner mitochondrial membrane. This transfer is mediated by several proteins including the steroidogenic acute regulatory (StAR) protein and Translocator Protein (TSPO, or peripheral benzodiazepine receptor [PBR])(Papadopoulos et al., 1990; Clark et al., 1994). Once cholesterol is

transferred to the inner mitochondrial membrane, its side chain is removed by the cytochrome P450 side chain cleavage enzyme (P450scc), yielding pregnenolone. Subsequent steps in the steroidogenic pathway occur within the endoplasmic reticulum and involve hydroxylation and dehydrogenation by several P450 enzymes, resulting in the various androgenic and estrogenic sex steroids (Miller, 1988; Payne and Hales, 2004; Ghayee and Auchus, 2007).

The major stimulator of gonadal steroidogenesis in both sexes is LH (Figure 1.2). The LH receptor is a G-protein coupled receptor that, upon activation, increases cellular adenylyl cyclase activity and intracellular cAMP concentrations leading to activation of protein kinase A (PKA), which then phosphorylates several proteins, including StAR (Ascoli et al., 2002). Studies in fish have confirmed that the cAMP-dependent/PKA pathway is also the major regulator of testicular steroidogenesis in these vertebrates (Wade and Van Der Kraak, 1991). Phosphorylation of StAR activates this protein and initiates the transfer of cholesterol from the cytosol to the mitochondria (Arakane et al., 1997). The PKA phosphorylation cascade also results in the increased transcription of genes important for steroidogenesis including StAR and many of the steroidogenic enzymes. Several hormones may modulate this process, and these include prostaglandins, leukotrienes, steroids, GH, IGF and EGF (Huhtaniemi and Toppari, 1995; Hull and Harvey, 2000; Akingbemi, 2005; Stocco et al., 2005).

Other hormone signaling pathways are important in modulating this PKA-stimulated steroidogenic response. These include protein kinase C (PKC) and

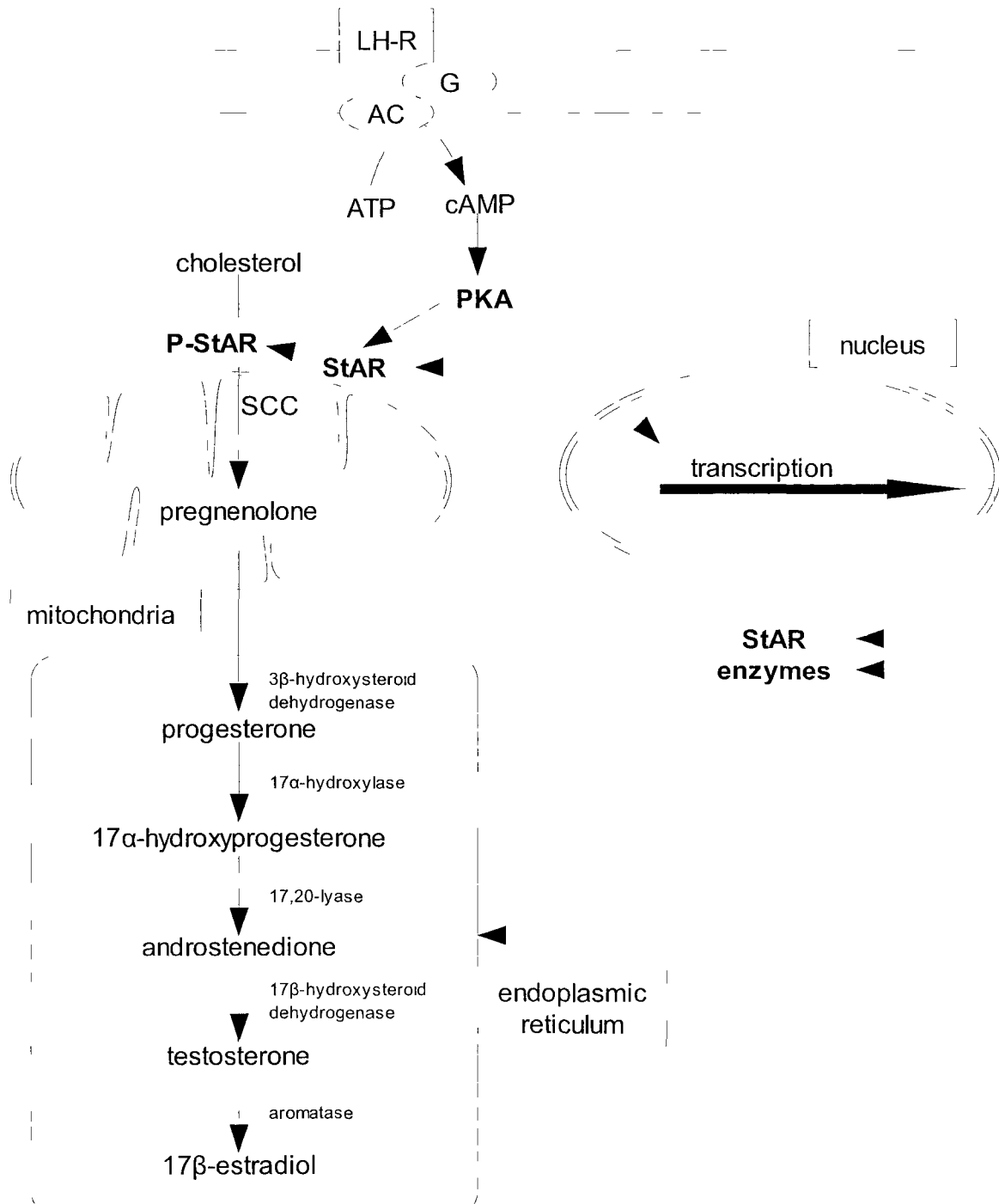


Figure 1.2. Testicular hormone-stimulated steroidogenesis. Luteinizing hormone receptor (LH-R) is a G-protein (G) coupled receptor stimulating adenylyl cyclase (AC) leading to increased intracellular cAMP concentrations and protein kinase A (PKA) activation. cAMP and PKA stimulate acute and chronic control of steroidogenesis (SCC - side chain cleavage, StAR - steroidogenic acute regulatory protein).

extracellular signal-regulated kinase 1/2 (ERK1/2), a mitogen-activated protein kinase (MAPK). Stimulation of PKC in MA-10 Leydig cells increases StAR protein transcript abundance, slightly increases steroid production, but does not increase StAR phosphorylation (Manna et al., 2007). Similarly, in goldfish, PKC activation and intracellular calcium levels increase basal testosterone production (Wade and Van Der Kraak, 1991). The PKC stimulation of StAR protein phosphorylation enhances steroid production in MA-10 Leydig cells in the presence of cAMP stimulation (Dyson et al., 2008; Manna et al., 2007). In contrast, gonadotropin-stimulated testosterone production is inhibited by PKC activation, but forskolin (an adenylate cyclase activator)-stimulated testosterone synthesis is not inhibited in goldfish testis fragments and ovarian follicles suggesting that PKC has effects at a step prior to the activation of adenylate cyclase in goldfish (Wade and Van Der Kraak, 1991; Van Der Kraak, 1992). In MA-10 Leydig cells, the PKA and PKC pathways are capable of phosphorylating ERK1/2 but with different consequences on the steroidogenic pathway. The inhibition of ERK decreases PKC-stimulated steroidogenesis but increases cAMP-stimulated increases in total StAR protein abundance and phosphorylation (Manna et al., 2007). The efficiency of the multiple signalling pathways regulating steroidogenesis also depends upon the distribution of important kinases and transport proteins within the Leydig cell. A coordinated localization of the proteins involved at the mitochondria, including PKA, StAR, ERK1/2, and TSPO, is necessary for the assembly of the cholesterol transport complex and phosphorylation of StAR to activate the acute steroidogenic response (Dyson et al.,

2008; Poderoso et al., 2008; Poderoso et al., 2009). This process also involves accessory proteins that recruit or tether these components at the mitochondria (Dyson et al., 2008). The role of MAPK/ ERK1/2 in fish testicular steroidogenesis has yet to be investigated. However, ERK1/2 is necessary for the cAMP/PKA stimulation of steroidogenesis in fish ovarian tissues (Méndez et al., 2005; Benninghoff and Thomas, 2006; Paul et al., 2010).

1.7 PPARs in the pituitary and gonads

PPAR α , β and γ expression is reported in various cell types within the mammalian testis and pituitary gland (Hase et al., 2002; Heaney et al., 2003; Corton and Lapinskas, 2005). Transcripts for all three PPARs are also detected in the gonads and pituitary gland of the goldfish (Mimeault et al., 2006; C. Cameron, unpublished results). Some functions for PPARs within these tissues are known in mammals.

Much of the research on PPARs in the pituitary is focused on the prospect of using PPAR γ ligands for the treatment of pituitary adenomas. Generally, PPAR γ activators induce cell cycle arrest and apoptosis in human and mouse pituitary tumor cells (corticotrophs, somatolactotrophs, gonadotrophs) and suppress hormone secretion from these tumors (Heaney, 2003; Heaney et al., 2003; Bogazzi et al., 2004). However, only limited success has been reported in clinical trials suggesting that thiazolidinedione (TZD, a specific mammalian PPAR γ agonist) treatment may only be effective in reducing serum cortisol in some Cushing's disease patients with

elevated circulating concentrations of pituitary adrenocorticotrophic hormone (ACTH) (Ambrosi et al., 2004; Suri and Weiss, 2005; Pecori Giraldi et al., 2006). Additionally, the suppressive effect of TZDs on pituitary tumors appears to be PPAR γ -independent (Emery et al., 2006). The only study to investigate the role of PPAR α in the pituitary reported an activation of the prolactin gene by the PPAR α agonist WY-14,643, but this response was not dependent upon DNA binding by PPAR α but on a protein-protein interaction between PPAR α and GHF-1 (Tolón et al., 1998). As much of the research in the pituitary has been performed with tumorigenic tissues, and these responses may not involve the receptor itself, there is no clear understanding of the role of PPARs in the normal pituitary.

There is evidence that PPARs are involved in the regulation of ovarian and testicular steroid hormone synthesis. This evidence is based upon treatments with TZDs, phthalate ester plasticizers, perfluorinated compounds, and fibrates. TZDs have been implicated in changes in intracellular cholesterol dynamics in Leydig cells (Freeman and Romero, 2003), increases in progesterone production (Froment et al., 2003) and reduced aromatase activity and transcript abundance in granulosa cells (Mu et al., 2001; Yanase et al., 2001), increased progesterone accumulation (Schoppee et al., 2002) and decreased androgen production in thecal cells (Schoppee et al., 2002; Veldhuis et al., 2002). In addition, the activities of enzymes responsible for the conversion of pregnenolone to androgens (3 β -HSD II and P450c17) are inhibited by troglitazone in yeast expressing these enzymes (Arlt et al., 2001). Impaired steroidogenesis has also been observed with phthalates. Rat

granulosa cells treated with di(2-ethylhexyl)phthalate (DEHP) decreased aromatase mRNA levels and depressed rates of estradiol secretion compared with controls, and evidence supported these effects occurred through activation of PPAR α and PPAR γ (Lovekamp-Swan et al., 2003). Female rats exposed to DEHP *in vivo* demonstrate decreased serum estradiol concentrations and display prolonged estrous cycles and lack of ovulation (Lovekamp-Swan and Davis, 2003). A wide variety of effects of phthalates on testicular function and steroid metabolism are reported, for some of which there is evidence of PPAR involvement (Corton and Lapinskas, 2005). Treatment of MA-10 mouse Leydig tumor cells with PPAR α agonists or mono(2-ethylhexyl) phthalate (MEHP) prevented cholesterol transport into mitochondria after hCG (human chorionic gonadotropin, a LH receptor agonist) stimulation, reduced levels of PBR expression (mRNA and protein) and inhibited progesterone synthesis (Gazouli et al., 2002). The same treatments *in vivo* resulted in reduced PBR mRNA in Leydig cells and depressed circulating testosterone levels (Gazouli et al., 2002). Male rat fetuses exposed to diisobutyl phthalate (DiBP) had decreased testicular total StAR protein and steroidogenic enzymes (P450_{scc}, CYP17), as well as decreased anogenital distance and testicular testosterone concentrations (Boberg et al., 2008). Perfluorinated compounds are also PPAR ligands, and perfluorodecanoic acid inhibits hCG-stimulated steroidogenesis as well as ligand-binding capacity, protein and mRNA levels of the mitochondrial cholesterol transport protein PBR in MA-10 Leydig cells (Boujrad et al., 2000). Fibrates also interfere with steroidogenesis. Treatment of rat Leydig cells *in vitro* with various fibrates, including

CLOa, ciprofibrate, GEM, and Wy-14,643, impaired hCG-stimulated testosterone secretion (Liu et al., 1996). Dietary Wy-14,643 increased serum estradiol concentrations and the incidence of Leydig cell tumors in male rats (Biegel et al., 2001). It is clear that many diverse PPAR ligands interfere with gonadal steroidogenesis. The variety of different mechanisms to explain these effects may be explained by differences in tissue or species sensitivity, or by a combination of effects of PPAR-dependent and PPAR-independent mechanisms.

1.8 Summary and hypotheses

Peroxisome proliferators are present in the environment at detectable levels and many of these PPs are capable of modulating the HPG axis in mammals. Studies by Mimeault et al. (2005) and Trudeau et al. (2005) showed that treatment of goldfish with GEM resulted in bioconcentration of GEM in the plasma, decreased circulating testosterone concentrations, and altered pituitary transcript levels for GH and Sg-II in exposed fish. These findings suggested that environmental PPs may impact the reproductive axis of wild fish species. A study of the mechanisms of this effect of GEM and fibrates in general on the goldfish is required. Despite a lack of information on the fish PPAR system and its involvement in reproduction, there is evidence to support these possibilities from studies in other vertebrates. Decreased plasma testosterone concentrations may be explained by effects of GEM at multiple sites within the HPG axis of the male goldfish, including the pituitary gland and testis. Each of these tissues will be studied *in vitro* to determine any direct effects of

GEM on endocrine function. This research will be guided by the following hypotheses:

Hypothesis 1: Fibrates affect the goldfish pituitary by decreasing the secretion of hormones important for the stimulation of testicular steroidogenesis, leading to a decrease in sex steroid synthesis and secretion, and a fall in circulating concentrations of testosterone.

Prediction 1: Fibrate treatment of dispersed pituitary cells will suppress either basal or stimulated LH or GH secretion from goldfish pituitary cells *in vitro*.

Hypothesis 2: Fibrates directly impair the steroidogenic pathway in the goldfish testis leading to decreases in sex steroid synthesis and secretion, and circulating concentrations of testosterone.

Prediction 2: Testosterone secretion from goldfish testis fragments cultured *in vitro* will be significantly impaired by fibrate treatment. This effect on steroidogenesis may include changes in basal rates of hormone secretion, but will be most evident during hormone-stimulated steroidogenesis (i.e.: hCG treatment).

Chapter 2: Effect of PPAR agonists on hormone secretion from goldfish (*Carassius auratus*) dispersed pituitary cells and testis fragments

ABSTRACT

Decreased plasma testosterone concentrations in gemfibrozil-exposed male goldfish may be explained by effects at various sites within the HPG axis. This study investigated the effects of gemfibrozil (GEM) and other PPAR agonists on hormone secretion from goldfish dispersed pituitary cells and testis fragments. Pituitary cells were either pre-exposed to PPAR agonists (4-12 h) prior to stimulation with sGnRH or co-treated with PPAR agonists and sGnRH for varied time periods (2-18 h). Significant changes in hormone secretion from pituitary cells were observed, including decreased LH and GH responses to sGnRH in rosiglitazone (ROS)-treated cells, and an increased GH response to sGnRH in GEM-treated cells. These findings, however, were not consistently observed across all experiments. In goldfish testis fragments, significantly decreased testosterone secretion was observed with fibrate and hCG treatments over 18 h. This effect was not apparent at all times of the year and was not always repeatable. When goldfish testis fragments were pre-exposed overnight to fibrates, a decreased steroidogenic response to hCG at 6 h was observed. The changes in hormone secretion from pituitary cells in response to PPAR agonists could not explain the observed decrease in plasma testosterone concentration of GEM *in vivo*. The decreased testosterone secretion from goldfish testicular fragments exposed to GEM is consistent with the observed effects of GEM *in vivo* and warrants further investigation (see Chapters 3 and 4).

2.1 INTRODUCTION

The hypothalamic-pituitary-gonadal (HPG) axis is responsible for the endocrine control of reproduction. Environmental pollutants may disrupt the HPG axis in exposed organisms with consequences for normal reproductive development and function. Peroxisome proliferator-activated receptors (PPARs) are regulators of energy metabolism, however there is evidence of their involvement in reproductive function at different sites within the HPG axis. Reported effects include decreased pituitary hormone secretion with thiazolidinediones (TZDs) (Heaney, 2003; Heaney et al., 2003; Bogazzi et al., 2004; Ambrosi et al., 2004), and decreased testicular and Leydig cell steroidogenesis in the presence of TZDs (Freeman and Romero, 2003), fibrates (Liu et al., 1996; Biegel et al., 2001), phthalates (Gazouli et al., 2002; Corton and Lapinskas, 2005), and perfluorinated compounds (Boujrad et al., 2000). Male goldfish exposed to waterborne gemfibrozil (GEM; a fibrate pharmaceutical and PPAR α ligand) for 2 weeks had decreased circulating testosterone concentrations (Mimeault et al., 2005). This study was designed to characterize this *in vivo* response by investigating the effects of GEM on the various components of the goldfish HPG axis *in vitro*.

Primary cultures of goldfish pituitary cells are an effective tool for the study of the control of pituitary hormone secretion (Chang et al., 1990ab). The secretion of luteinizing hormone (LH), an anterior pituitary glycoprotein hormone important for gonadal function, is complex but is stimulated by gonadotropin-releasing hormone (GnRH; salmon GnRH and chicken GnRH-II in the goldfish) and inhibited by

dopamine (DA) (Trudeau, 1997; Yaron et al., 2003). The role of growth hormone (GH), another anterior pituitary hormone, includes the control of growth and reproduction. In the goldfish, the release of GH is under stimulatory control by growth hormone releasing hormone (GHRH), GnRH, DA, and is inhibited by somatostatin-14 and IGF-I (Chang et al., 1996; Canosa et al., 2007). Cultured goldfish testis fragments are used in the study of the regulation of steroidogenesis as well as the influence of toxicants on this process (Wade and Van Der Kraak, 1991; Evanson and Van der Kraak, 2001). The sex steroids, including testosterone (T), are synthesized in Leydig cells within the interstitial space in the testes. In fish, as in most vertebrates, T is important for the development of secondary sexual characteristics, behaviour, and spermatogenesis (Borg, 1994). The functions of PPARs in the fish pituitary and testes have not been studied although transcripts for all 3 PPAR isoforms are detected in these tissues in the goldfish (Mimeault et al., 2006; C. Cameron, unpublished results).

This study investigated the effects of PPAR ligands on the endocrine function of goldfish pituitary cells and testis fragments *in vitro*. This approach was chosen to assess any direct effects of GEM on either of these tissues that could explain the observed decline in plasma testosterone in goldfish exposed *in vivo*. Other PPAR ligands with demonstrated effects on these tissues in other species were also investigated. These ligands include other PPAR α (bezafibrate, WY-14,643) and PPAR γ (rosiglitazone) ligands.

2.2 MATERIALS AND METHODS

2.2.1 Fish

Common goldfish, *Carassius auratus*, were purchased from a tropical fish supplier (Aleong's International, Mississauga, ON). Fish were acclimated at $18 \pm 1^\circ\text{C}$ for at least 2 weeks in 70-L fiberglass tanks that received dechloraminated City of Ottawa municipal water. Fish were fed a commercial trout pellet (Martin Mills, Elmira, ON) once daily. All experiments were performed under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhered to guidelines established by the Canadian Council on Animal Care for the use of animals in research and teaching.

2.2.2 Chemicals and Reagents

Human chorionic gonadotropin (hCG), forskolin, bezafibrate (BEZ), gemfibrozil (GEM), WY-14,643, and testosterone were purchased from Sigma-Aldrich (St. Louis, MO). Rosiglitazone (ROS) was purchased from Cayman Chemical (Burlington, ON). Salmon gonadotropin-releasing hormone (sGnRH) was purchased from Bachem Americas (Torrence, CA). Frozen aliquots of hCG (in saline) and sGnRH (in 0.1 N acetic acid) were thawed and doses freshly prepared in culture medium. Forskolin, BEZ, GEM, WY-14,643, and ROS were first solubilized in dimethyl sulfoxide (DMSO). Testosterone was solubilized in 99% ethanol. Controls also received solvent, and maximum concentrations of DMSO and ethanol did not exceed 0.2% and 0.5%, respectively.

2.2.3 Pituitary cell dispersion, plating and static culture

Methods for pituitary cell dispersion and static culture were similar to those of Chang et al., 1990a. Fish were anesthetized in MS-222 (>125 mg/L) prior to decapitation and pituitary glands were removed and placed in ice-cold dispersion medium (M199 with Hanks' salts, 25 mM HEPES, 26.2 mM sodium bicarbonate, 0.3% BSA, 100 000 U/L penicillin, 100 mg/L streptomycin, pH 7.2). Pituitary glands were washed 3-times, diced into fragments (~1 mm³), and digested at room temperature with gentle shaking in dispersion media with trypsin (25000 U/mL, 40 min, with gentle shaking), trypsin inhibitor (25000 U/mL, 5 min), and DNase II (0.1 mg/10 mL, 5 min). The digested fragments were then washed sequentially with Ca²⁺-free medium (Ca²⁺-free HBSS, 25 mM HEPES, 26.2 mM sodium bicarbonate, 0.3% BSA, 100 000 U/L penicillin, 100 mg/L streptomycin, pH 7.2) containing 2, 1, and 0 mM EGTA. Fragments were mechanically dispersed by gentle suction and extrusion using a plastic transfer pipet and filtered nylon mesh (BD Falcon Cell Strainer, 40 µm). Cells were collected by centrifugation at 200 x g for 10 min at 4°C and resuspended in the Ca²⁺-free medium. Cell yield and viability were determined by counting in a hemocytometer in the presence of trypan blue; generally >90% of cells excluded trypan blue.

Cells were plated at a density of 2.5 x 10⁵ cells/well in 24-well tissue culture plates (Corning International) in 1 ml of plating medium (M199 with Earle's salts, 25 mM HEPES, 26.2 mM NaHCO₃, 100 000 U/L penicillin, 100 mg/L streptomycin, pH 7.2). After 1 h, horse serum was added to a final concentration of 1% and cells were

cultured overnight at 28°C under 5% CO₂ and saturated humidity. Prior to beginning experiments, cells were washed with test medium (M199 with Hanks' salts, 25 mM HEPES, 26.2 mM NaHCO₃, 0.1% BSA, 100 000 U/L penicillin, 100 mg/L streptomycin, pH 7.2) and allowed to rest for 1 h after medium change.

A range of designs was employed for the static pituitary cell culture experiments. In experiments where sGnRH was used to stimulate hormone secretion, PPAR agonists were applied either at the same time (co-exposure) or cells were pre-treated with PPAR agonists followed by stimulation with 100 nM sGnRH. This concentration of sGnRH was chosen as it results in maximal LH secretion over 2 h (Chang et al., 1990a).

At the end of each experiment, media was recovered from each well, frozen on dry ice and stored at -80°C prior to radioimmunoassay for LH and GH. Radioimmunoassays for GH were performed by Dr. J. Chang at the University of Alberta, and for LH were performed by Dr. J. Chang (University of Alberta) or Dr. E. Zhao (University of Ottawa).

2.2.4 Testis fragment culture

Testis fragments were incubated in modified Cortland's saline (0.12 mM NaCl, 5.1 mM KCl, 2.97 mM NaH₂PO₄, 1.56 mM CaCl₂, 0.98 mM MgCl₂, 0.93 mM MgSO₄, 0.1% glucose, 0.1% bovine serum albumin, 100 U/mL penicillin, 0.1 mg/mL streptomycin, pH 7.6) as previously described (Wade and Van Der Kraak, 1991). Goldfish were anesthetized and decapitated as above (2.2.3), and testes were removed and placed into modified Cortland's saline. Whole-body and testis weight

were recorded, and GSI ([gonad mass/body mass] x 100) was calculated. Testes were minced and washed with fresh Cortland's saline. Two fragments totaling 20-25 mg were placed into each well of a 24-well tissue culture plate (Corning) containing 1 ml of modified Cortland's saline. The fragments were washed again prior to treatment, and cultured at 18°C. Tissue was either incubated in the presence of PPAR agonists and hCG for 18 h (co-exposure) or treated for 18 h with PPAR agonists followed by a 6 h stimulation with hCG (pre-exposure). At the end of the experiment, the medium was centrifuged (4°C, 20000 x *g*, 3 min) and aliquots were stored at -80°C until analyzed for testosterone content by radioimmunoassay (McMaster et al., 1992).

2.2.5 Statistics

Data were analyzed by ANOVA (1-way, 2-way or randomized block where appropriate) with Holm-Sidak used for pair-wise comparisons. Differences were considered significant when $p < 0.05$. Where data did not meet assumptions of normality or equal variance, logarithmic transformations were used prior to statistical analysis. SigmaStat software (SPSS Inc.) was used for all statistical analyses.

2.3 RESULTS

2.3.1 Pituitary cell culture

Co-exposure with 100 nM sGnRH and 10 μ M GEM for 12 h significantly decreased basal LH secretion (Fig. 2.4B). Effects of GEM on basal LH secretion were not observed in other experiments (Figs 2.1, 2.2A, 2.4A, 2.6A, 2.6B). An

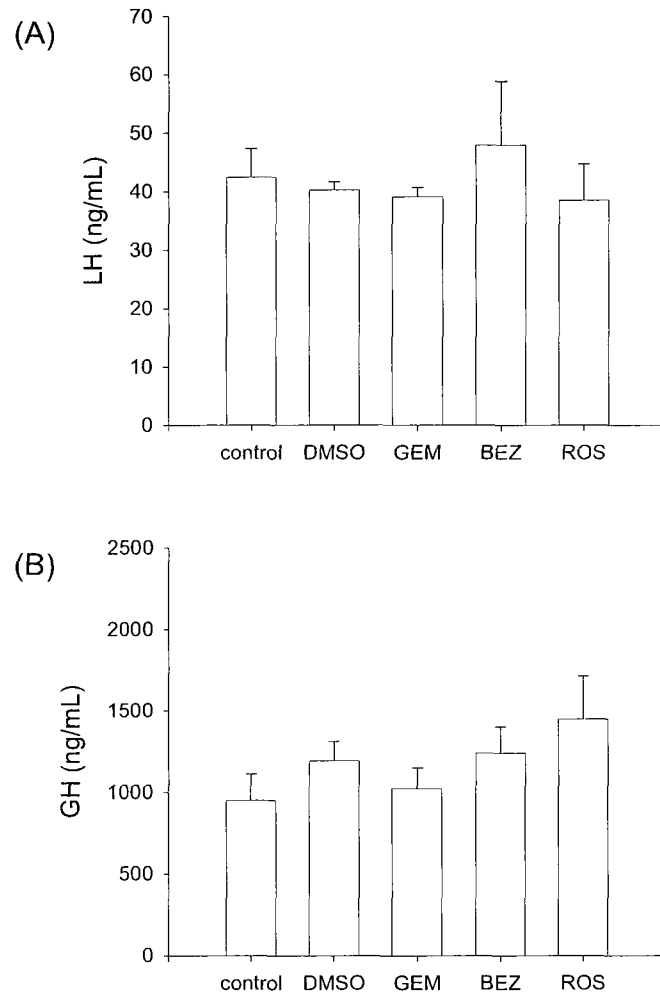


Figure 2.1. Basal (A) LH and (B) GH secretion from dispersed goldfish pituitary cells in September treated with PPAR agonists ([GEM] gemfibrozil, [BEZ] bezafibrate, [ROS] rosiglitazone) at 10 μ M for 16 h. Dimethyl sulfoxide (DMSO) used as vehicle for PPAR agonists (DMSO = 0.1% DMSO, control = no vehicle, n = 3, mean \pm SEM).

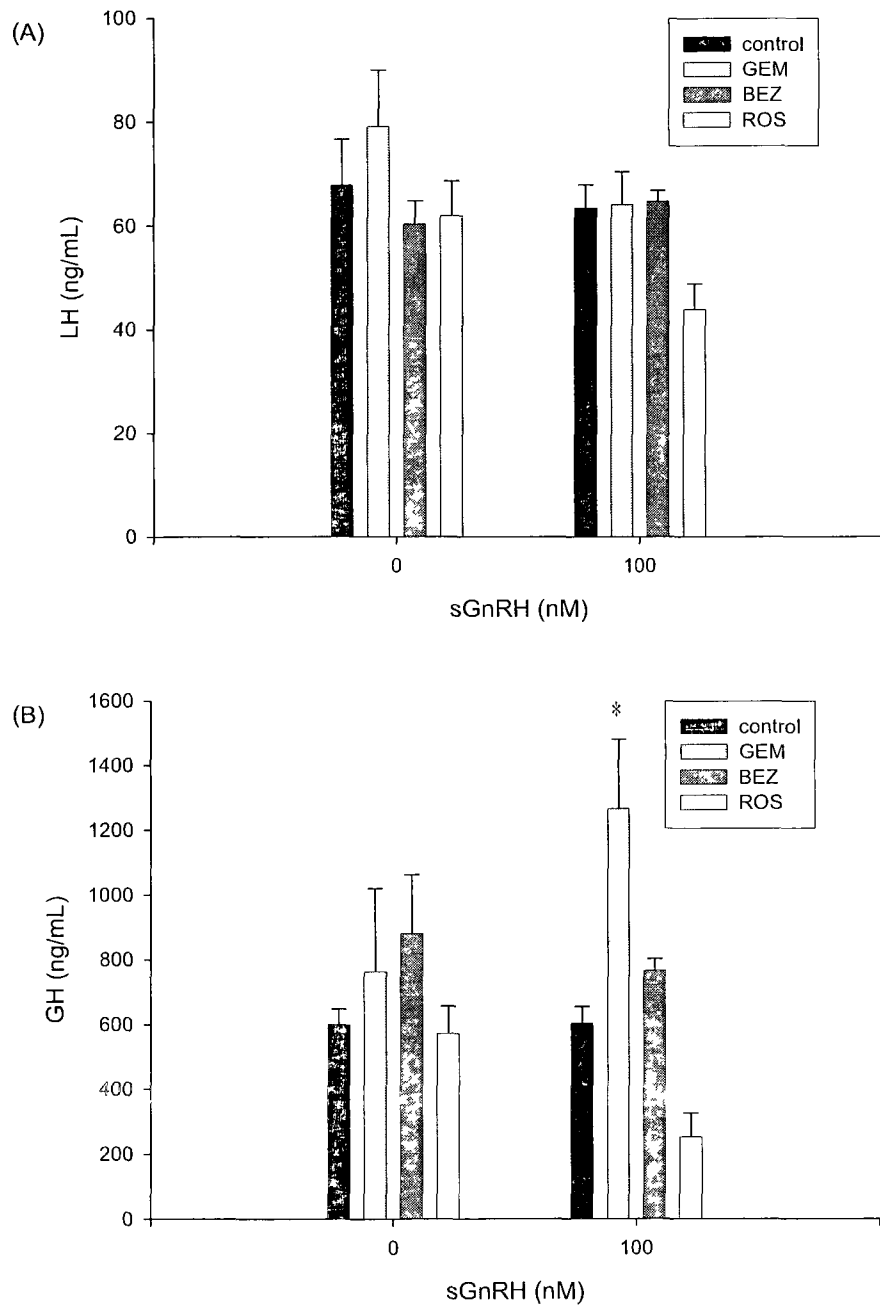


Figure 2.2. (A) LH and (B) GH secretion during 6 h co-exposure with sGnRH from dispersed goldfish pituitary cells in December previously treated with PPAR agonists at 10 μ M for 4.5 h ([GEM] gemfibrozil, [BEZ] bezafibrate, [ROS] rosiglitazone, $n = 3$, mean \pm SEM, 2-way ANOVA with GnRH [$p = 0.86$] and treatment [$p = 0.004$] as factors, * $p < 0.05$ versus control).

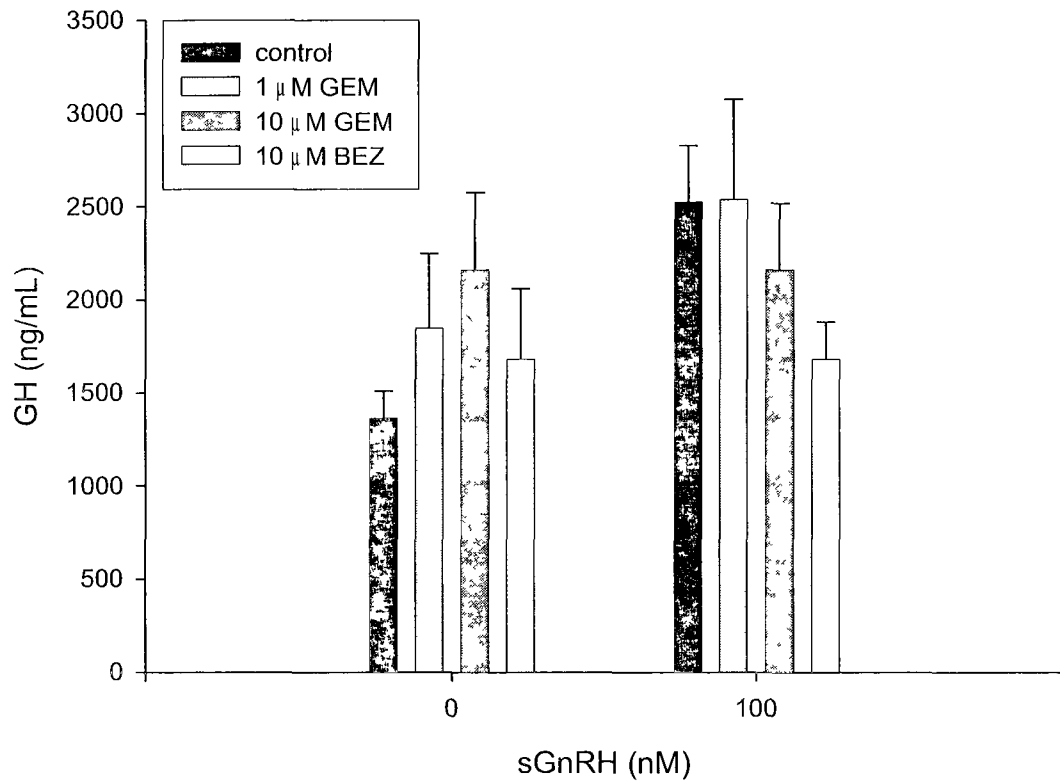


Figure 2.3. GH secretion from dispersed goldfish pituitary cells in March co exposed with PPAR agonists ([GEM] gemfibrozil, [BEZ] bezafibrate) and sGnRH for 12 h (n = 3, mean \pm SEM).

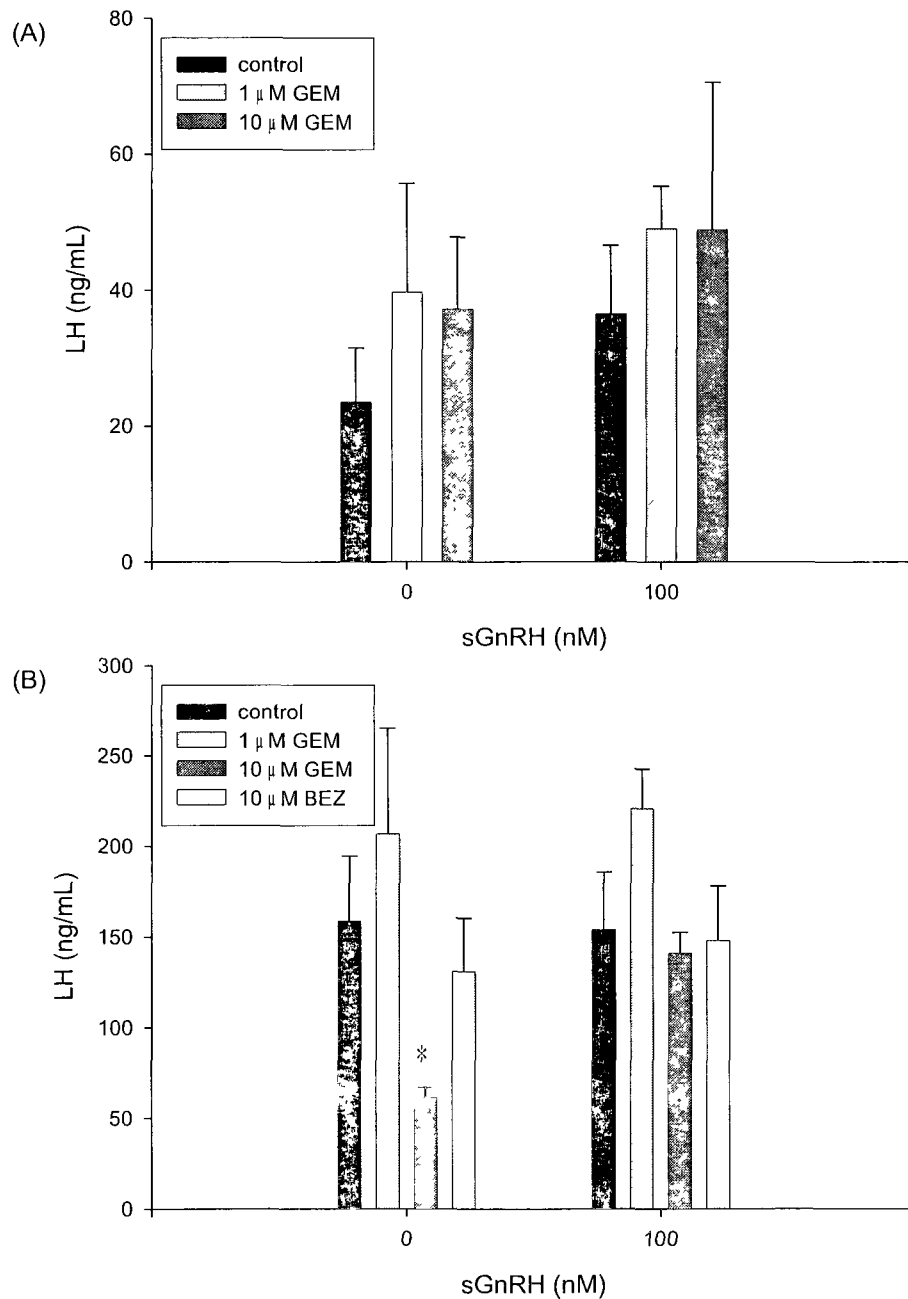


Figure 2.4. LH secretion from dispersed goldfish pituitary cells in March (A) treated 12 h with gemfibrozil (GEM) then treated with sGnRH for 3 h or (B) co-exposed to fibrates (GEM, [BEZ] bezafibrate) and sGnRH for 12 h (n = 3, mean ± SEM , p < 0.05 versus control).

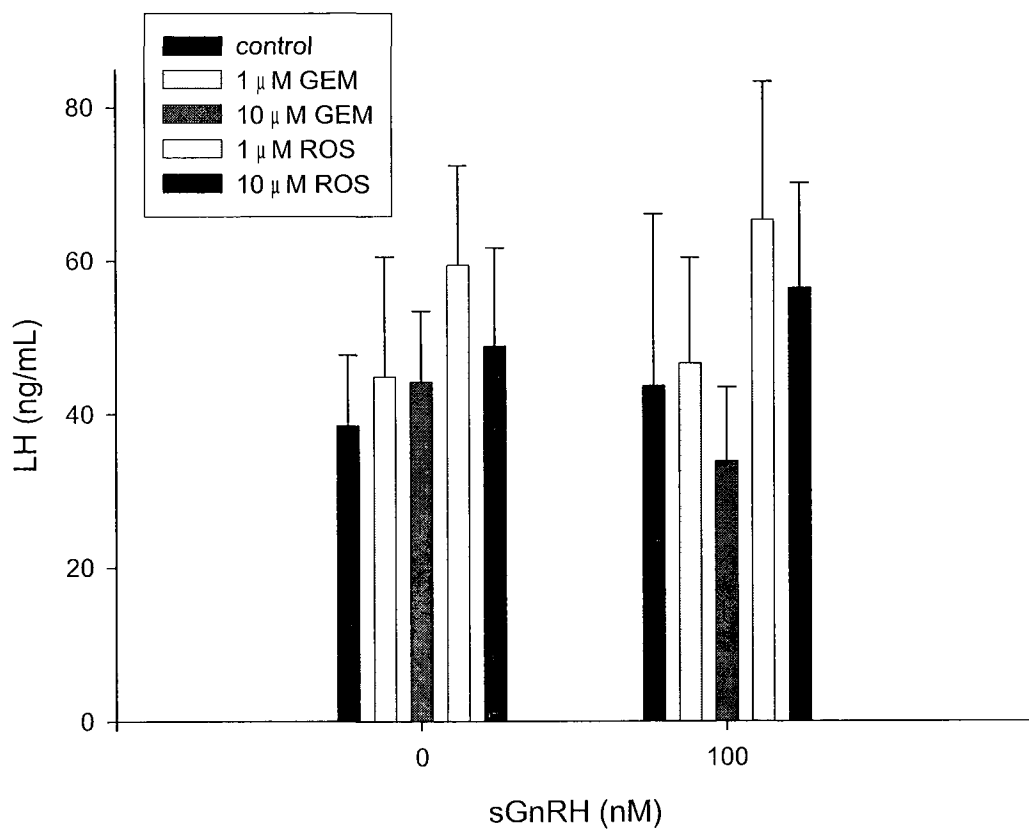


Figure 2.5. LH secretion from dispersed goldfish pituitary cells in December treated with PPAR agonists ([GEM] gemfibrozil, [ROS] rosiglitazone) 18 h then treated 2 h with sGnRH (n = 3, mean ± SEM).

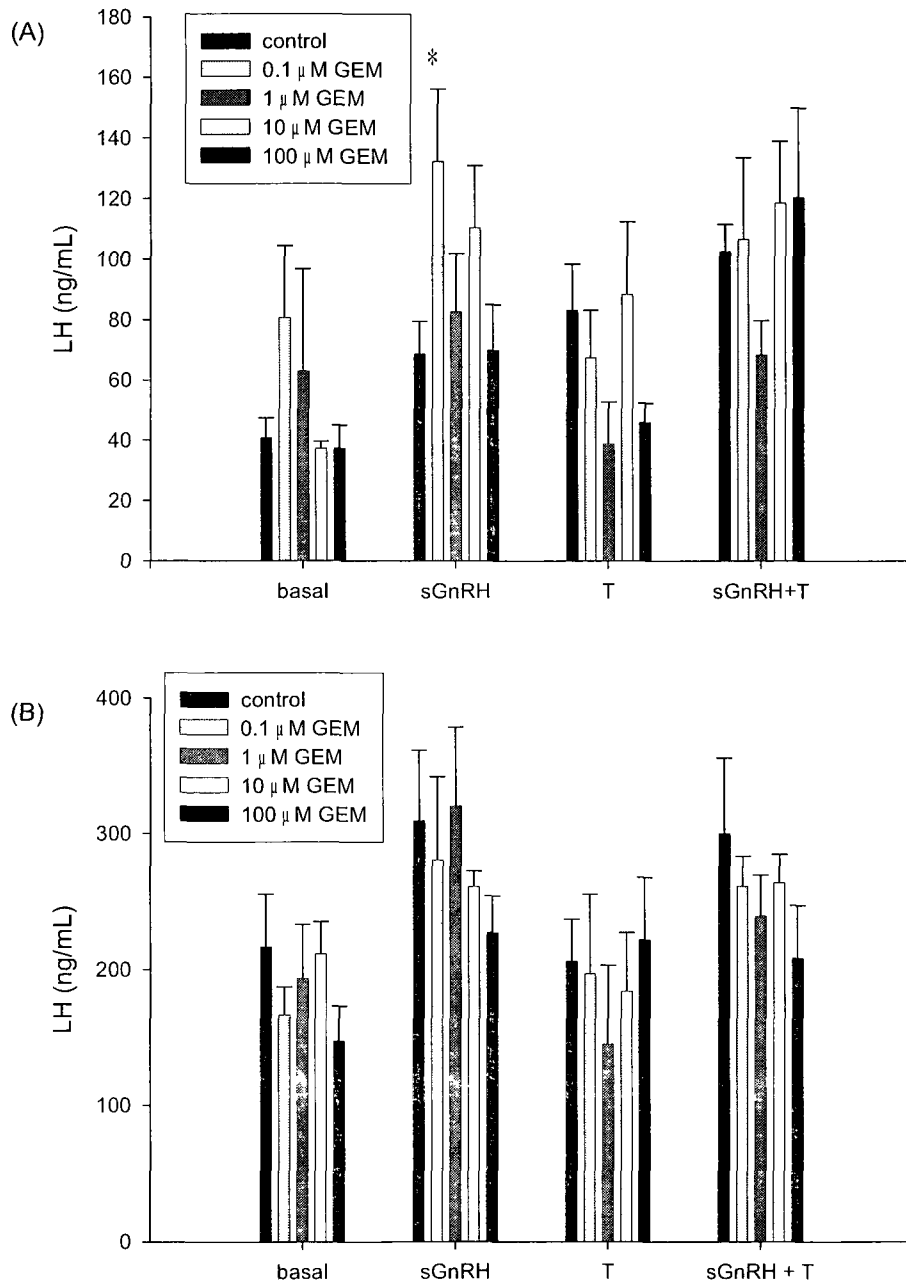


Figure 2.6. LH secretion from dispersed goldfish pituitary cells in February exposed to gemfibrozil (GEM) and testosterone (T; 1 nM) for (A) 18 h followed by 4 h treatment with 100 nM sGnRH and (B) 18 h with 100 nM sGnRH (n = 3, mean \pm SEM, * p < 0.05 versus control).

increase in sGnRH-stimulated LH output was observed with 0.1 μM GEM treatment but this effect was not observed at higher doses (Fig. 2.6A), or in other experiments (Figs 2.2, 2.4, 2.5, 2.6B). Further experiments involving agonist pre-treatment followed by sGnRH stimulation failed to show any significant effect on LH secretion (Figs 2.4A, 2.5). The inclusion of testosterone (1 nM) in culture media did not significantly affect LH secretion either alone or in combination with sGnRH and GEM (Figs 2.6A, 2.6B).

GH secretion from non-stimulated goldfish pituitary cells over 16 h was not affected by GEM, BEZ or ROS at concentrations of 10 μM (Fig. 2.1). When cells were pre-treated with PPAR agonists for 4.5 h followed by a 6 h sGnRH stimulation, inhibition of GH was observed with 10 μM ROS, and GH secretion increased with GEM (10 μM) treatment (Fig. 2.2B). No changes in GH secretion were observed when cells were co-exposed for 12 h with agonists (GEM or BEZ) and 100 nM sGnRH (Fig. 2.3).

2.3.2 Testis fragment culture

Both basal and hCG-stimulated testosterone secretion from testis fragments were not affected by GEM after 18 h in culture during the months of June (Fig. 2.7; basal not detectable, not shown), November (Fig. 2.8), or February (Fig. 2.9). Forskolin-stimulated testosterone production was also not affected by GEM treatment in February (Fig. 2.9). In March, an 18 h treatment with 100 μM GEM or WY-14,643 increased basal testosterone output and suppressed hCG-stimulated secretion (Fig. 2.10). Similar experiments (18 h co-exposure with hCG) at this time

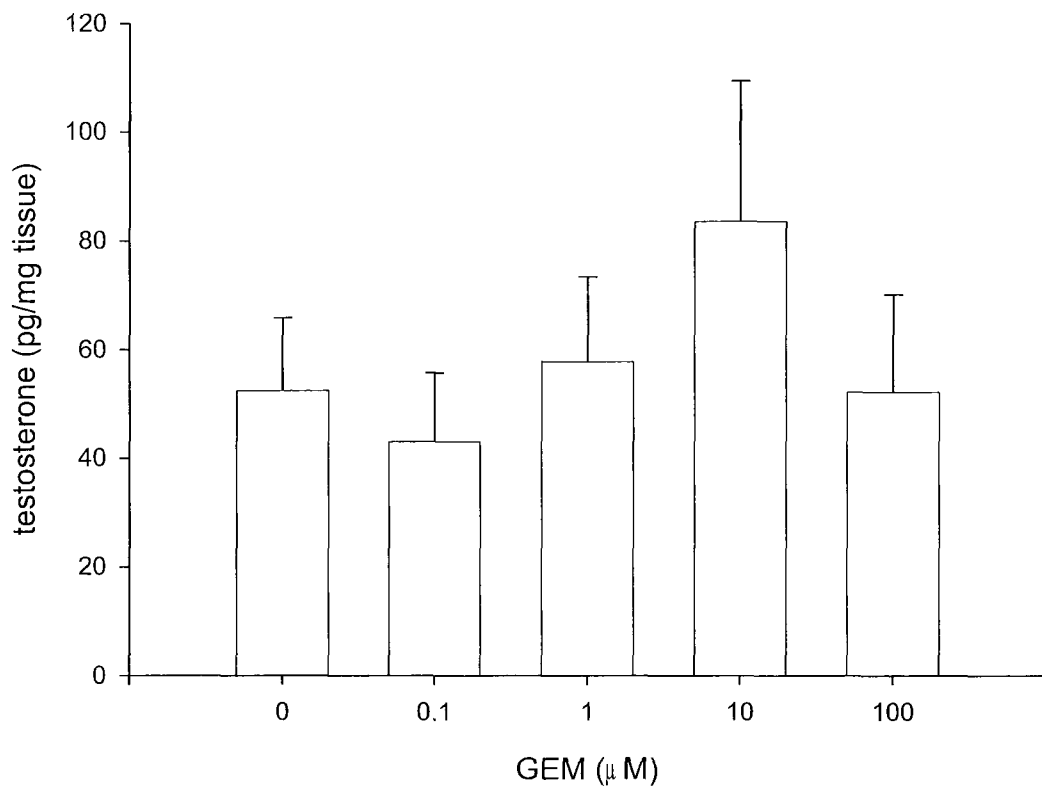


Figure 2.7. 18 h hCG-stimulated (1 IU/mL) testosterone secretion from goldfish testis fragments in June treated with gemfibrozil (GEM) (n = 5, mean ± SEM).

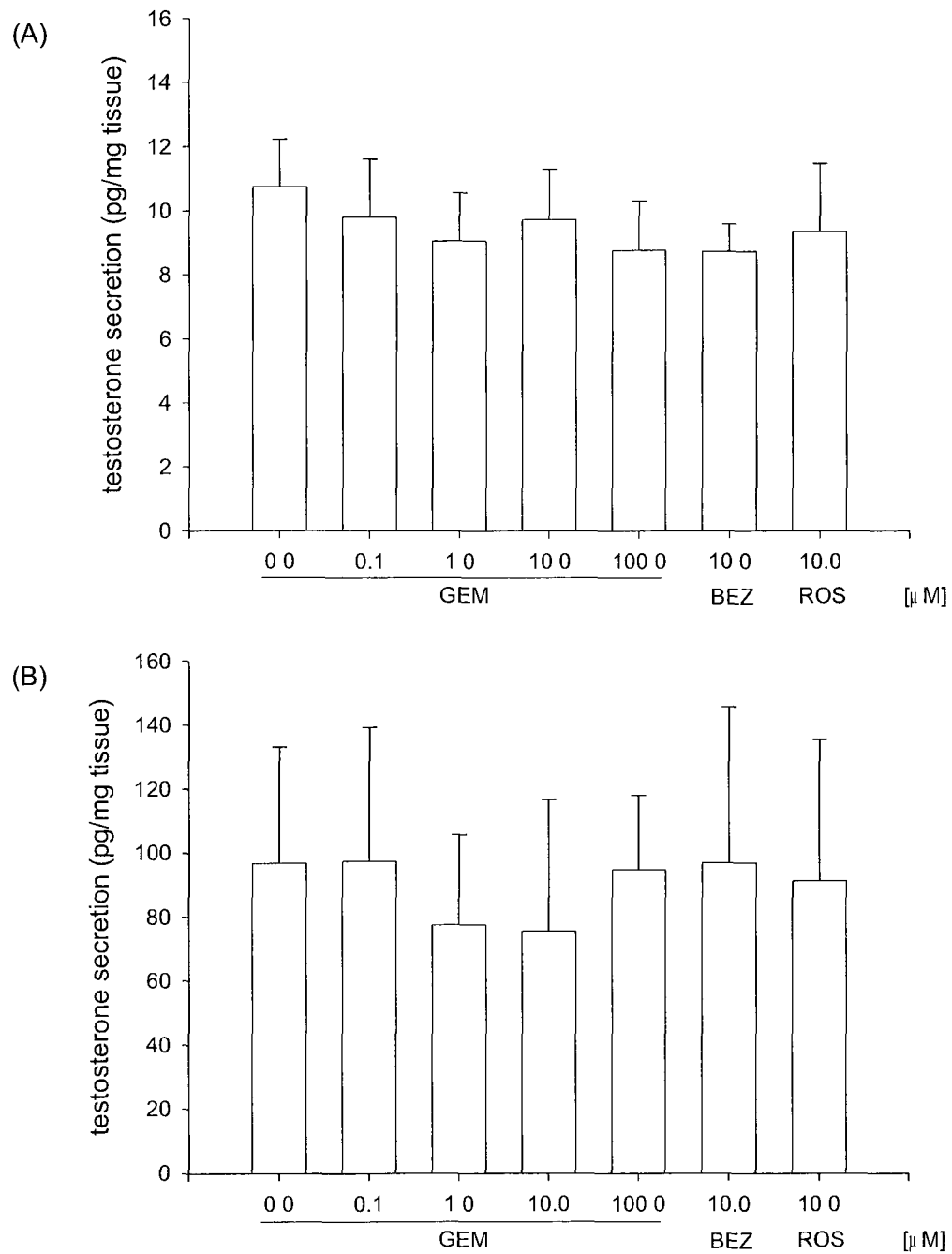


Figure 2.8. 18 h (A) basal and (B) hCG-stimulated (1 IU/mL) testosterone secretion from goldfish testis fragments in November treated with PPAR agonists (gemfibrozil [GEM], bezafibrate [BEZ], or rosiglitazone [ROS]) (n = 4, mean \pm SEM).

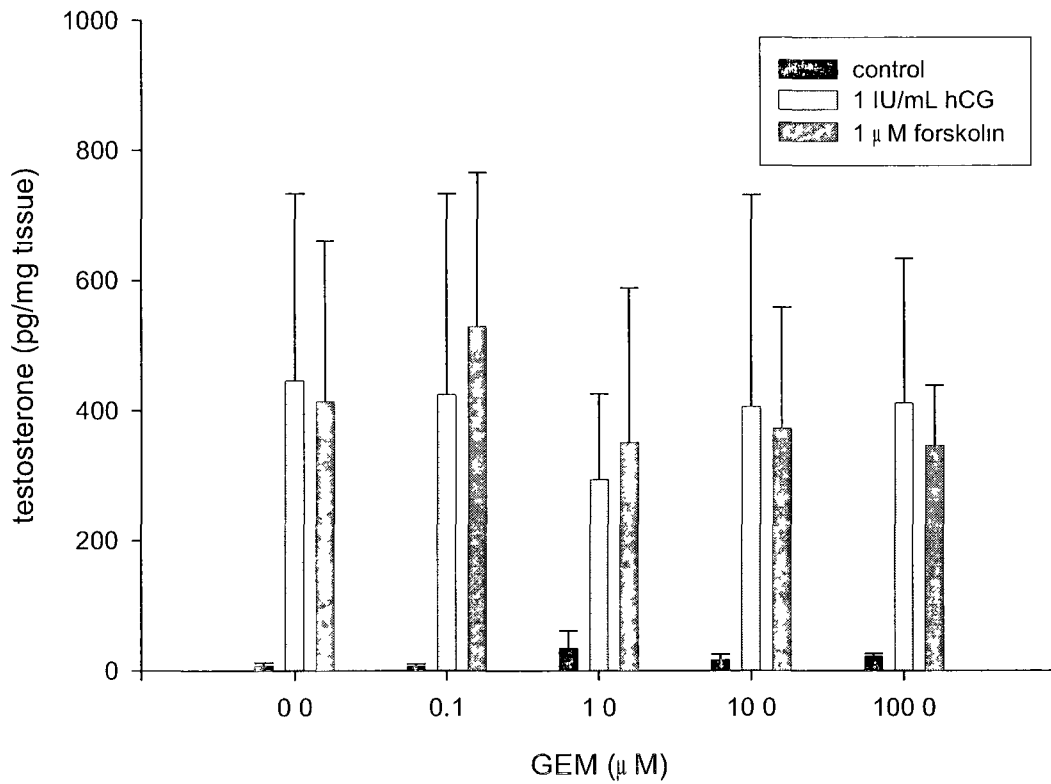


Figure 2.9. Testosterone secretion from goldfish testis fragments in February treated with gemfibrozil (GEM) and hCG or forskolin for 18 h ($n = 3$, mean \pm SEM).

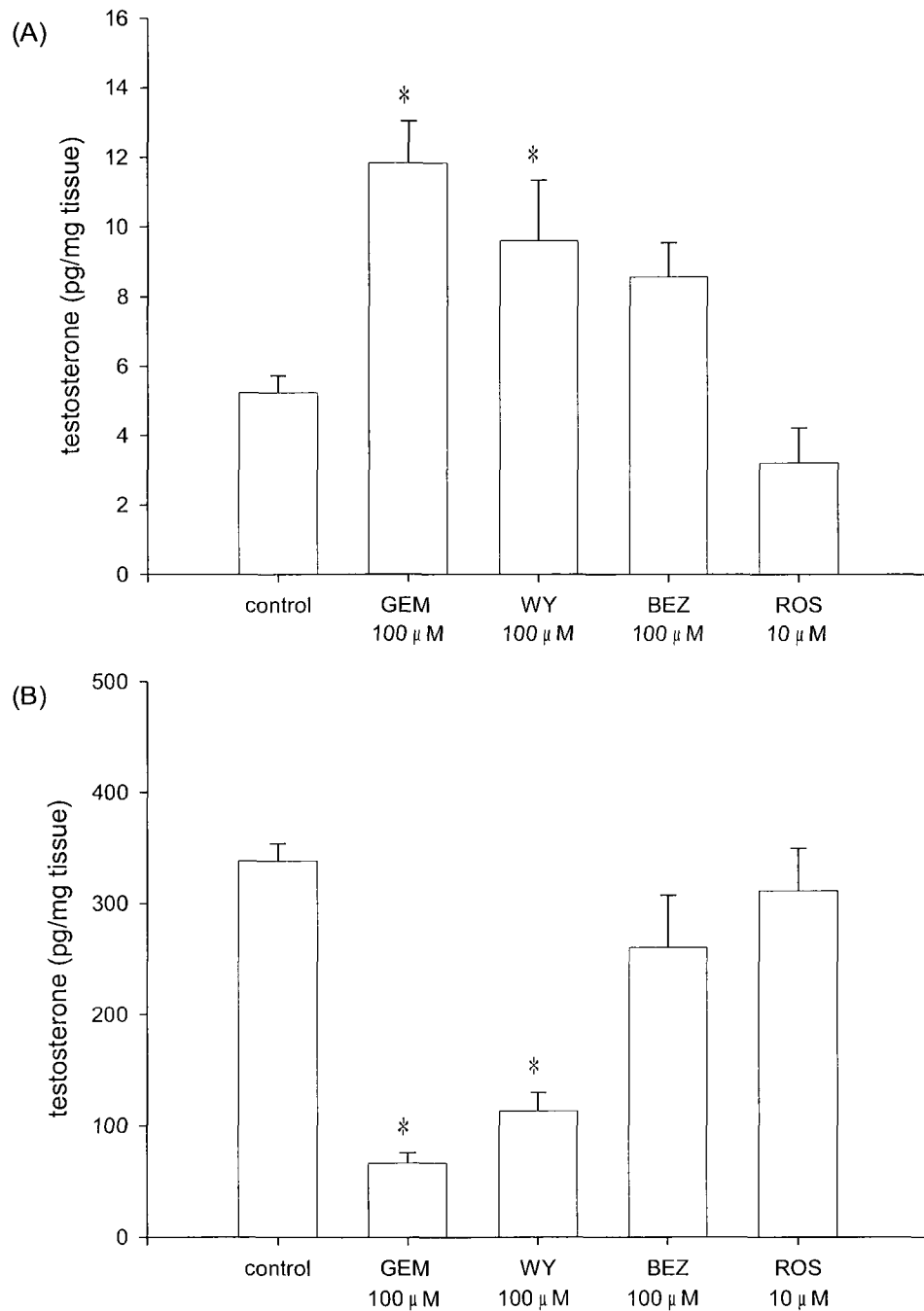


Figure 2.10. 18 h (A) basal and (B) hCG-stimulated (1 IU/mL) testosterone secretion from goldfish testis fragments in March treated with PPAR agonists (gemfibrozil [GEM], WY-14,643 [WY], bezafibrate [BEZ], rosiglitazone [ROS]) (n = 4, mean ± SEM, * p < 0.05 versus control).

of year (March – May), failed to produce similar results. In June, overnight pre-exposure to 100 μ M GEM did not affect 6 h basal testosterone secretion, however the 6 h response to 1 IU/mL hCG was significantly reduced (Fig. 2.11).

2.4 DISCUSSION

The objective of these *in vitro* studies was to investigate the effects of PPAR agonists on the pituitary and testes in isolation for evidence of direct effects on these tissues. In particular, evidence was sought that would explain the decreases in plasma testosterone concentrations in goldfish treated with GEM. The few observed effects of PPAR ligands on hormone secretion from goldfish pituitary cells were either not repeatable or were generally not consistent with the hypothesis of the pituitary-mediated decreases in circulating testosterone. However, goldfish testis fragments pre-exposed to GEM released lower quantities of testosterone during a subsequent 6 h hCG treatment and this finding is consistent with the observed effects in male goldfish *in vivo* (Mimeault et al., 2005). A direct inhibitory effect of GEM on the steroidogenic pathway in the goldfish testis *in vivo* is a mechanism by which plasma testosterone concentrations could be decreased.

Common goldfish are seasonal spawners. At northern latitudes, goldfish spawn March through June, with gonadal regression occurring during summer and early fall, followed by gonadal recrudescence during the late fall and winter in the approach to the next spawning season. This seasonal cycle is regulated by water temperature and photoperiod. The approach to spawning is characterized by a

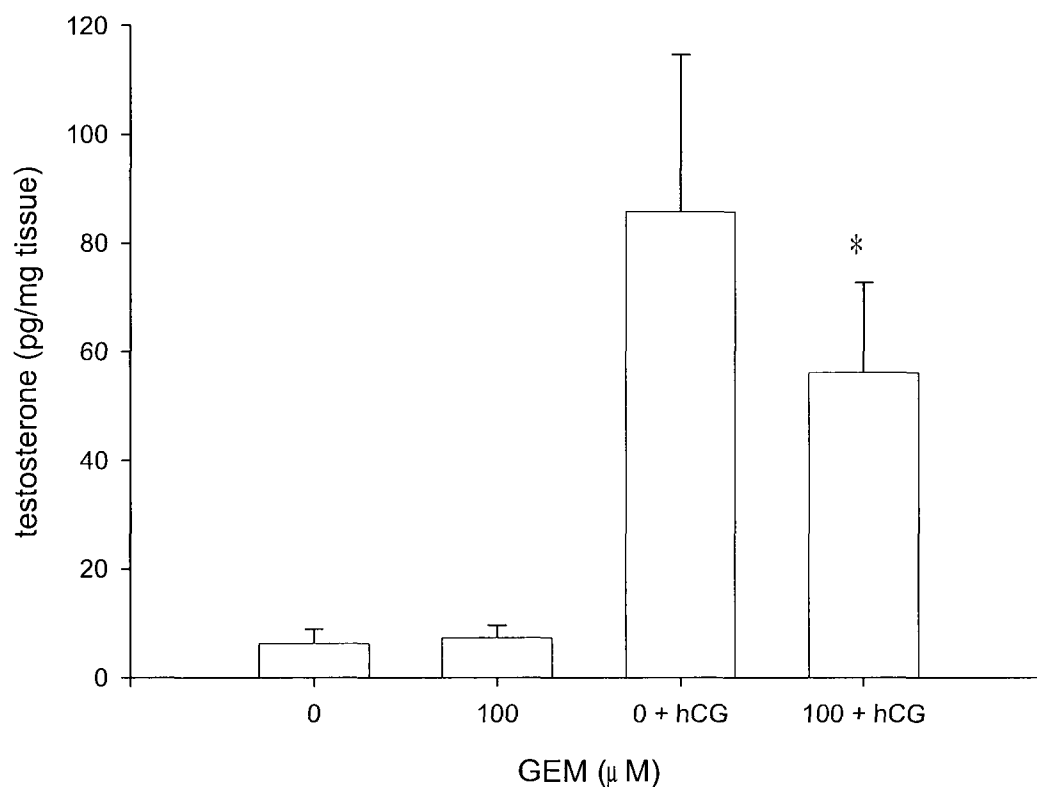


Figure 2.11. Basal and hCG-stimulated (1 IU/mL) 6 h testosterone secretion from goldfish testis fragments in June previously treated overnight with gemfibrozil (GEM) (n = 4, mean ± SEM, * p < 0.05 versus control).

significant up-regulation of the HPG axis resulting in gonadal growth and maturation. In general, this involves changes in the expression of hypothalamic factors (e.g.: GnRH, DA), changes in pituitary sensitivity to these hypothalamic factors, increases in pituitary gland hormone content and secretion (e.g.: LH, GH), enhanced gonadal sensitivity to gonadotropic hormones, increased gonadal steroidogenesis, and positive feedback control at the brain and pituitary by steroid hormones (Trudeau, 1997). The post-spawning period (summer to fall) is characterized by a down-regulation of the HPG axis and gonadal regression.

In several pituitary cell culture experiments sGnRH failed to stimulate hormone secretion above basal levels. This finding may be attributed to the fact that fish in several of the experiments were sexually regressed. The response to sGnRH is highly seasonal and is drastically reduced in sexually regressed goldfish (Lo and Chang, 1998; Johnson and Chang, 2002). The response to sGnRH is typically observed in short (2 h) static culture experiments and in perfusion studies (Chang et al., 1990ab). However, perfused goldfish pituitary cells are rapidly desensitized to sGnRH within minutes (Lo and Chang, 1998; Johnson et al., 2002; Klausen et al., 2008). While sGnRH-treated cells may secrete significantly higher quantities of hormone for a short period of time, the desensitization to sGnRH may return the rates of secretion in treated cells back to basal levels quickly. During the course of a long-term incubation, the quantity of hormone (e.g.: LH) secreted into medium is quite large, compared to the quantity secreted during the short period of time in which sGnRH-treated vs. non-treated differ, resulting in an apparent similarity in

rates of hormone secretion (JP Chang, personal communication).

GEM treatment of goldfish pituitary cells resulted in an increase in sGnRH-stimulated GH, as well as both an increase and decrease in sGnRH-stimulated LH production. The effects of this pharmaceutical were inconsistent and did not support the hypothesis that a decrease in gonadotropic hormone secretion would lead to a decrease in stimulation of gonadal steroidogenesis. Therefore, the alternative hypothesis of a direct effect of GEM on steroidogenesis in the testis of the male goldfish was tested.

In a previous study, male goldfish exposed to environmental concentrations of GEM (1.5 µg/L) in the water for 2 weeks in November had decreased plasma testosterone concentrations compared with the controls (Mimeault et al., 2005). This effect of 1.5 µg/L waterborne GEM on plasma testosterone concentrations in male goldfish was found to be dependent upon the time of the year when the fish were tested. Decreases in testosterone concentrations were not observed in similar experiments performed in March (increased testosterone) or June (no changes) (Woodhouse, 2005). At pharmacological concentrations (1500 µg/L) however, circulating testosterone concentrations were always decreased compared with the controls (Woodhouse, 2005). The initial design of *in vitro* experiments with goldfish testis fragments was an 18 h fibrate co-exposure with hCG-stimulation. Decreased hCG-stimulated testosterone secretion was observed with PPAR α agonists in March, but not at other times of the year, suggesting a seasonal component to this response. This seasonal influence on the *in vitro* response of goldfish testis

fragments to GEM effect did not necessarily coincide with the seasonal response observed *in vivo*. This discrepancy could be due to several factors including differences in exposure time and short-term hCG-stimulation (*in vitro*) versus plasma testosterone concentrations in the absence of external stimulation (*in vivo*). The *in vitro* response to hCG is seasonal and coincides with the seasonal reproductive cycle of the goldfish, with increases in hCG-stimulated (1 IU/mL) 18 h testosterone secretion from ~55 pg/mg in late June to 300+ pg/mg in the period of March through April. Seasonal changes in PPAR transcript abundance have been observed in some fish species (Batista-Pinto et al., 2009). In the goldfish, a trend for increased PPAR α transcript abundance was observed from November through March and June, however this relationship was not significant (Woodhouse, 2005).

Certain experiments during the period in which goldfish testis fragments are highly sensitive to hCG treatment (e.g.: February to May) failed to demonstrate any response of the steroidogenic pathway to PPAR agonists (data not shown). This lack of effect suggested that co-incubation of fragments with GEM and hCG could have masked the effects of GEM. The response to fibrates which involves ligand binding to PPAR α , PPAR-DNA binding, activation of transcription, and increased protein translation would be relatively slow, requiring several hours. At the same time, the rapid stimulatory effect of hCG on cAMP synthesis and steroid production in steroidogenic tissues may prevent the detection of any further changes occurring within the tissue. In experiments that instead involved an 18 h pre-exposure to PPAR ligands followed by 6 h hCG-stimulation, a more consistent and significant

inhibition of hCG-stimulated testosterone secretion from goldfish testis fragments was observed. This model may also better reflect the effect of such a toxicant *in vivo*, where animals experience a chronic exposure to fibrates in water, and then must respond to seasonal increases in LH. For these reasons, the experiments in the following chapters adopted this model involving a pre-exposure to PPAR ligand followed by stimulation with hCG or other compounds.

The inhibition of hCG-stimulated steroidogenesis in goldfish testis fragments is in agreement with other studies of the effects of fibrates on steroidogenesis. Several fibrates at similar concentrations used in this study, including GEM, inhibit hCG-stimulated testosterone secretion in pre-exposed Leydig cells isolated from mouse testis and MA-10 Leydig cells (Liu et al., 1996; Gazouli et al., 2002). A reduction in mitochondrial cholesterol transport as a result of decreased expression of peripheral-type benzodiazepine receptor (PBR) following WY-14,643 treatment was observed both *in vitro* in MA-10 Leydig cells and *in vivo* in male mice, but not in PPAR α -null mice (Gazouli et al., 2002). This suggests a down-regulation of PBR gene expression by PPAR α as a mechanism for disrupted steroidogenesis. A similar mechanism could explain the decreased testosterone synthesis observed in goldfish testis fragments treated with GEM.

The lack of evidence for an effect of GEM on goldfish pituitary cells and the inhibition of hCG-stimulated testosterone synthesis in testis fragments suggests a direct effect of this fibrate on testicular steroidogenesis in the goldfish. Pre-exposure of testis fragments is a better model for the study of this effect, rather than a co-

exposure with the fibrate and hCG. Further studies (see Chapters 3 and 4) will investigate the mechanism by which GEM interferes with the steroidogenic pathway in the goldfish testis.

Chapter 3: Effect of gemfibrozil on *in vitro* testicular steroidogenesis in the goldfish, *Carassius auratus*

ABSTRACT

Fibrate pharmaceuticals, including gemfibrozil (GEM), are detected in the aquatic environment but their potential for effects on aquatic organisms have yet to be fully characterised. This study investigated the effects of GEM on *in vitro* steroidogenesis using testes fragments prepared from the goldfish *Carassius auratus*. Eighteen hour pre-incubation of the fragments with GEM (100 μM) or fenofibrate (FEN; 100 μM) resulted in impaired hormone (human chorionic gonadotropin, hCG) and forskolin-stimulated testosterone secretion over 6 h. The formation of cAMP in the presence of hCG was not altered by GEM-treatment. Testosterone secretion from GEM- and FEN-treated testis fragments was restored in the presence of a membrane-permeable cholesterol analog (25-hydroxycholesterol, 50 μM) and pregnenolone (100 nM). The restoration of testosterone synthesis by precursors bypassing the P450 side chain cleavage enzyme suggested that the inhibition of steroidogenesis by GEM and FEN may result from a decrease in the availability of mitochondrial cholesterol for steroid synthesis. Real-time PCR analysis of transcripts for proteins involved in mitochondrial cholesterol transport (StAR and PBR) did not reveal differences between treatments. Gemfibrozil treatment did not affect total tissue StAR protein observed using western blots, suggesting that the activation or function of the StAR protein could be affected, or that GEM altered the abundance or function of another protein required for cholesterol transport.

3.1 INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors with roles in lipid and energy homeostasis, cell proliferation, apoptosis, and reproductive development and function (Lee et al., 2003; Desvergne and Wahli, 1999; Gazouli et al., 2002; Schoppee et al., 2002; Gonzalez et al., 1998; Heaney et al., 2003). Several synthetic PPAR ligands are detected in the aquatic environment. These include lipid-lowering fibrate pharmaceuticals, phthalate ester plasticizers, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, pesticides, and alkylphenols (Miao et al., 2002; Fromme et al., 2002; Cajaraville et al., 2003; Fent et al., 2006; Christen et al., 2010; Corcoran et al., 2010). Environmental exposure to peroxisome proliferators may potentially affect the growth, development, and/or reproductive success of aquatic organisms.

Receptors sharing high sequence identity with the mammalian α , β and γ PPAR isoforms have been reported in a number of fish species (Boukouvala et al., 2004; Leaver et al., 2005; Leaver et al., 2007; Tsai et al., 2008), including the goldfish *Carassius auratus* (Mimeault et al., 2005). Fish PPARs are transcriptionally active on mammalian PPAR response elements with both synthetic and endogenous PPAR ligands. This PPAR functionality was demonstrated in a variety of species including pufferfish (*Takifugu rubripes*) (Kondo et al., 2007), rainbow trout (*Oncorhynchus mykiss*) (Liu et al., 2005), plaice (*Pleuronectes platessa*) and gilthead sea bream (*Sparus aurata*) (Leaver et al., 2005), and sea bass (*Dicentrarchus labrax*) (Boukouvala et al., 2004). Some of the physiological roles of

PPARs appear to be conserved in fish, as PPAR agonists result in physiological responses similar to those described in mammals, such as the induction of fatty acyl-CoA oxidase and lauric acid hydroxylase activities (Scarano et al., 1994; Ruyter et al., 1997; Haasch et al., 1998; Sabourault et al., 1999; Du et al., 2004; Weston et al., 2009). However, relatively little is known about the function of PPARs in fish, and to date the functions of PPARs in the gonads have not been investigated in any fish species.

There is evidence that PPARs are involved in the regulation of ovarian and testicular steroid hormone synthesis in rodents. Altered steroidogenic activity has been demonstrated following treatment with various PPAR ligands including fibrates, thiazolidinediones, and phthalate ester plasticizers. Reported effects include altered steroidogenic enzyme expression or activity, changes in sex steroid production, and reproductive impairment (Boujrad et al., 2000; Lovekamp and Davis, 2001; Biegel et al., 2001; Gazouli et al., 2002; Lovekamp-Swan et al., 2003; Freeman and Romero, 2003). The variety of reported effects of PPAR ligands on gonad function in mammals suggests that the role of PPARs in these tissues is complex. The lack of consensus on the roles of PPARs in the reproductive axis of mammals makes predictions of the roles of PPARs in fish reproduction difficult.

A previous study demonstrated that waterborne gemfibrozil (GEM), a fibrate pharmaceutical, bioconcentrates in goldfish plasma and that this exposure is associated with decreased concentrations of testosterone in plasma (Mimeault et al., 2005). The present study investigates the effects of fibrates on *in vitro* testosterone

production by goldfish testis fragments to investigate the mechanism of the depressed circulating testosterone observed *in vivo*.

3.2 MATERIALS AND METHODS

3.2.1 Fish

Common goldfish, *Carassius auratus*, were purchased from a commercial supplier (Aleong's International, Mississauga, ON). Fish were acclimated at 18 ± 1 °C for at least 2 weeks in 70-L fiberglass tanks that received dechloraminated City of Ottawa municipal water. Fish were fed a commercial trout pellet (Martin Mills, Elmira, ON) once daily. All experiments were performed during the prespawning/spawning period (April – June) with male goldfish having a gonado-somatic index (GSI = [testis wt./body wt.] x 100) greater than 3%. All experiments were performed under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhered to guidelines established by the Canadian Council on Animal Care for the use of animals in research and teaching.

3.2.2 Chemicals and Reagents

Human chorionic gonadotropin (hCG), forskolin (activator of adenylyl cyclase), 25-hydroxycholesterol (membrane-permeable cholesterol), pregnenolone (steroid hormone precursor), and GEM were purchased from Sigma-Aldrich (St. Louis, MO). Frozen aliquots of hCG in saline were thawed and doses were freshly prepared in culture medium. Gemfibrozil and forskolin were first solubilized in dimethyl sulfoxide (DMSO); pregnenolone and 25-hydroxycholesterol were

solubilized in 99% ethanol. Control incubations received solvent, and maximum concentrations of DMSO and ethanol did not exceed 0.2% and 0.5%, respectively. These solvent concentrations were previously demonstrated to be without effect on testosterone output from goldfish testis fragments under similar conditions (Wade and Van Der Kraak, 1991).

3.2.3 Incubation of Goldfish Testis Fragments

Testis fragments were incubated in modified Cortland's saline (0.12 mM NaCl, 5.1 mM KCl, 2.97 mM NaH₂PO₄, 1.56 mM CaCl₂, 0.98 mM MgCl₂, 0.93 mM MgSO₄, 0.1% glucose, 0.1% bovine serum albumin, 100 U/mL penicillin, 0.1 mg/mL streptomycin, pH 7.6) as previously described (Wade and Van Der Kraak, 1991). Goldfish were anesthetized in MS-222 (>125 mg/L), decapitated, and testes removed and placed into modified Cortland's saline. Whole-body and testis weights were recorded, and GSI was calculated. Testes were minced and washed with fresh Cortland's saline. Two fragments totaling 20-25 mg were placed into each well of a 24-well tissue culture plate (Corning) containing 1 mL modified Cortland's saline. Fragments were washed again prior to beginning treatments, and incubated at 18°C. There were 3-4 replicate wells for each treatment from each fish, and each experiment was repeated at least 3-times with different fish. This replication was performed to increase technical replication, as testis fragments from the same individual fish may secrete considerably different amounts of testosterone, and to ensure biological replication for statistical analyses. Following overnight (18 h) treatment with fibrates, the medium was replaced with fresh Cortland's saline

containing the same fibrate as well as stimulators of steroidogenesis. These included hCG, forskolin, 25-hydroxycholesterol, and pregnenolone. After a 6 h stimulation, the medium was centrifuged (20000 x g, 3 min, 4°C) and supernatants were stored at -80°C until analysis for testosterone content by radioimmunoassay (McMaster et al., 1992). Testis fragments were frozen in liquid nitrogen and stored at -80°C.

Where cAMP content was estimated, tissue fragments were stimulated with hCG in the presence of IBMX (1 mM; a phosphodiesterase inhibitor). After 3 h, tissue fragments and aliquots of culture medium were separately boiled for 10 min and stored at -80°C. Tissue cAMP was extracted by sonication (3 x 10 s) on ice in 9 volumes of 10% trichloroacetic acid (TCA). Following centrifugation (5600 x g, 20 min, 4°C), supernatants were washed 3-times with 5 volumes of water-saturated diethyl ether, and heated to 50°C for 10 min. Tissue extracts were diluted in cAMP assay buffer and the media was directly assayed for cAMP using a commercial cAMP EIA kit (Biomedical Technologies Inc., Stoughton, MA)(Evanson and Van der Kraak, 2001).

3.2.4 RNA extraction, cDNA synthesis, real-time PCR

Testis fragment RNA was extracted with TRIzol reagent (Invitrogen, Burlington, ON) according to the manufacturer's instructions, following bead homogenization of tissue in TRIzol in a Retsch Mixer Mill MM400 (Newtown, PA). Following DNase I (Invitrogen) treatment of 1µg RNA, cDNA synthesis (Superscript II, Invitrogen) was performed according to the manufacturer's instructions. Primers

for goldfish StAR, PBR and EF-1 α (internal control) were designed using Primer3 software (Table 3.1; <http://fokker.wi.mit.edu/primer3/input.htm>). Real-time PCR was performed with a commercial kit (Brilliant SYBR Green QPCR Master Mix, Stratagene; La Jolla, CA) on a Stratagene Mx3000 thermocycler (15 min at 95°C, 40x [30 s each at 95°C, 58°C, 72°C]). Samples were assayed in duplicate for each set of primers. Data for each sample was normalized to control gene transcript abundance ($2^{-\Delta Ct} = 2^{-(\text{Target Ct} - \text{EF-1}\alpha \text{ Ct})}$, Ct = cycle threshold) prior to statistical analysis. For purposes of graphical presentation of data, the normalized data for each sample is expressed as the fold-change versus the average control response.

Table 3.1. Nucleotide primers used for real-time PCR.

	GenBank #	forward primer (5'-3')	reverse primer (5'-3')	size (bp)
EF-1 α	AB056104	GATTGTTGCTGGTGGTGTG	GCAGGGTTGTAGCCGATTT	216
StAR	AY877430	AAGTTGGAAGTGATGCTGGA	CTTGTTTGACATTGGGGTTC	100
PBR	AY877431	TTGTGTGGAAAGAGCTTGA	GTGAGCGCCAAAGAAGATG	113

3.2.5 Western blotting

Protein was extracted from frozen testis fragments by sonication on ice (2 x 10 s) in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Tween 20, pH 7.4) with protease inhibitors (1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mg/l leupeptin, 2 mg/l aprotinin), followed by centrifugation at 10000 x g for 10 min at 4°C. The protein concentration in the

supernatant was estimated using the bicinchoninic acid (BCA) protein assay (Sigma). Equal amounts of protein from each sample (20 µg) were separated by SDS-PAGE (5% stacking and 10% separating gel), transferred to a PVDF membrane (1 h @ 20 V, semi-dry transfer; BioRad [Hercules, CA]) and incubated overnight at 4°C with 1:3000 anti-fish StAR protein antibody (Kortner and Arukwe, 2007) (a kind gift from Dr. A. Arukwe, Department of Biology, Norwegian University of Science and Technology, Trondheim, Norway).

The polyclonal anti-fish StAR protein antibody was raised in rabbit against a 16 amino acid synthetic antigen (H₂N-MPEQRGVVRAENGPTC-CONH₂). In an NCBI Protein BLAST, this antigen was 100% similar to the predicted amino acid sequence of StAR protein in several fish species (*Salmo salar*, *Salvelinus fontinalis*, *Oncorhynchus mykiss*, *Haplochromis burtoni*, *Epinephelus coioides*, *Sparus aurata*, *Dicentrarchus labrax*, *Micropterus salmoides*, *Micropogonias undulatus*). Full-length goldfish StAR protein cDNA and protein sequences are not available. The partial goldfish StAR protein cDNA sequence that is available does not include the region corresponding to the synthetic antigen. When compared to predicted amino acid sequences of StAR protein in common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*), two species that are closely related to the goldfish, the antigen was 87% similar (14/16 amino acid residues in common). This StAR protein antibody has been demonstrated to positively immunostain ovarian follicular cells at the boundary of previtellogenic oocytes from Atlantic salmon and Atlantic cod (*Gadus morhua*) (Kortner and Arukwe, 2007; Vang et al., 2007). The StAR protein antibody

recognizes a 30 kDa protein in western blots of protein extracts from brain and head kidney of Atlantic salmon (Lyssimachou and Arukwe, 2007).

Western blot detection was performed using an anti-rabbit IgG horseradish peroxidase conjugate and an ECL Plus Detection Kit (GE Healthcare). Membranes were visualized using Chemi Doc camera and software (Bio-Rad Laboratories, Mississauga, ON).

3.2.6 Statistics

As each animal was represented among all treatments within each experiment, data were analyzed by either one-way repeated measures ANOVA or paired t-test where appropriate. Logarithmic transformations of data were used where data did not meet the assumptions of normality or equal variance. Where differences were indicated by ANOVA, pair-wise comparisons were made using the Holm-Sidak method, where $p < 0.05$ was considered a significant difference. SigmaStat software (SPSS Inc.) was used for all statistical analyses.

3.3 RESULTS

Overnight treatment with 100 μM but not 1 or 10 μM GEM significantly inhibited testosterone secretion from goldfish testis fragments treated for 6 h with hCG (Fig. 3.1). Similarly, forskolin-stimulated steroidogenesis was impaired by 10 and 100 μM GEM treatment (Fig. 3.2). Inhibition of testosterone synthesis by GEM was not observed when testis fragments were stimulated with 50 μM 25-hydroxycholesterol (Fig. 3.3) or 100 nM pregnenolone (Fig. 3.4). Increased

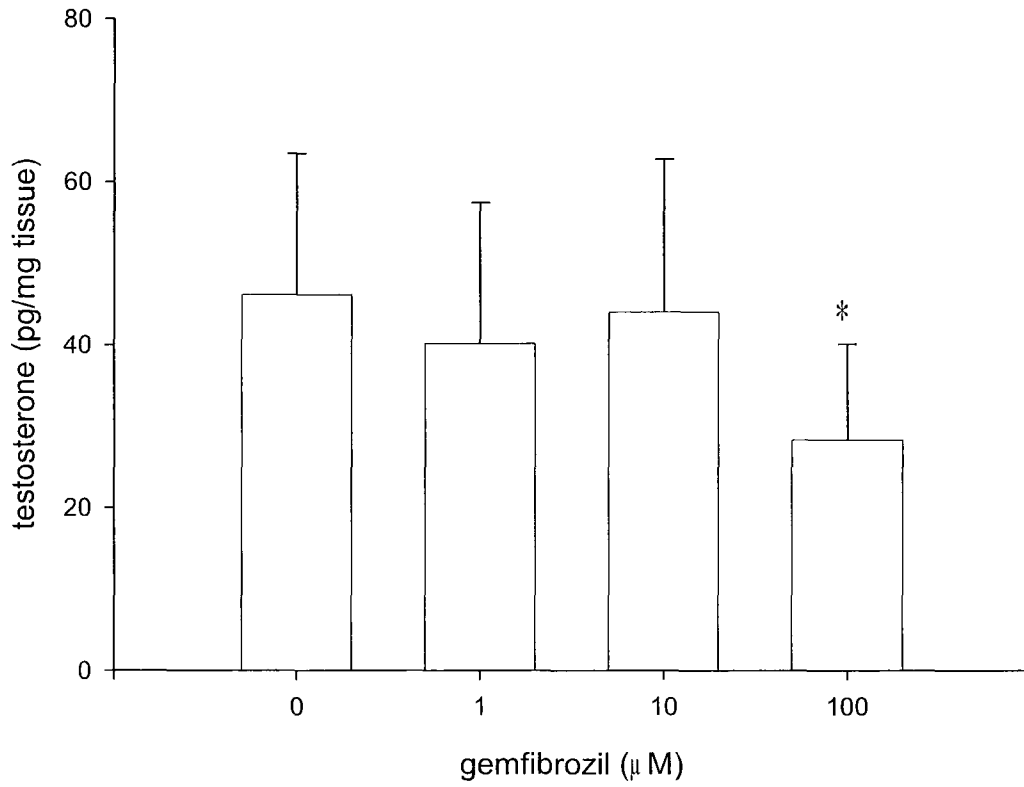


Figure 3.1. Testosterone secretion from goldfish testis fragments during a 6 h incubation with 1 IU/mL hCG and gemfibrozil following an overnight gemfibrozil incubation (n = 6, mean \pm SEM, * p < 0.05 versus control).

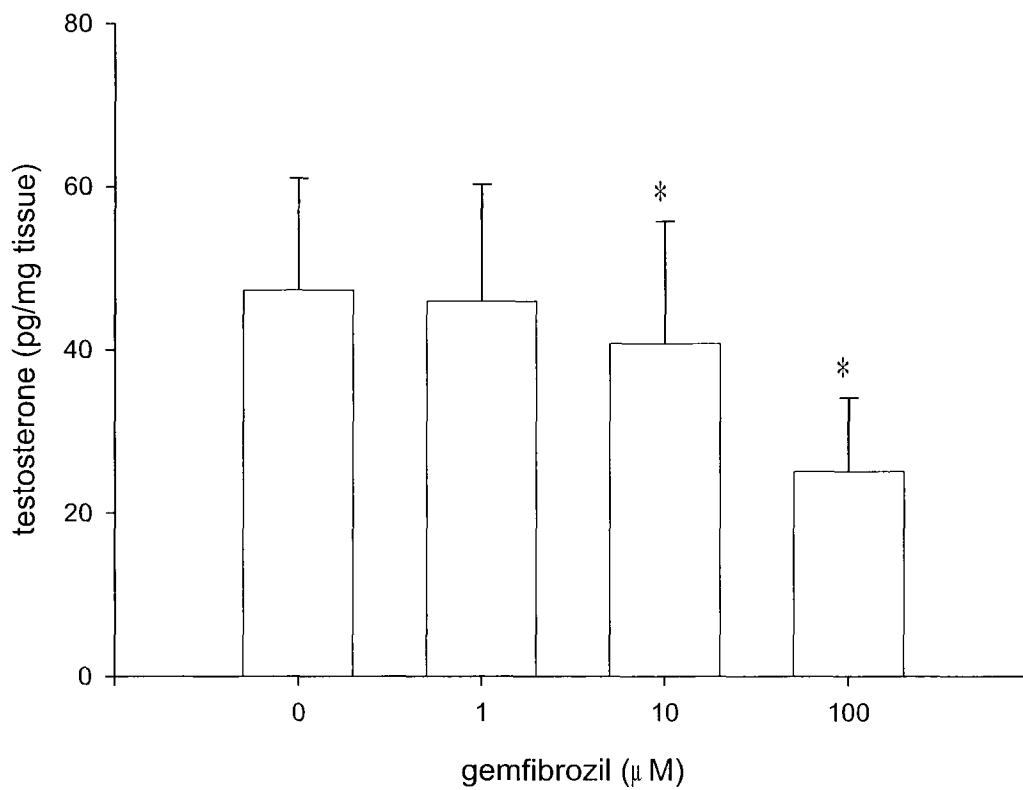


Figure 3.2. Testosterone secretion from goldfish testis fragments during a 6 h incubation with 1 μM forskolin and gemfibrozil following an overnight gemfibrozil incubation (n = 6, mean ± SEM, * p < 0.05 versus control).

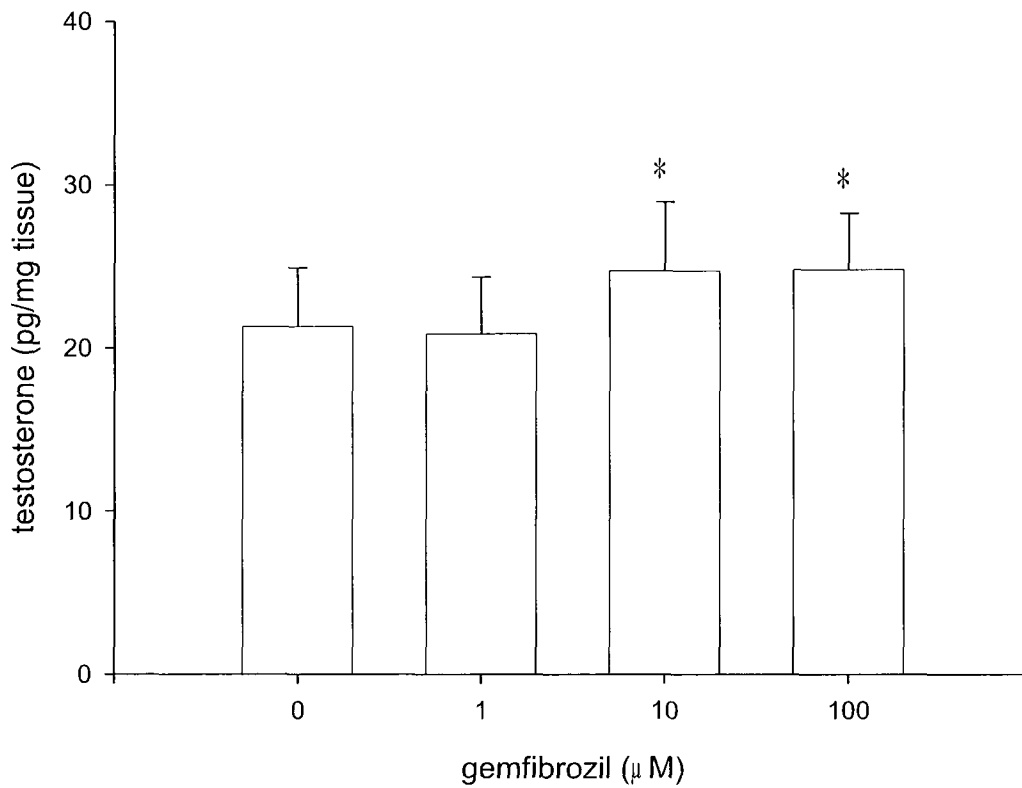


Figure 3.3. Testosterone secretion from goldfish testis fragments during a 6 h incubation with 50 μ M 25-hydroxycholesterol and gemfibrozil following an overnight gemfibrozil incubation (n = 4, mean \pm SEM, * p < 0.05 versus control).

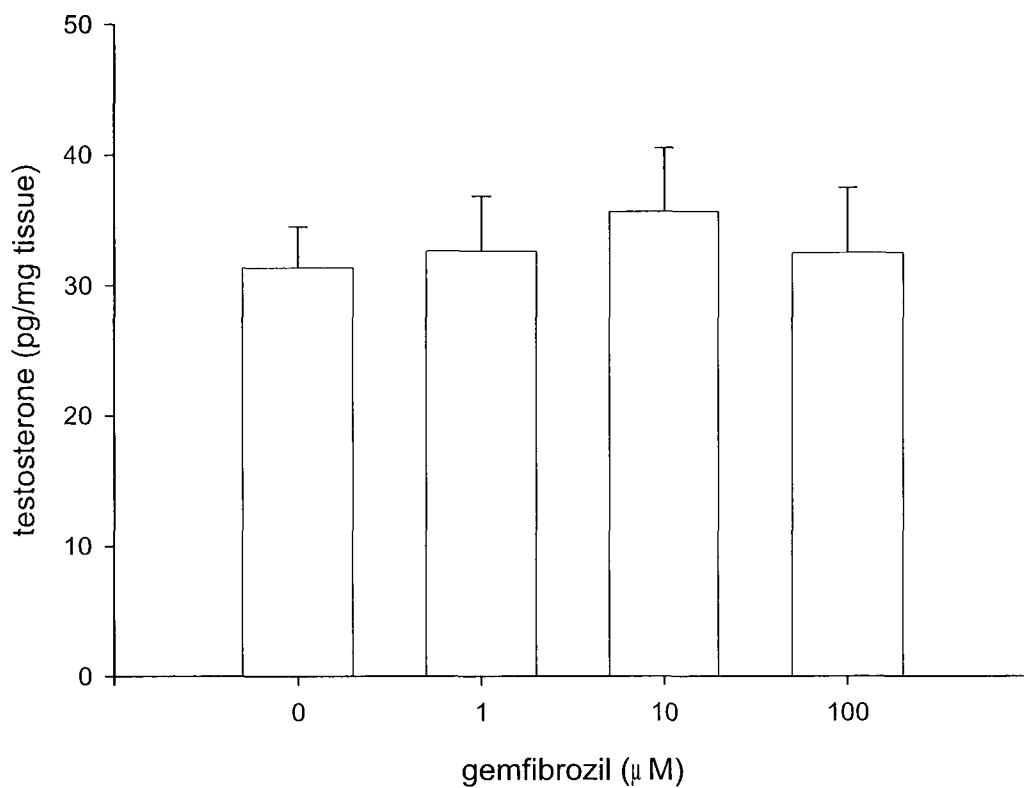


Figure 3.4. Testosterone secretion from goldfish testis fragments during a 6 h incubation with 100 nM pregnenolone and gemfibrozil following an overnight gemfibrozil incubation (n = 4, mean ± SEM).

fragments treated with 10 μ M and 100 μ M GEM (Fig. 3.3). Human CG-stimulated testosterone output was decreased from goldfish testis fragments treated 100 μ M fenofibrate (FEN; Fig. 3.5A) but not with WY-14,643, clofibrate, or bezafibrate at this concentration (Figs 3.5B, 3.5C, 3.5D). Similar to GEM, forskolin-stimulated testosterone secretion was inhibited by overnight treatment with 10 μ M and 100 μ M FEN (Fig. 3.6A), and this inhibitory effect was not evident in tissue incubated with 25-hydroxycholesterol (Fig. 3.6B).

Tissue fragments treated with 100 μ M GEM overnight and stimulated for 3 h with hCG in the presence of IBMX secreted significantly less testosterone than control fragments (Fig. 3.7A). However, cAMP concentrations in the media and tissue fragments did not differ significantly between these groups (Fig. 3.7B, 3.7C).

Treatment of goldfish testis fragments with 1 IU/mL hCG for 18 h significantly increased testosterone secretion and StAR protein transcript abundance (Fig. 3.8). However, this increase in transcript did not result in increased StAR protein content at 18 h (Fig. 3.9).

Transcript abundance for StAR and PBR protein content in testis fragments stimulated with hCG for 6 h did not reveal any differences between control and GEM-treated tissues (100 μ M) (Fig. 3.10). Basal testosterone synthesis and StAR protein levels were unchanged in control and GEM-treated tissues (Fig. 3.11). The 6 h response to hCG was impaired in GEM-treated tissues as measured by testosterone synthesis; however concentrations of StAR protein showed no differences (Fig. 3.11).

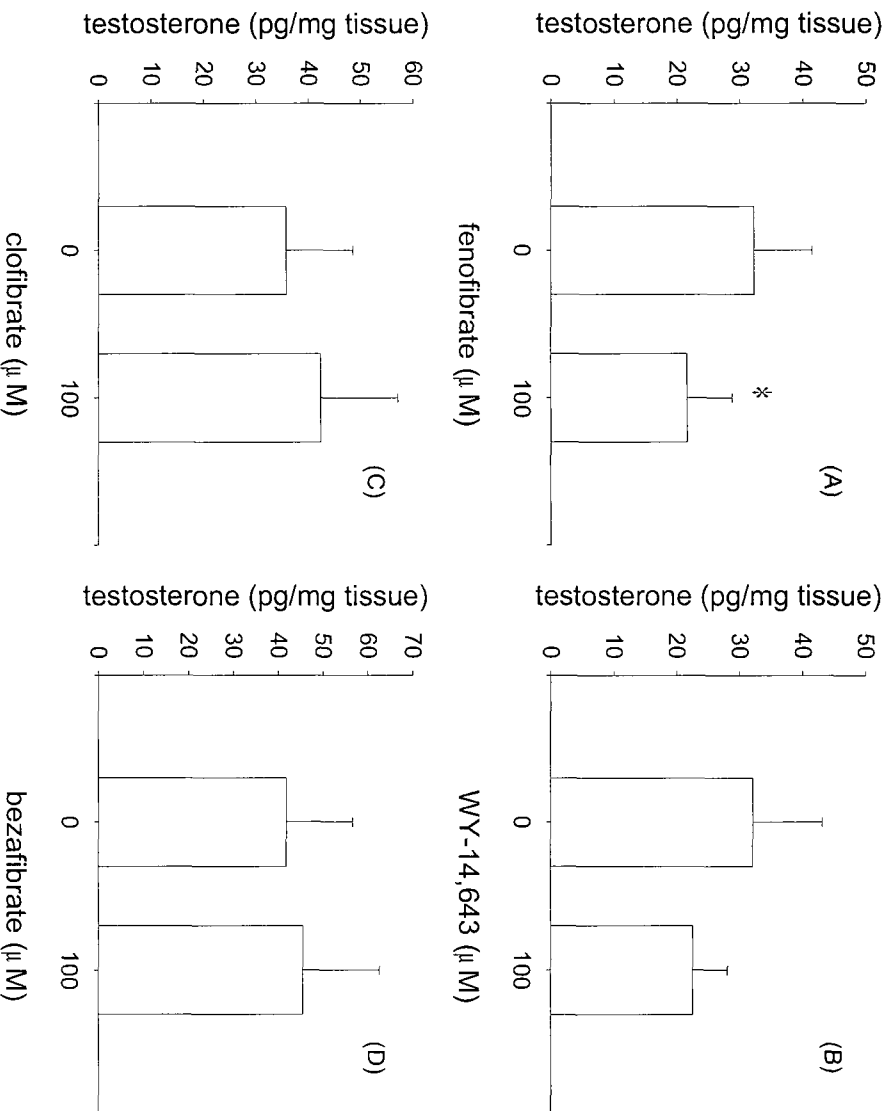


Figure 3.5. Testosterone secretion from goldfish testis fragments during a 6 h incubation with 1 IU/mL hCG and fibrates following an overnight fibrate incubation. Fibrates include fenofibrate (A, n = 6), WY-14,643 (B, n = 6), clofibrate (C, n = 3), and bezafibrate (D, n = 3) (mean \pm SEM, * p < 0.05 versus control).

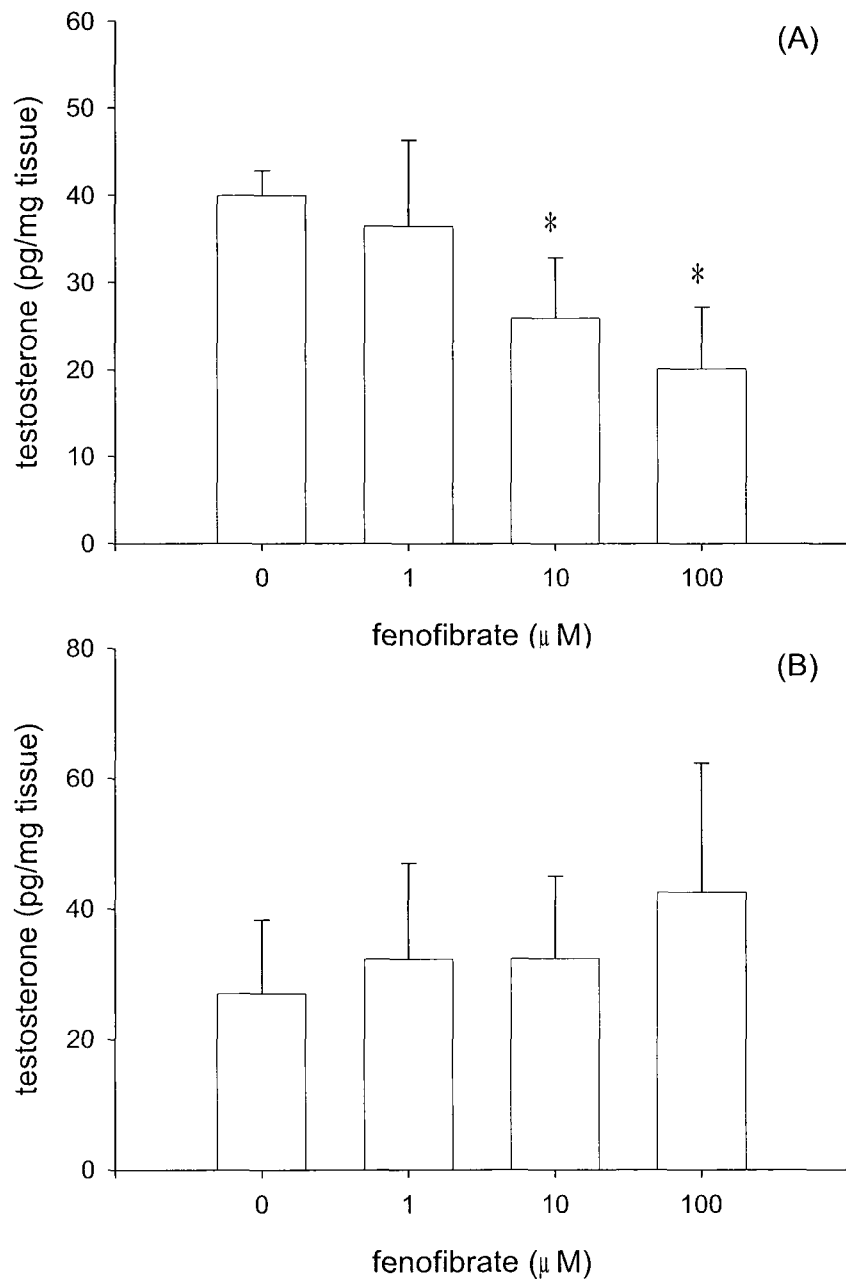


Figure 3.6. Testosterone secretion from goldfish testis fragments during a 6 h incubation with fenofibrate and (A) 1 μ M forskolin or (B) 50 μ M 25-hydroxycholesterol following an overnight fenofibrate incubation (n = 3, mean \pm SEM, * p < 0.05 versus control).

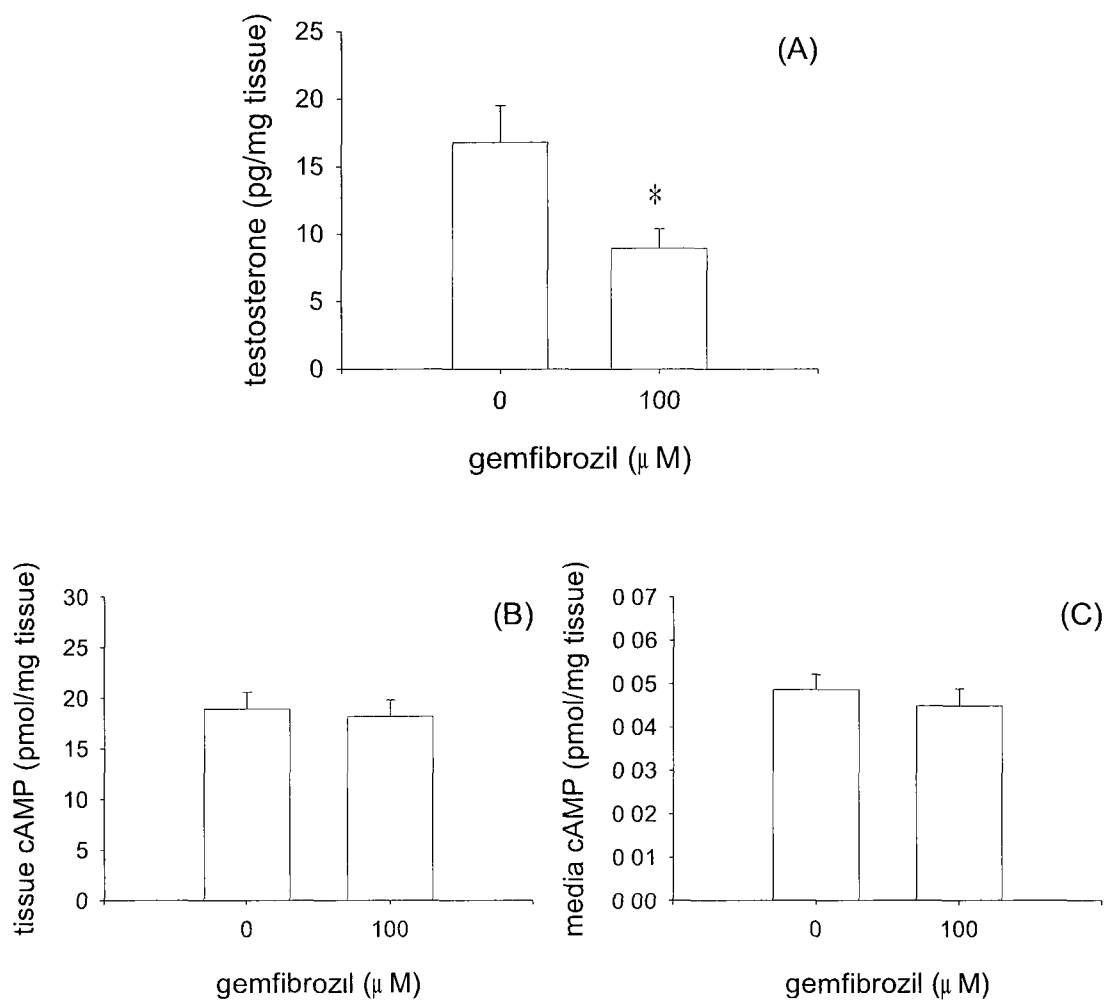


Figure 3.7. Testosterone secretion (A) and cAMP concentrations in tissue (B) and media (C) from goldfish testis fragments cultured overnight with gemfibrozil then incubated with 1 IU/mL hCG, gemfibrozil and 1 mM IBMX for 3 h ($n = 9$, mean \pm SEM, * $p < 0.05$ versus control).

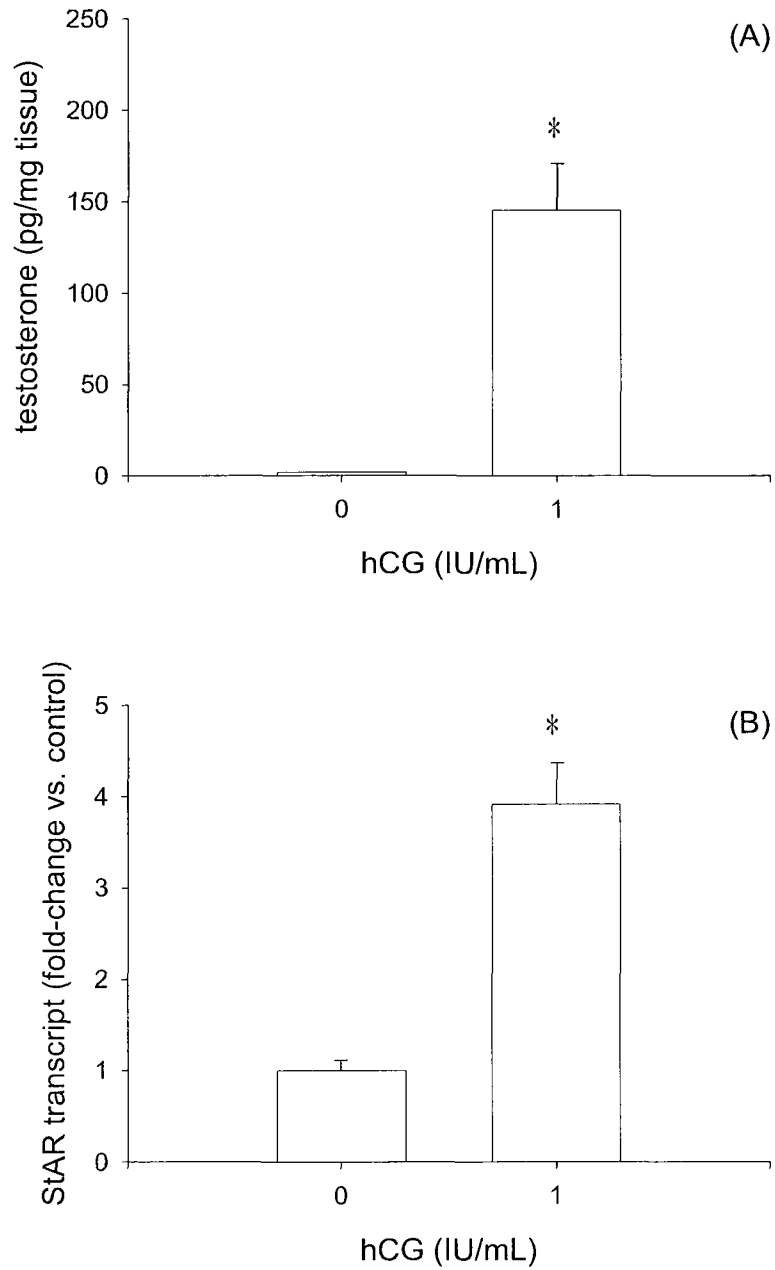


Figure 3.8. Basal and hCG-stimulated goldfish testis fragment (A) testosterone secretion and (B) StAR protein transcript abundance (relative to elongation factor 1 α) after an 18 h incubation (n = 4, mean \pm SEM, * p < 0.05 versus control).

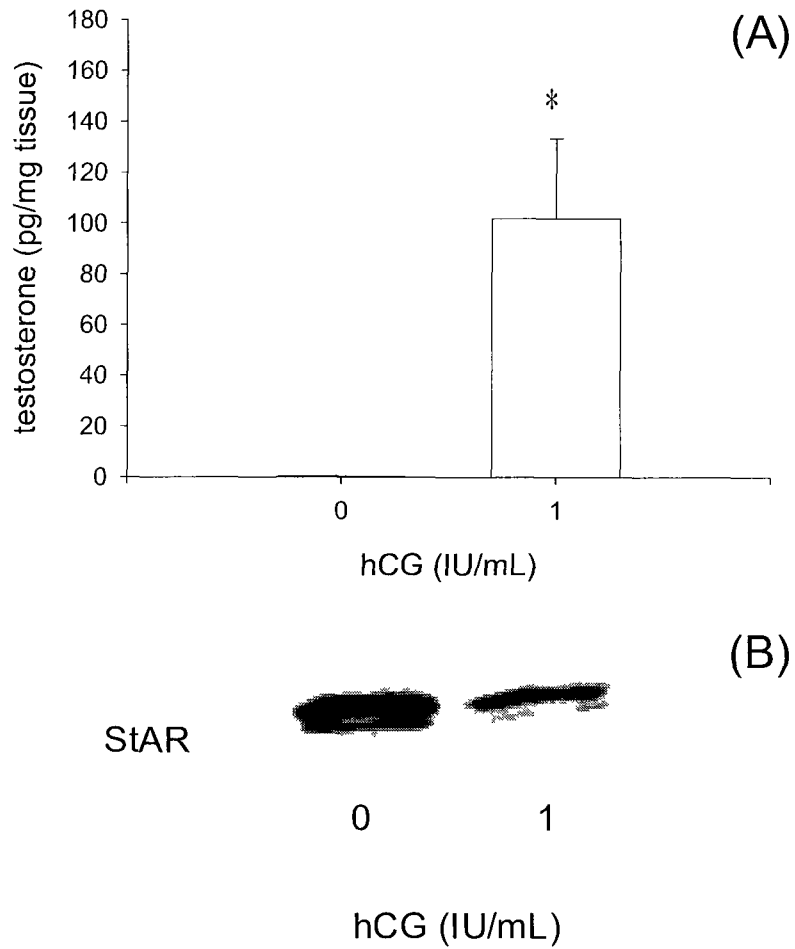


Figure 3.9. Basal and hCG-stimulated goldfish testis fragment (A) testosterone secretion ($n = 3$, mean \pm SEM, * $p < 0.05$ versus control) and (B) a representative StAR protein western blot after an 18 h incubation.

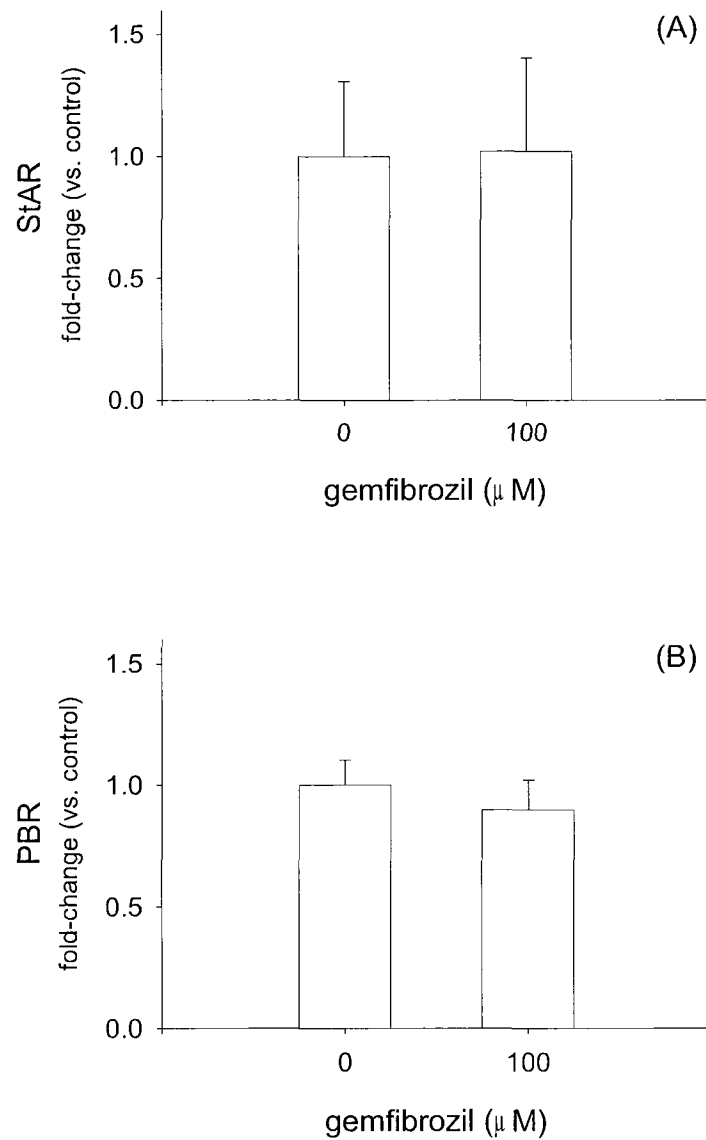


Figure 3.10. Transcript abundance (relative to elongation factor 1 α) for (A) StAR protein and (B) PBR following overnight gemfibrozil and 6 h hCG (1 IU/mL) and gemfibrozil treatment ($n = 5$, mean \pm SEM).

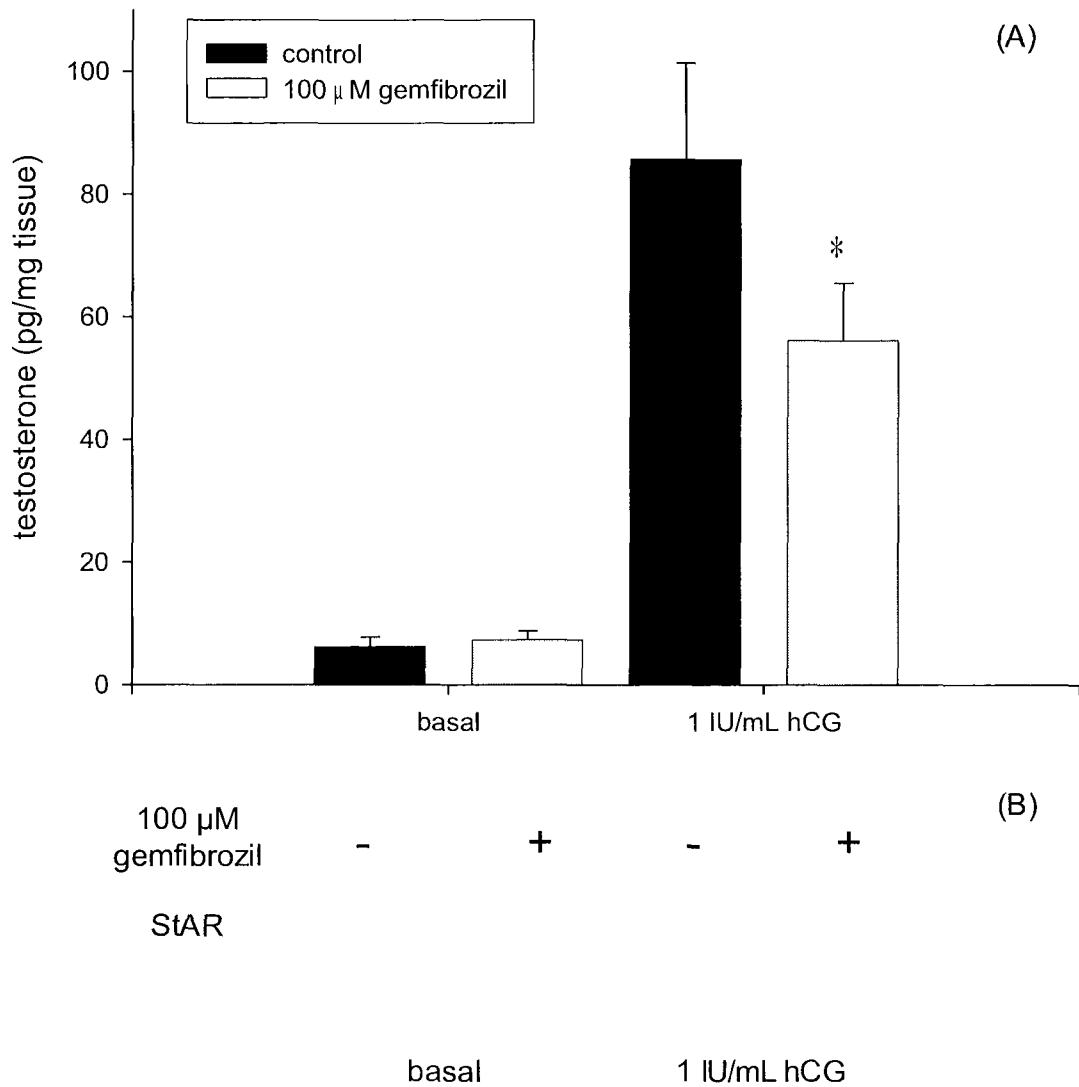


Figure 3.11. Basal and hCG-stimulated (A) testosterone secretion and (B) western blot for StAR protein during a 6 h incubation with hCG and gemfibrozil following an overnight gemfibrozil treatment (n = 4, mean \pm SEM, * p < 0.05 versus control).

3.4 DISCUSSION

Previously, decreased plasma testosterone content was observed in male goldfish exposed to environmental concentrations (1.5 µg/L) of GEM over a 2 week period (Mimeault et al., 2005). Thus, the effects of GEM on the HPG axis of the male goldfish were investigated in this study *in vitro*. The inhibition of hCG-stimulated steroidogenesis in goldfish testis fragments, combined with a lack of effect on *in vitro* pituitary hormone secretion (Chapter 2), suggest that GEM has indeed direct effects on the testis *in vivo*.

A strong steroidogenic response to 18 h hCG treatment was observed in goldfish testis fragments *in vitro*, as testosterone output and the induction of StAR protein gene transcription were significantly increased. However, no change in StAR protein level occurred following this 18 h hCG treatment. The implication is that either tissue concentrations of StAR protein are slow to respond under these *in vitro* conditions or that an increased turnover of newly synthesized StAR protein is not detectable by western blot methods. A 30 kDa protein was detected in western blots in goldfish testis fragment preparations using the anti-fish StAR protein antibody. This is identical to fish StAR protein western blots performed with brain and head kidney from Atlantic salmon (Lyssimachou and Arukwe, 2007). In Fig 3.9 a doublet is observed for StAR protein and this could be the result of increased gel resolution (improved separation of protein within the sample) and the post-translational processing of StAR protein precursors. Information on the post-translational processing of StAR protein in fish tissues is not available. In mouse MA-10 Leydig

cells however, StAR protein is synthesized as a 37 kDa cytosolic protein that is processed to a 32 kDa protein before further processing at the mitochondria to the active 30 kDa form (Clark et al., 1994). Multiple bands of approximately 30 kDa are also sometimes observed in western blots for StAR protein in MA-10 Leydig cells (Clark et al., 1994; Manna et al., 2006; Manna et al., 2007). The dual bands for StAR protein observed in goldfish testis fragments likely represent forms of this protein at various stages of intracellular processing (possibly the equivalents of the 32 kDa and 30 kDa forms observed in MA-10 Leydig cells).

Experiments where goldfish testis fragments were co-exposed to fibrates and hCG failed to demonstrate a consistent effect on testosterone production (Chapter 2). The approach of overnight pre-exposure to fibrates, followed by a 6 h hCG stimulation, resulted in a consistent and repeatable effect on hCG-stimulated steroidogenesis. Similar results were observed in studies with cultured rainbow trout head kidney cells exposed to fibrates and stimulated with ACTH (Mimeault et al., in preparation). The lack of effect in cultures co-exposed to fibrates and hCG may be due to a delay in the action of the fibrate, which needs to be absorbed into the tissue fragments and into cells, combined with the rapid response to hCG/ACTH which acts upon a cell-surface receptor. The administration of fibrates prior to hCG/ACTH may allow for the effects of the fibrates to be realized in advance of the maximal stimulation of the steroidogenic pathway by these hormones.

Both GEM and FEN inhibited testosterone synthesis (100 μ M, and 10 μ M and 100 μ M, respectively). WY-14,643 was previously shown to inhibit testosterone

synthesis in goldfish testis fragments at 100 μM (Chapter 2). A trend suggesting a similar response was observed in the current study but this response was not statistically significant. Both clofibrate and bezafibrate were without effect on hCG-stimulated testosterone secretion. In rainbow trout head kidney cells, clofibrate (400 μM) and FEN (40 and 400 μM) inhibited ACTH-stimulated cortisol release, while GEM and bezafibrate were without effect (Mimeault et al., in preparation). While the range of concentrations for inhibition of steroidogenesis is similar, a different set of fibrates was effective in each of these models. It is not clear whether this difference is related to a species- (goldfish vs trout) or tissue- (testes vs head-kidney) specific effect. Differences in PPAR subtype expression could explain differences in the response.

Various stimulators of testosterone synthesis (hCG, forskolin, 25-hydroxycholesterol, pregnenolone) were used to determine the step within the steroidogenic pathway at which GEM exerted its effect. Any stimulator found to restore steroid production suggests an impairment of the steroidogenic pathway prior to the step at which the stimulator activates the pathway. Treatment with hCG (an LH analogue) activates the G-protein coupled LH receptor resulting in increased intracellular cAMP and leads to increased steroid production (see Fig. 1.2). Forskolin activates adenylyl cyclase directly and increases intracellular cAMP independent of LH receptor function. The cholesterol analogue 25-hydroxycholesterol stimulates testosterone synthesis as it is a membrane-permeable form of cholesterol that does not require activated mitochondrial transport to reach the side chain cleavage

enzyme within the mitochondria. Pregnenolone stimulates steroid production as it is a steroid hormone precursor, the product of the side chain cleavage enzyme, that bypasses the requirement for cholesterol processing within the mitochondria.

Both hCG- and forskolin-stimulated testosterone secretion were impaired, while the formation of cAMP in testis fragments in response to hCG was not affected by GEM treatment. The inhibition of forskolin-stimulated testosterone synthesis by GEM suggested an effect distal to LH receptor availability and/or function. Importantly, stimulators that bypassed the requirement for mitochondrial cholesterol transport, that is 25-hydroxycholesterol and progesterone, restored testosterone synthesis to control levels in GEM-treated fragments. Similarly, both hCG- and forskolin-stimulated steroidogenesis were inhibited by FEN, and testosterone output was restored in the presence of 25-hydroxycholesterol. These results support the hypothesis that GEM and FEN impair cholesterol transport into the mitochondria, possibly having effects on either the abundance or function of StAR and/or PBR protein.

PBR was identified as a target for impaired steroidogenesis with PPAR agonist treatment. MA-10 Leydig tumor cells treated with PPAR α agonists or mono (2-ethylhexyl) phthalate (MEHP) had impaired cholesterol transport into the mitochondria after hCG stimulation, reduced levels of PBR expression (mRNA and protein) and reduced progesterone synthesis (Gazouli et al., 2002). *In vivo*, the same treatments resulted in reduced PBR mRNA abundance in mouse testis and depressed circulating testosterone levels (Gazouli et al., 2002). Also, in MA-10

Leydig cells, perfluorodecanoic acid inhibited hCG-stimulated steroidogenesis and PBR ligand-binding capacity, protein level and mRNA (Boujrad et al., 2000). However, in this and a previous study in goldfish (Woodhouse, 2005), changes in PBR transcript levels were not apparent following treatments with GEM. Detecting changes in PBR protein level was not possible in these studies as fish antibodies are not available and this protein remains a possible target of fibrates in the goldfish testis.

Steroidogenesis is dependent upon the transfer of cholesterol to the side-chain cleavage enzyme (P450_{scc}) at the inner mitochondrial membrane, and this step is largely dependent upon StAR protein. As the rate-limiting step in steroidogenesis, StAR protein is often identified as a potential site of action for toxicants disrupting steroidogenesis, including a number of human pharmaceuticals (Hontela, 2006; Gravel and Vijayan, 2006). The effect of fibrates on testicular StAR protein expression has not been studied. However, other toxicants known to activate PPARs, including phthalates and perfluorinated compounds, have been implicated in the disruption of this protein. Decreases in circulating testosterone and testicular StAR protein transcript and protein abundance following treatment with various phthalates was reported in fetal rats exposed *in utero* (Shultz et al., 2001; Barlow et al., 2003; Borch et al., 2006; Boberg et al., 2008). Cultured Leydig cells respond similarly to phthalate exposure. Dibutyl phthalate inhibited progesterone synthesis in MLTC-1 Leydig cells, and this was associated with down-regulation of StAR protein expression (Wang et al., 2007). Treatment of male rats with perfluorododecanoic

acid resulted in decreased testosterone concentrations and StAR protein transcript and total protein abundance (Shi et al., 2007; Shi et al., 2009). Data inconsistent with these results are also reported, where steroid concentrations and StAR expression increased in response to DBP in male rats (Ryu et al., 2007; Ryu et al., 2008). Factors including age at time of exposure and sampling, method of delivery, dosage, and exposure time may explain differences observed between studies. In addition, it is not clear in all cases whether these effects involve PPAR-dependent or PPAR-independent mechanisms.

Transcript and total protein abundance for StAR protein in goldfish testis fragments were not affected by GEM-treatment. Other potential effects of GEM could include a change in the phosphorylation status and function of StAR protein, or changes in the abundance or activity/phosphorylation of other proteins required for mitochondrial cholesterol transport. Recent studies are beginning to shed light on the complexity of mitochondrial cholesterol transport in Leydig cell lines. This transport involves interactions between several proteins at the mitochondria membrane including intracellular kinases such as PKA and ERK1/2, kinase anchoring proteins, and transport proteins such as dStAR, PBR (TSPO), and VDAC (Poderoso et al., 2008; Dyson et al., 2008; Poderoso et al., 2009). Mitochondrial cholesterol transport in fishes is not as well-studied, but likely involves a similar level of complexity and a similar complement of proteins. Gemfibrozil may inhibit steroidogenesis by interfering with the formation of this complex at the mitochondria.

In summary, GEM and FEN inhibited hCG-stimulated testosterone synthesis

in goldfish testis fragments *in vitro*. This inhibition appeared to result from decreased mitochondrial cholesterol transport. However, transcripts for StAR and PBR remained unaffected, as well as levels of StAR total protein. Other possibilities that require further investigation are the involvement of other proteins that comprise the mitochondrial cholesterol transporter, and the activation or phosphorylation of StAR and other proteins involved in cholesterol transport.

Chapter 4: Extracellular signal-regulated kinase 1/2 and fibrate inhibition of *in vitro* steroidogenesis in the goldfish, *Carassius auratus*

ABSTRACT

This study investigated interactions between several pathways regulating testicular steroidogenesis in the goldfish including human chorionic gonadotropin (hCG)/protein kinase A (PKA), protein kinase C (PKC), extracellular signal-regulated kinase 1/2 (ERK 1/2), and peroxisome proliferator-activated receptors (PPARs). Treatment for 18 h with 100 μ M GEM inhibited hCG-stimulated testosterone synthesis over 6 h in goldfish testis fragments *in vitro*. Stimulation of PKC with phorbol 12-myristate 13-acetate (PMA) for 6 h inhibited hCG-stimulated testosterone synthesis and inhibition of PKC with bisindolylmaleimide (BIS) was without effect. Treatment with PMA and BIS did not affect the inhibition of steroidogenesis by GEM suggesting that the PKC pathway is not involved. Inhibition of ERK1/2 signaling with U0126 also suppressed hCG-stimulated steroidogenesis. PMA treatment significantly increased phosphorylated ERK 1/2 (P-ERK 1/2) in goldfish testis fragments. StAR protein transcript abundance and total protein were not modified by treatment with hCG, PMA, U0126 or GEM. In mitochondrial fractions, hCG treatment significantly increased phospho-ERK 1/2 as compared to unstimulated testis fragments and those treated with PMA alone, hCG + U0126 and hCG + GEM. The amount of StAR protein detected in mitochondrial fractions was not affected by these treatments. In agreement with studies in mammalian species, P-ERK 1/2 is required for hCG-stimulated steroidogenesis in the goldfish testis. This

may represent a requirement for increased mitochondrial P-ERK 1/2 for cholesterol transport. The inhibitory effect of GEM was not dependent on the PKC pathway. GEM treatment reduced mitochondrial P-ERK 1/2 in hCG-stimulated goldfish testis fragments. This reduction in mitochondrial P-ERK 1/2 may be the mechanism by which GEM impairs hCG-stimulated mitochondrial cholesterol transport and steroidogenesis.

4.1 INTRODUCTION

The major stimulatory signaling pathway regulating testicular steroidogenesis is cAMP-dependent protein kinase A (PKA); other signaling pathways constitute a relatively small percentage of the response. However, other pathways including protein kinase C (PKC) and mitogen activated protein kinases (MAPK) may significantly modulate basal or PKA-stimulated steroid synthesis. Stimulation of PKC in MA-10 Leydig cells enhances cAMP-stimulated steroidogenesis and ERK 1/2 phosphorylation is required for PKC- and cAMP-stimulated steroidogenesis (Jo et al., 2005; Manna et al., 2007; Dyson et al., 2008). Phosphorylated mitochondrial ERK 1/2 is required for the PKA-stimulated increase in mitochondrial cholesterol transport (Poderoso et al., 2008; Poderoso et al., 2009).

In goldfish testis fragments, increased PKC activation and intracellular calcium levels increase basal testosterone production (Wade and Van Der Kraak, 1991). In contrast, PKC activation in goldfish testis fragments and ovarian follicles inhibits hCG-stimulated testosterone production but does not inhibit testosterone synthesis stimulated by an adenylate cyclase activator, forskolin, suggesting that PKC has inhibitory effects at a step prior to the activation of adenylate cyclase in goldfish (Wade and Van Der Kraak, 1991; Van Der Kraak, 1992). The role of ERK 1/2 in testicular steroidogenesis in fish has not yet been investigated. In fish ovarian cells, ERK 1/2 has been reported to be both stimulatory and inhibitory to PKA-stimulated steroid production. In co-cultures of ovarian granulosa and thecal cells from Atlantic croaker (*Micropogonias undulatus*) (Benninghoff and Thomas, 2006)

and common carp (*Cyprinus carpio*) (Paul et al., 2010), phosphorylated ERK 1/2 is required for cAMP- and PKA-stimulation of steroidogenesis. In isolated brown trout (*Salmo trutta*) ovarian thecal layers, however, the inhibition of LH-induced steroidogenesis by insulin and IGF-I is dependent upon ERK 1/2 phosphorylation (Méndez et al., 2005).

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that regulate the rate of nuclear transcription of target genes. The ligand binding affinities and transcriptional activities of PPARs are enhanced following phosphorylation by kinases such as PKA, PKC, and MAPK (Juge-Aubry et al., 1999; Lazennec et al., 2000; Blanquart et al., 2004; Diradourian et al., 2005; Gray et al., 2005; Burns and Vanden Heuvel, 2007; Burgermeister and Seger, 2008). PPARs and their ligands may also be directly involved in phosphorylation cascades, inducing the phosphorylation of other signaling molecules within the cell (Rokos and Ledwith, 1997; Jiang et al., 2004; Gardner et al., 2005; Burgermeister and Seger, 2008). In this way, PPARs may have effects independent of their functions in nuclear transcription on cellular processes such as steroidogenesis.

Previous studies have demonstrated the effects of fibrates, peroxisome proliferator-activated receptor (PPAR)- α ligands, on steroid production in fish. These include a decrease in circulating testosterone concentration in goldfish exposed to waterborne gemfibrozil (GEM) (Mimeault et al., 2005), decreased ACTH-stimulated cortisol secretion from rainbow trout (*Oncorhynchus mykiss*) head kidney cells *in vitro* (Mimeault et al., in preparation), and decreased hCG-stimulated testosterone

synthesis in goldfish testis fragments *in vitro* (Chapter 3). Fibrates may exert their effect on steroidogenesis in the goldfish by disrupting the phosphorylation cascade responsible for stimulating the pathway rather than by altering the rate of transcription of a particular target gene. This study investigated the role of PKC and ERK 1/2 in testicular steroidogenesis and the potential interactions of these pathways with the inhibition of hCG-stimulated steroidogenesis by GEM in the goldfish testis.

4.2 MATERIALS AND METHODS

4.2.1 Fish

Common goldfish, *Carassius auratus*, were purchased from a tropical fish supplier (Aleong's International, Mississauga, ON). Fish were acclimated to 18 ± 1 °C for at least 2 weeks in 70-L fiberglass tanks that received dechloraminated City of Ottawa municipal water. Fish were fed a commercial trout pellet (Martin Mills, Elmira, ON) once daily. All experiments were performed during the prespawning/spawning period (April – June) with male goldfish having a gonado-somatic index (GSI = [testis wt./body wt.] x 100) greater than 3%, which is indicative of this seasonal period. All experiments were performed under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee and adhered to guidelines established by the Canadian Council on Animal Care for the use of animals in research and teaching.

4.2.2 Chemicals and reagents

Human chorionic gonadotropin (hCG) and gemfibrozil (GEM) were purchased from Sigma-Aldrich (St. Louis, MO). Frozen aliquots of hCG in saline were thawed and doses freshly prepared in culture medium. Phorbol 12-myristate 13- acetate (PMA; a stimulator of PKC), bisindolylmaleimide (BIS; an inhibitor of PKC), and U0126 (inhibitor of MEK 1/2, inhibits ERK 1/2 phosphorylation) were purchased from EMD/Calbiochem (Gibbstown, NJ). Gemfibrozil, PMA, BIS and U0126 were solubilized in dimethyl sulfoxide (DMSO) as concentrated stocks. Controls received solvent and maximum concentrations of DMSO did not exceed 0.2% in any experiment. These solvent concentrations were previously demonstrated to not affect testosterone output from goldfish testis fragments under similar conditions (Wade and Van Der Kraak, 1991).

4.2.3 Incubation of goldfish testis fragments

Testis fragments were incubated in modified Cortland's saline (0.12 mM NaCl, 5.1 mM KCl, 2.97 mM NaH₂PO₄, 1.56 mM CaCl₂, 0.98 mM MgCl₂, 0.93 mM MgSO₄, 0.1% glucose, 0.1% bovine serum albumin, 100 U/mL penicillin, 0.1 mg/mL streptomycin, pH 7.6) as previously described (Wade and Van der Kraak, 1991). Goldfish were anesthetized in MS-222 (>125 mg/L), decapitated, and the testes removed and placed into modified Cortland's saline. Whole-body and testis weight were recorded, and GSI calculated. Testes were minced and washed with fresh Cortland's saline. Two fragments totaling 20-25 mg were placed into each well of a 24-well tissue culture plate (Corning) containing 1 mL modified Cortland's saline.

Fragments were washed again prior to beginning treatments and incubated at 18°C. There were 3-4 replicate wells for each treatment from each fish, and each experiment was repeated at least 3 times with different fish. Following overnight (18 h) treatment with fibrates, the medium was replaced with fresh Cortlands's saline containing the same fibrate as well as inhibitors and stimulators of steroidogenesis (hCG, PMA, BIS, U0126) at pre-determined concentrations. In experiments involving U0126 treatment, the testis fragments were pre-incubated in U0126 for 1 h (to ensure inhibition of MEK 1/2) prior to adding further treatments. Following each experiment, the medium was removed and centrifuged (4°C, 20000 g for 3 min) and supernatants stored at -80°C until analyzed for testosterone content by radioimmunoassay (McMaster et al., 1992). Testis fragments were frozen in liquid N₂ and stored at -80°C.

4.2.4 Western blotting

Protein samples for western blotting were extracted from frozen testis fragments by sonication on ice (2 x 10 s; Contes Micro-Ultrasonic Cell Disrupter) in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Tween 20, pH 7.4) with protease inhibitors (1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mg/l leupeptin, 2 mg/l aprotinin), followed by centrifugation at 10,000 x g for 10 min at 4°C. The pellet was discarded and the protein concentration of the supernatant was estimated (see below).

In experiments where protein in cellular fractions (cytosolic and mitochondrial) was of interest, samples were prepared by differential centrifugation using fresh

tissue following established methods (Clark et al., 1994; Manna et al., 1999; Manna et al., 2002). Following treatment testis fragments were disrupted with a teflon homogenizer in TSE buffer (10 mM Tris, 250 mM sucrose, 100 mM EDTA, pH 7.4, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mg/L leupeptin, 2 mg/L aprotinin) and the homogenate was centrifuged (600 x *g*, 4°C, 25 min). The supernatant was transferred to a new tube and centrifuged (10000 x *g*, 4°C, 30 min). The resulting pellet (mitochondrial fraction, resuspended in TSE buffer) and the supernatant (cytosolic fraction) were stored separately at -80°C.

The protein concentration of the tissue extract was estimated using the bicinchoninic acid (BCA) protein assay (Sigma) prior to loading samples for electrophoresis. Equal amounts of protein from each sample (15-25 µg) were separated by SDS-PAGE (5% stacking and 10% separating gel), transferred to a PVDF membrane (1 h @ 20 V, semi-dry transfer; BioRad [Hercules, CA]), blocked with 5% skim milk in TBS-T (Tris-buffered saline with Tween-20; 25 mM Tris base, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween-20, pH 8), and incubated overnight at 4°C with primary antibody in 5% BSA in TBS-T. The anti-fish StAR protein antibody (a kind gift from Dr. A. Arukwe, Department of Biology, Norwegian University of Science and Technology, Trondheim, Norway) was used at a dilution of 1:3000 (Kortner and Arukwe, 2007). The phospho-ERK 1/2 and total ERK 1/2 antibodies (Cell Signaling, Danvers, MA) were diluted to 1:1000. These antibodies recognize ERK 1 (44 kDa) and ERK 2 (42 kDa) with a characteristic double banding pattern. Both of the ERK 1/2 antibodies are identified as cross-reactive with zebrafish by the manufacturer

and have been used in other fish species including rainbow trout (Ebner et al., 2007). Amino acid sequence identities for ERK 1 and ERK 2 between zebrafish and human are 91% and 94%, respectively. In addition, the goldfish ERK 2 amino acid sequence is 94% similar to human ERK 2. The phospho-ERK 1/2 antibody is polyclonal and was raised against a synthetic phosphopeptide encompassing the phosphorylation sites within the ERK protein (Thr 202 and Tyr 204 in ERK 1, Thr 185 and Tyr 187 in ERK 2). The total-ERK 1/2 antibody is polyclonal and was raised against a synthetic peptide at the C-terminus, away from the phosphorylation sites of ERK 1 and ERK 2. Detection of blots was performed using an anti-rabbit IgG horseradish peroxidase conjugate and an ECL Plus Detection Kit (GE Healthcare). For the detection of subsequent proteins, western blots were repeated following stripping of bound antibody from the membranes by placing them in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 0.1 M 2-mercaptoethanol, 2% SDS) at 50°C for 20 min, followed by 2 large-volume TBS-T washes. Membranes were visualized using the Chemi Doc camera and software (Bio-Rad Laboratories, Mississauga, ON) and captured tif images were analyzed using ImageJ software (ImageJ, National Institutes of Health; <http://rsbweb.nih.gov/ij/>; methods of analysis, <http://www.lukemiller.org/journal/2007/08/quantifying-western-blot-without.html> and <http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gels>) with total-ERK 1/2 band intensity serving as the control.

4.2.5 RNA extraction, cDNA synthesis, real-time PCR

Testis fragment RNA was extracted with TRIzol reagent (Invitrogen,

Burlington, ON), following bead homogenization of tissues in TRIzol on a Retsch Mixer Mill MM400 (Newtown, PA, USA). Following DNase I (Invitrogen) treatment of 1µg RNA, cDNA synthesis (Superscript II, Invitrogen) was performed. Primers for goldfish StAR and EF-1α (internal control) were designed using Primer3 software (Table 3.1; <http://fokker.wi.mit.edu/primer3/input.htm>). Real-time PCR was performed using a commercial kit (Brilliant SYBR Green QPCR Master Mix, Stratagene; La Jolla, CA) on a Stratagene Mx3000 thermocycler (15 min at 95°C, 40x [30 s each at 95°C, 58°C, 72°C]). Samples were assayed in duplicate for each set of primers. Data for each sample was normalized to control gene transcript abundance ($2^{-\Delta Ct} = 2^{-(\text{StAR } Ct - \text{EF-1}\alpha \text{ Ct})}$, Ct = cycle threshold) prior to statistical analysis. For purposes of graphical presentation of data, the normalized data for each sample is expressed as the fold-change versus the average control response.

4.2.6 Statistics

In experiments where each animal was represented among all treatments data were analyzed by either one-way repeated measures ANOVA or paired t-test. Two-way ANOVA was used where appropriate. Data transformations (logarithmic or square-root) were used where data did not meet the assumptions of normality or equal variance. Where differences were indicated by ANOVA, pair-wise comparisons were made using the Holm-Sidak method, where $p < 0.05$ was considered a significant difference. SigmaStat software (SPSS Inc.) was used for all statistical analyses.

4.3 RESULTS

4.3.1 Effects of hCG, GEM and the PKC pathway

Following an 18 h incubation with 100 μ M GEM treatment, basal (non-hCG-stimulated) 6 h testosterone secretion from goldfish testis fragments was not different from control (no addition) in the presence of GEM, PMA (500 nM) or BIS (500 nM) (Fig. 4.1). hCG-Stimulated testosterone secretion was inhibited in the presence of GEM (100 μ M; 64% of control), PMA (500 nM; 77.6% of control), PMA with GEM (49.1% of control), and BIS (500 nM) with GEM (59.5% of control), but not with BIS alone (106% of control)(Fig. 4.2).

4.3.2 Effects of hCG, GEM, and the PKC and ERK 1/2 pathways

Basal testosterone secretion over 6 h was not affected by either PMA (500 nM), U0126 (10 μ M), or the combination of these (Fig. 4.3). Fragments that had been treated with GEM (100 μ M) for 18 h secreted significantly more testosterone over 6 h than those that were not (Fig. 4.3), however, no significant effect of GEM was observed when the individual control, PMA, or U0126 groups were considered. GEM (100 μ M), PMA (500 nM) and U0126 (10 μ M) inhibited hCG-stimulated testosterone secretion (Fig. 4.4). The combination of PMA and U0126 was significantly more potent in the inhibition of testosterone synthesis than PMA alone in both control and GEM-treated testis fragments (Fig. 4.4). Treatment with PMA (500 nM) stimulated a significant increase in ERK 1/2 phosphorylation in both non-stimulated and hCG-stimulated goldfish testis fragments (Fig. 4.5). Phosphorylated ERK 1/2 was decreased by U0126 (10 μ M) treatment in hCG-stimulated testis

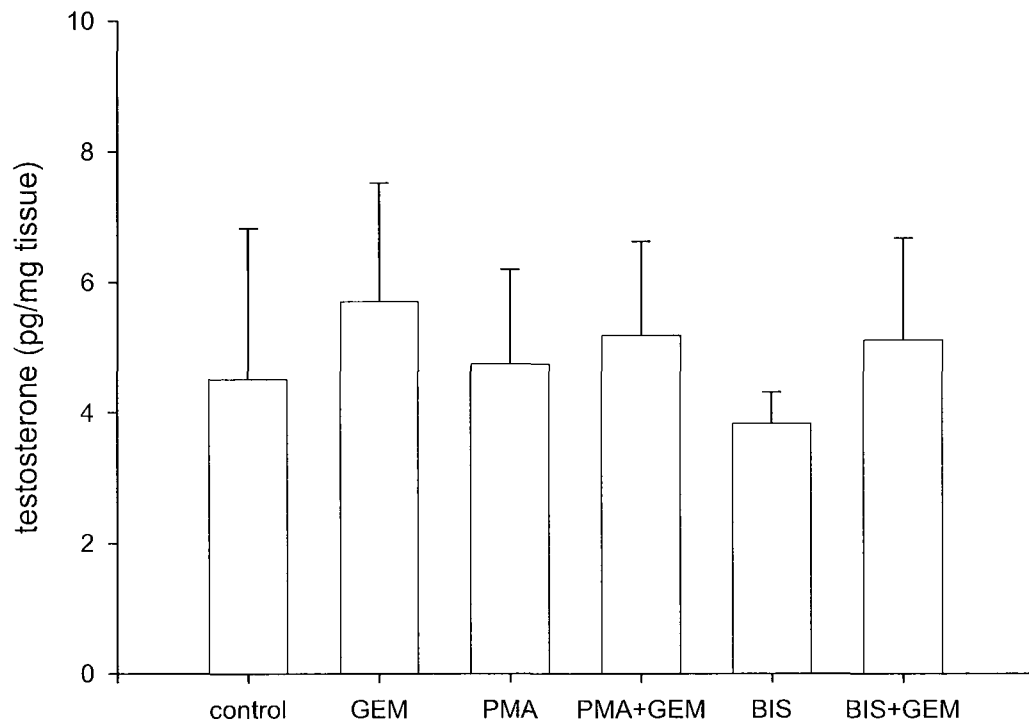


Figure 4.1. Basal testosterone secretion from goldfish testis fragments treated with gemfibrozil (GEM; 100 μ M) for 18 h followed by 6 h treatment with GEM, phorbol 12-myristate 13-acetate (PMA; 500 nM) and bisindolylmaleimide (BIS; 500 nM) (mean \pm SEM, n = 4).

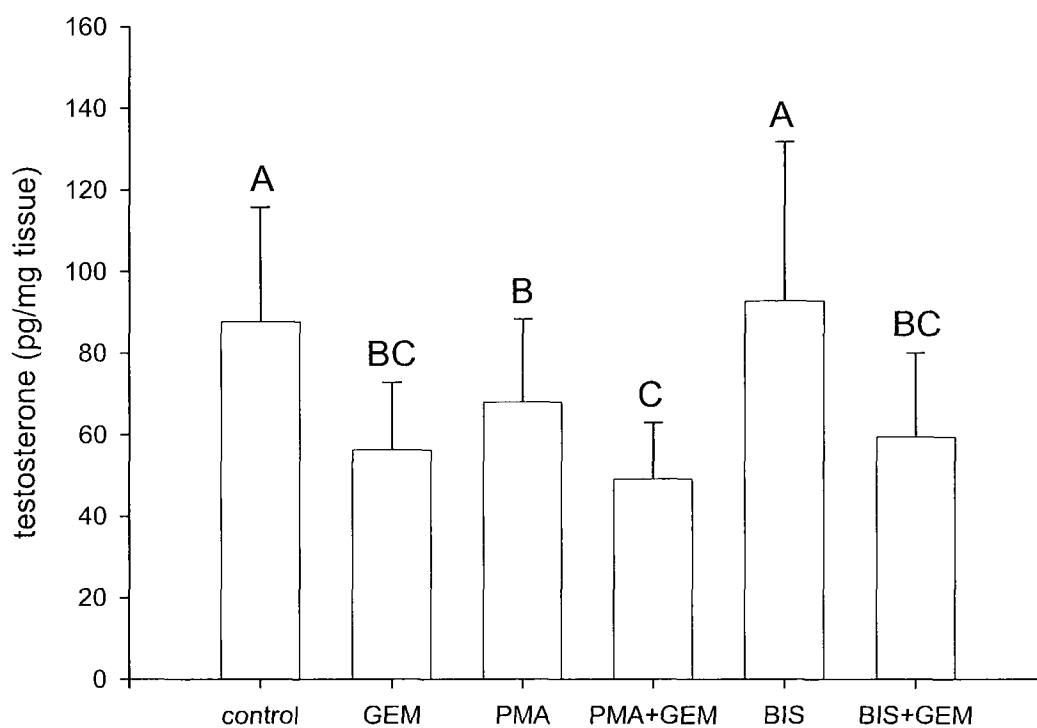


Figure 4.2. hCG-stimulated (1 IU/mL) testosterone secretion from goldfish testis fragments treated with gemfibrozil (GEM; 100 μ M) for 18 h followed by 6 h treatment with hCG, GEM, phorbol 12-myristate 13-acetate (PMA; 500 nM) and bisindolylmaleimide (BIS; 500 nM). Different letters indicate statistical significance (mean \pm SEM, n = 4, p < 0.05).

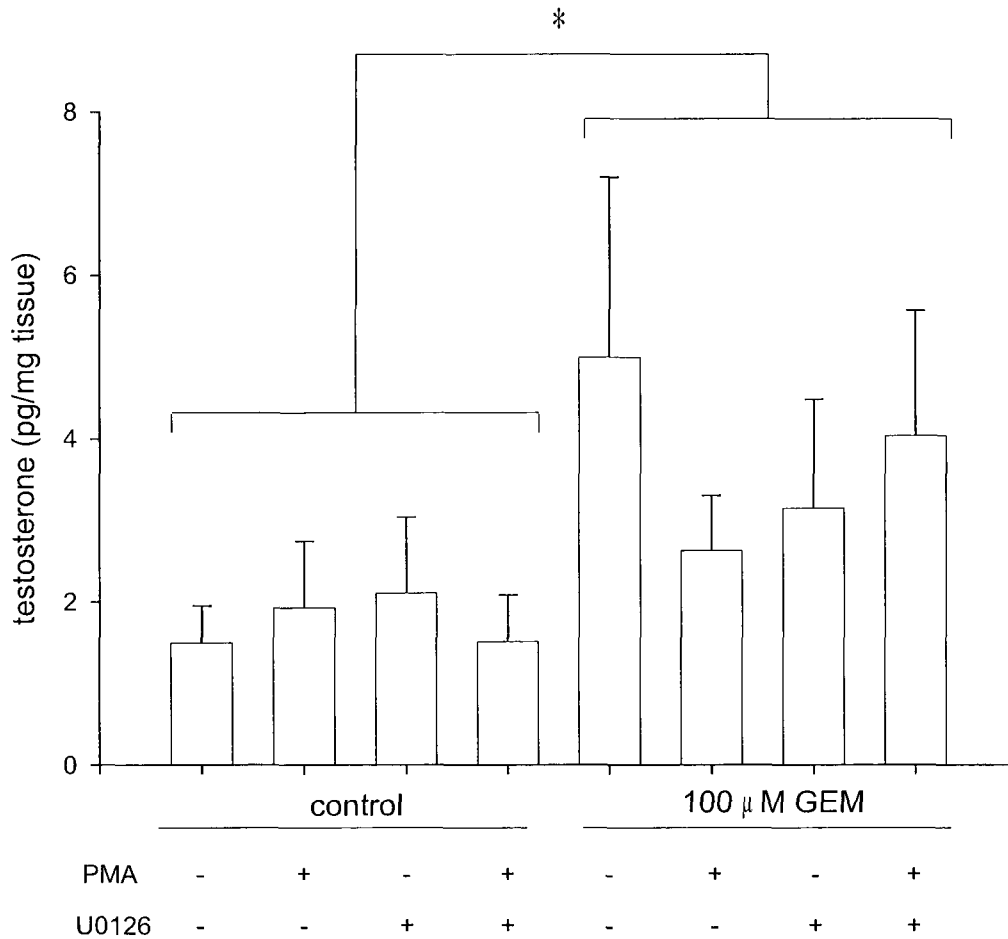


Figure 4.3. Basal testosterone secretion from goldfish testis fragments treated with gemfibrozil (GEM; 100 μ M) for 18 h followed by 6 h treatment with GEM, phorbol 12-myristate 13-acetate (PMA; 500 nM) and U0126 (10 μ M). U0126 treatment applied 1 h prior to hCG and PMA (mean \pm SEM, n = 4, 2-way ANOVA on square-root transformed data with GEM [p = 0.038] and PMA/U0126 combination [p = 0.472] as factors, * p = 0.038).

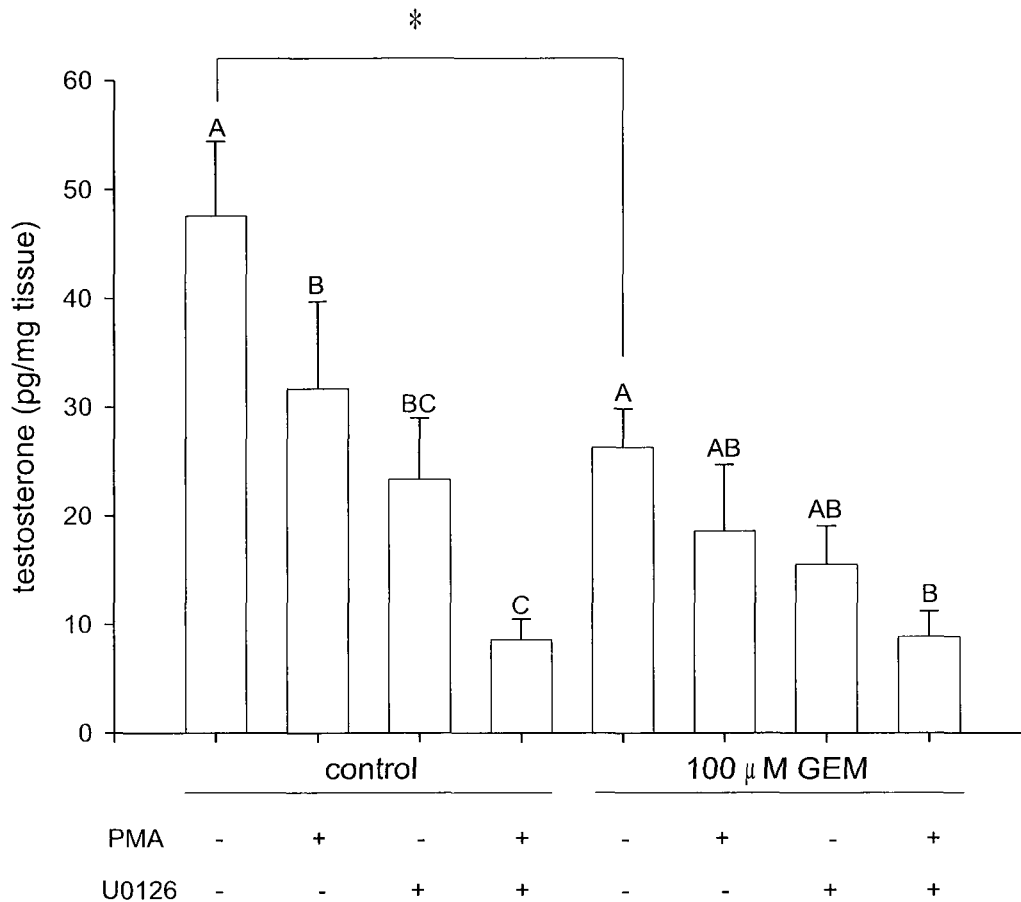


Figure 4.4. hCG-stimulated (1 IU/mL) testosterone secretion from goldfish testis fragments treated with gemfibrozil (GEM; 100 μ M) for 18 h followed by 6 h treatment with hCG, GEM, phorbol 12-myristate 13-acetate (PMA; 500 nM) and U0126 (10 μ M). U0126 treatment applied 1 h prior to hCG and PMA. Different letters indicate statistical significance within levels of GEM (mean \pm SEM, n = 4, 2-way ANOVA with GEM [$p = 0.009$] and PMA/U0126 combination [$p < 0.001$] as factors, * $p < 0.05$).

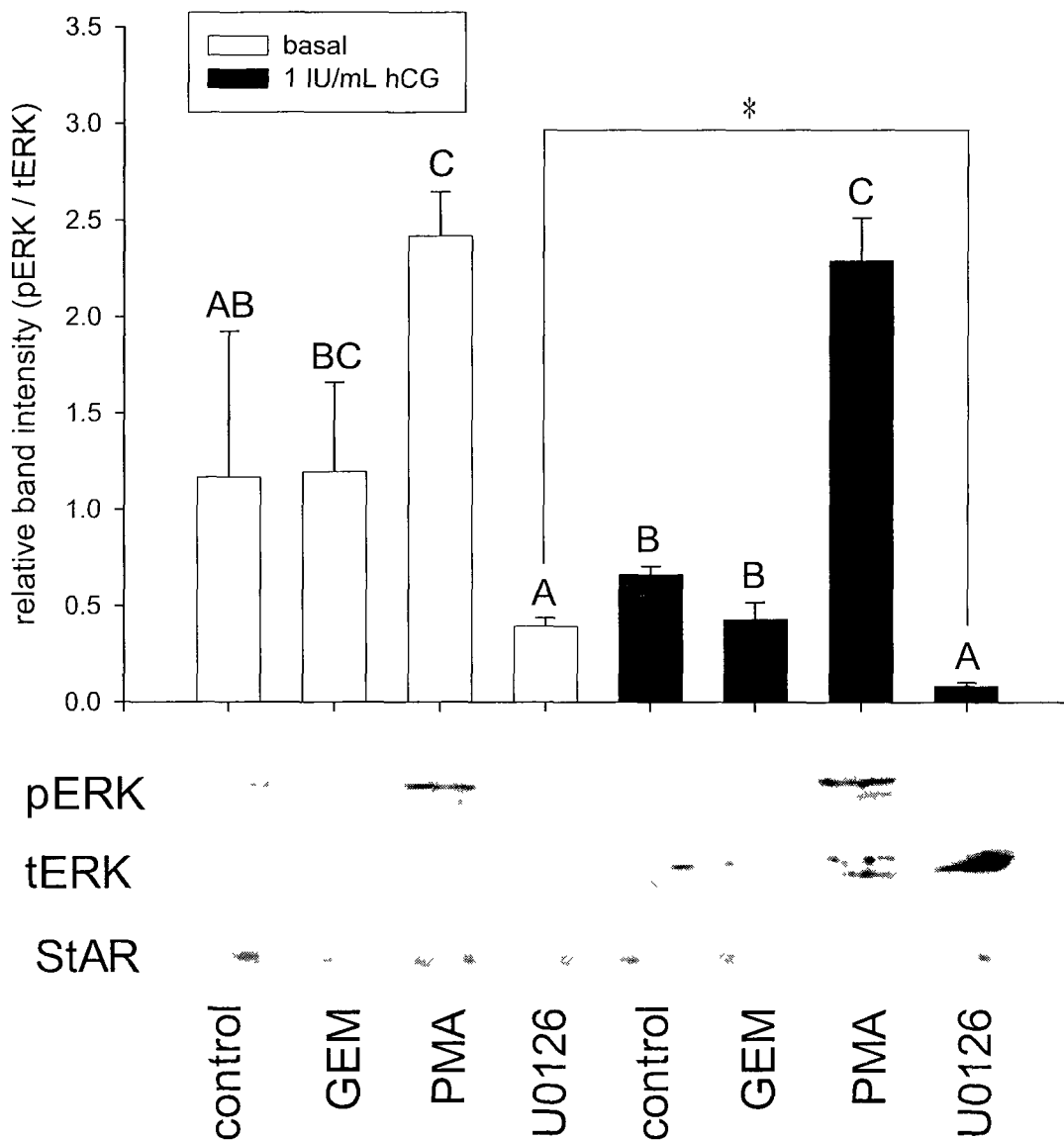


Figure 4.5. Phospho-ERK 1/2 and StAR western blot of goldfish testis fragments treated 18 h with GEM (100 μ M) followed by 6 h hCG (1 IU/mL), GEM, phorbol 12-myristate 13-acetate (PMA; 500 nM), and U0126 (10 μ M) treatment. U0126 treatment applied 1 h prior to hCG and PMA. Representative western blots shown. Different letters indicate statistical significance ($p < 0.05$) within levels of hCG (mean \pm SEM, $n = 3$, 2-way ANOVA on log-transformed data with hCG [$p = 0.007$] and treatment [$p < 0.001$] as factors, * $p < 0.05$ hCG within treatment).

fragments (Fig. 4.5). Treatment with GEM did not alter levels of P-ERK 1/2, and no significant changes in StAR protein levels were observed. Basal (Fig. 4.6) and hCG-stimulated (Fig. 4.7) StAR protein transcript abundance was not affected by treatment with GEM, PMA, or U0126. Testosterone synthesis in goldfish testis fragments was significantly inhibited by U0126 (10 μ M) when added 1 h prior to stimulation by either hCG (1 IU/mL) or forskolin (1 μ M) (Fig. 4.8).

4.3.3 Effects of hCG, GEM, and PKC on phospho-ERK 1/2 in subcellular fractions

Phosphorylated ERK 1/2 was not significantly affected by treatment in cytosolic extracts from goldfish testis fragments. A non-significant decrease in P-ERK 1/2 was observed with U0126 (10 μ M) in hCG- and forskolin-treated fragments (82.2% and 91.7% decreases, respectively; Fig. 4.9). In mitochondrial fractions, hCG (1 IU/mL) treatment significantly increased phospho-ERK 1/2 as compared to non-hCG stimulated testis fragments (Fig. 4.10). Levels of phospho-ERK 1/2 in testis fragments treated with PMA (500 nM) alone, hCG + U0126 (10 μ M) and hCG + GEM (100 μ M) were not different from the basal treatment group but were significantly reduced as compared to testis fragments stimulated with hCG (Fig. 4.10). U0126 (10 μ M) also significantly reduced phospho-ERK 1/2 levels in testis fragments treated with forskolin (1 μ M). The amount of StAR protein detected in mitochondrial fractions was not statistically different among any of the treatment groups (Fig. 4.11).

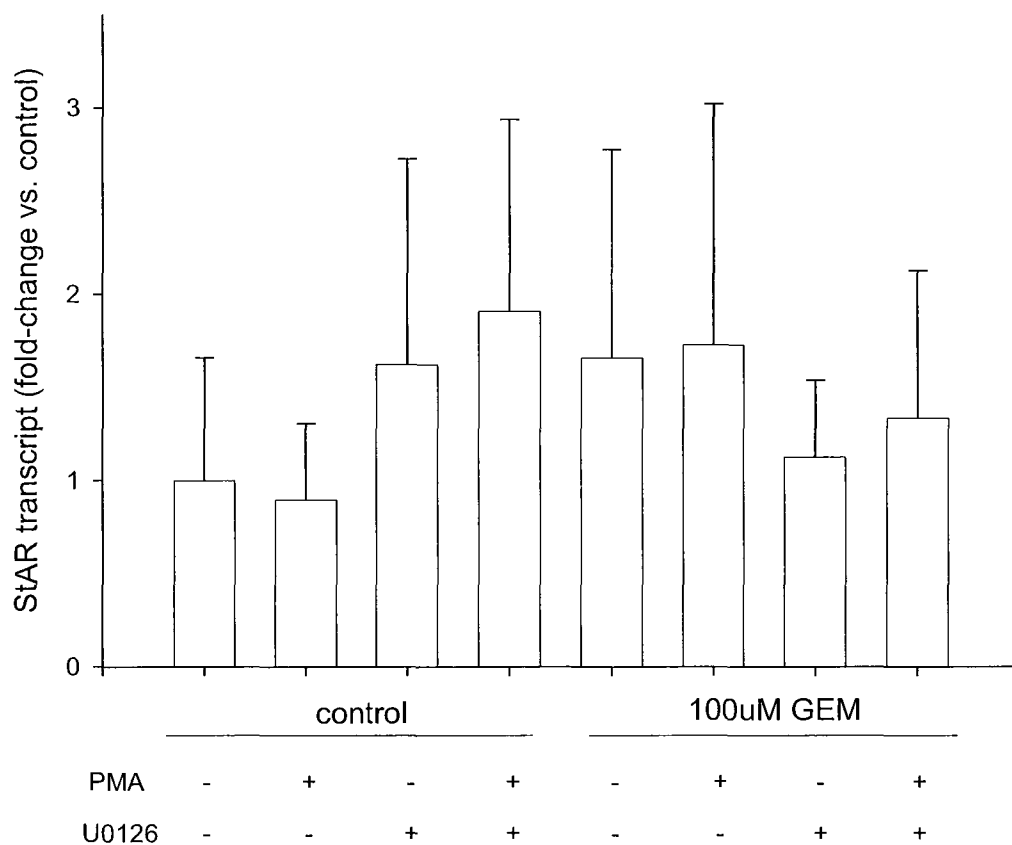


Figure 4.6. Basal StAR protein transcript abundance in goldfish testis fragments treated with gemfibrozil (GEM; 100 μ M) for 18 h followed by 6 h treatment with GEM, phorbol 12-myristate 13-acetate (PMA; 500 nM) and U0126 (10 μ M) (mean \pm SEM, n = 3, control = elongation factor 1 α , 2-way ANOVA on log-transformed data with GEM [p = 0.786] and PMA/U0126 combination [p = 0.911] as factors).

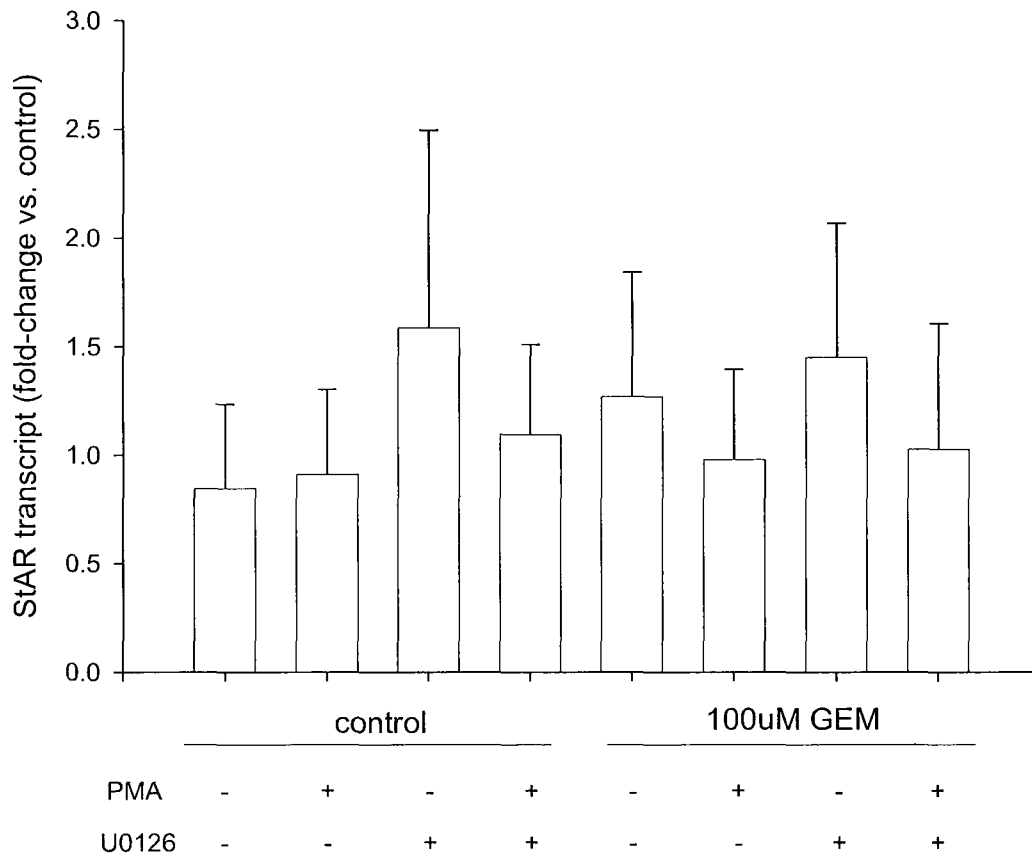


Figure 4.7. hCG-stimulated (1 IU/mL) StAR protein transcript abundance in goldfish testis fragments treated with gemfibrozil (GEM; 100 μ M) for 18 h followed by 6 h treatment with hCG, GEM, phorbol 12-myristate 13-acetate (PMA; 500 nM) and U0126 (10 μ M). U0126 treatment applied 1 h prior to hCG and PMA (mean \pm SEM, n = 3, control = elongation factor 1 α , 2-way ANOVA on log-transformed data with GEM [p = 0.880] and PMA/U0126 combination [p = 0.848] as factors).

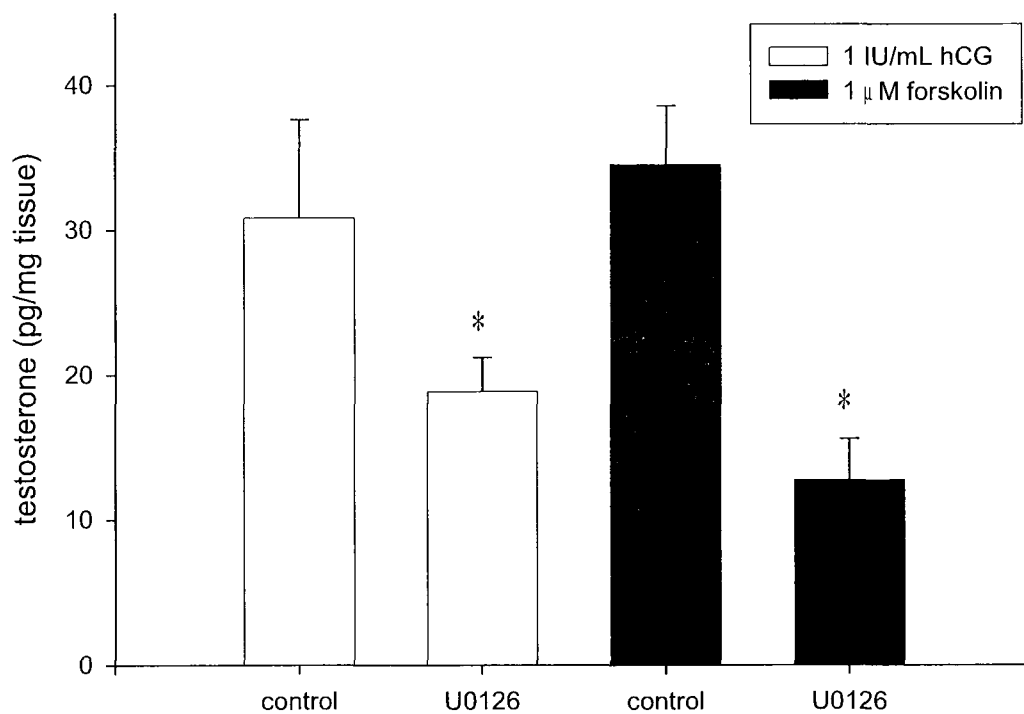


Figure 4.8. 6 h hCG (1 IU/mL)- and forskolin (1 μM)-stimulated testosterone secretion from goldfish testis fragments treated with U0126 (10 μM) for 1 h prior to stimulation (mean ± SEM, n = 6, * p < 0.05 versus respective control).

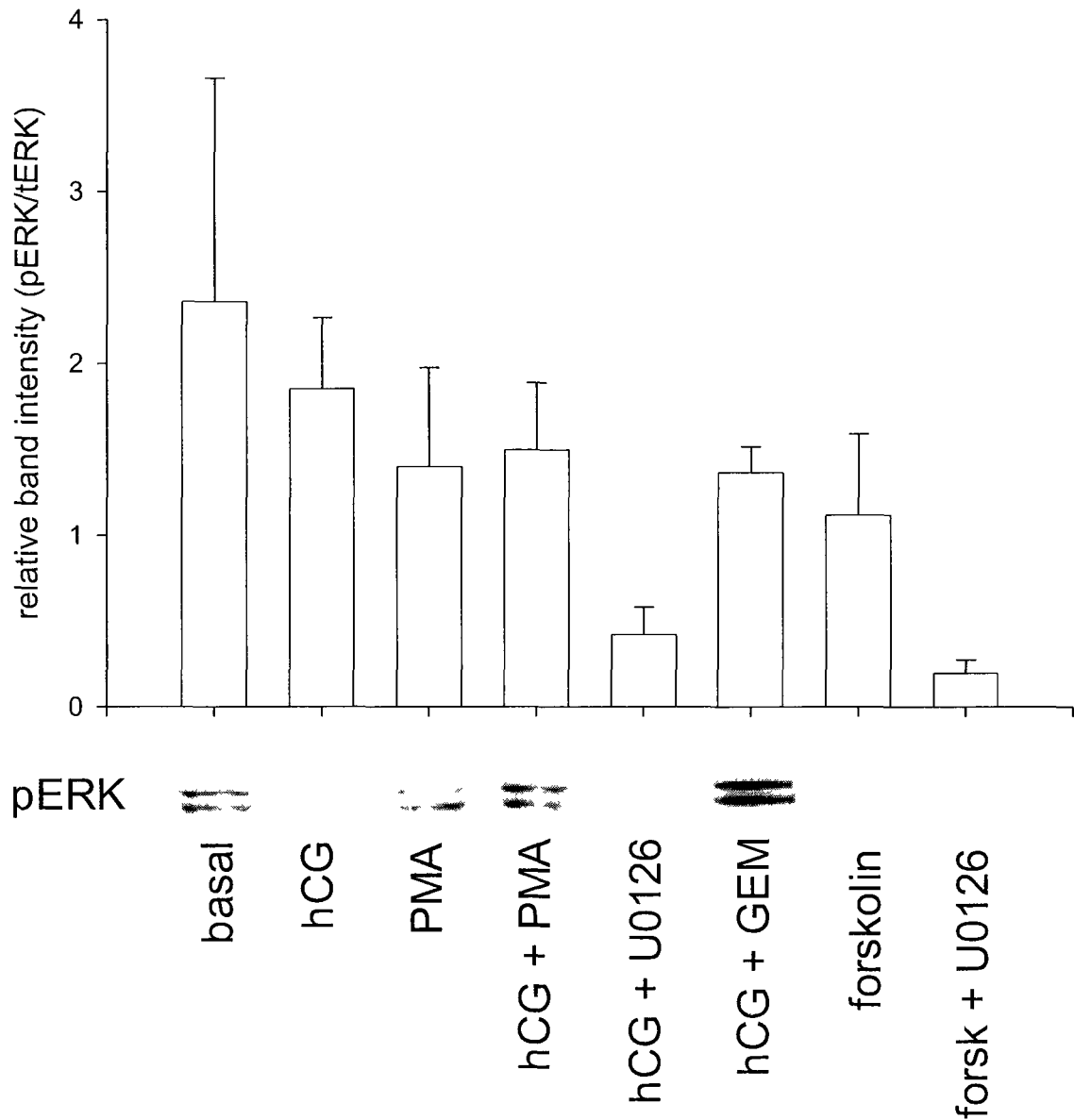


Figure 4.9. Phospho-ERK 1/2 in cytosolic fractions of goldfish testis fragments treated 18 h with GEM (100 μ M) followed by 2 h treatment with hCG (1 IU/mL), GEM, phorbol 12-myristate 13-acetate (PMA; 500 nM), forskolin (1 μ M) and U0126 (10 μ M). U0126 treatment applied 1 h prior to hCG, PMA or forskolin. Representative western blot shown significance (mean \pm SEM, $n = 3$, $p < 0.05$).

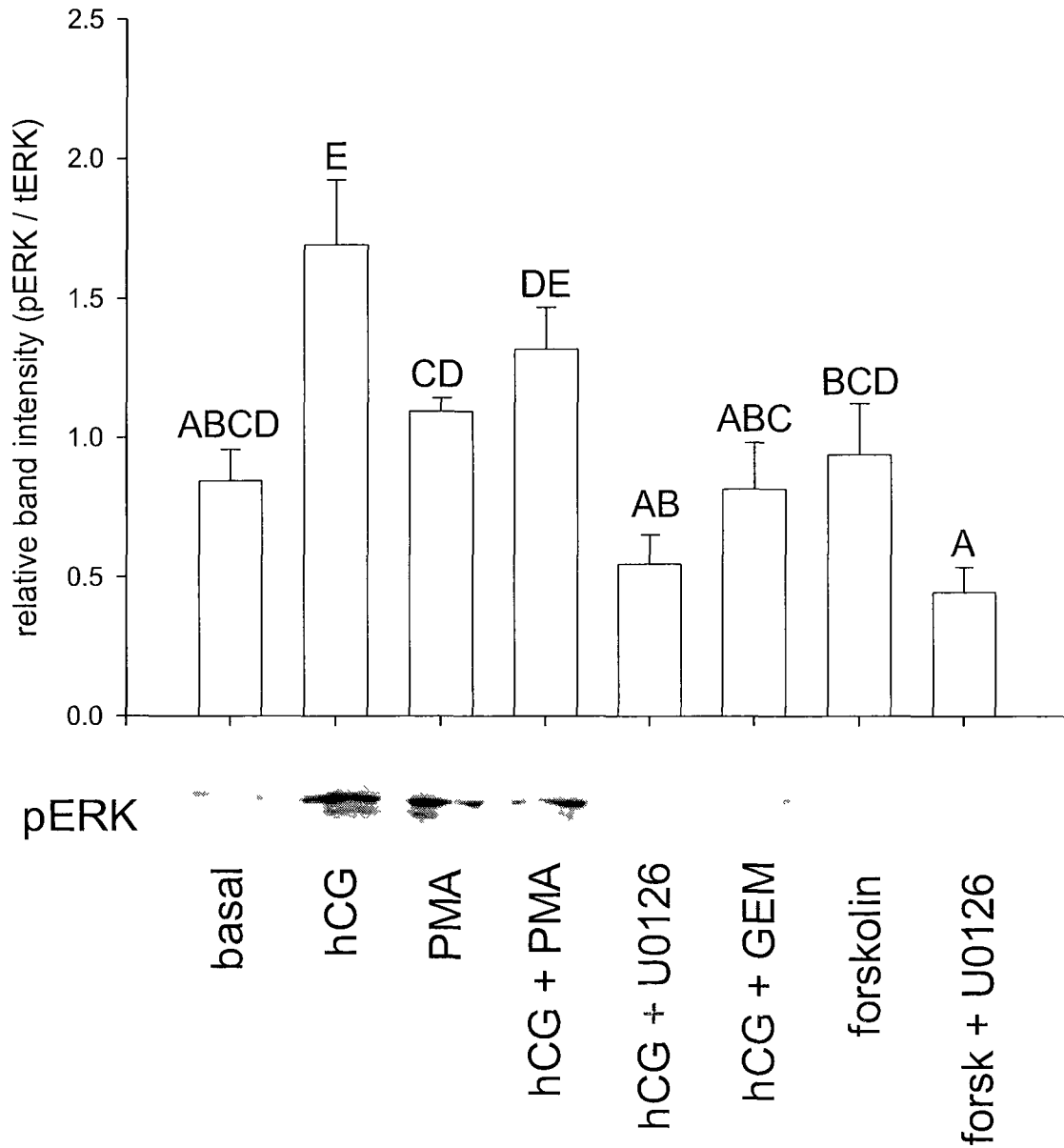


Figure 4.10. Phospho-ERK 1/2 in mitochondrial fractions of goldfish testis fragments treated 18 h with GEM (100 μ M) followed by 2 h treatment with hCG (1 IU/mL), GEM, phorbol 12-myristate 13-acetate (PMA; 500 nM), forskolin (1 μ M) and U0126 (10 μ M). U0126 treatment applied 1 h prior to hCG, PMA or forskolin. Representative western blot shown. Different letters indicate statistical significance (mean \pm SEM, n = 3, p < 0.05).

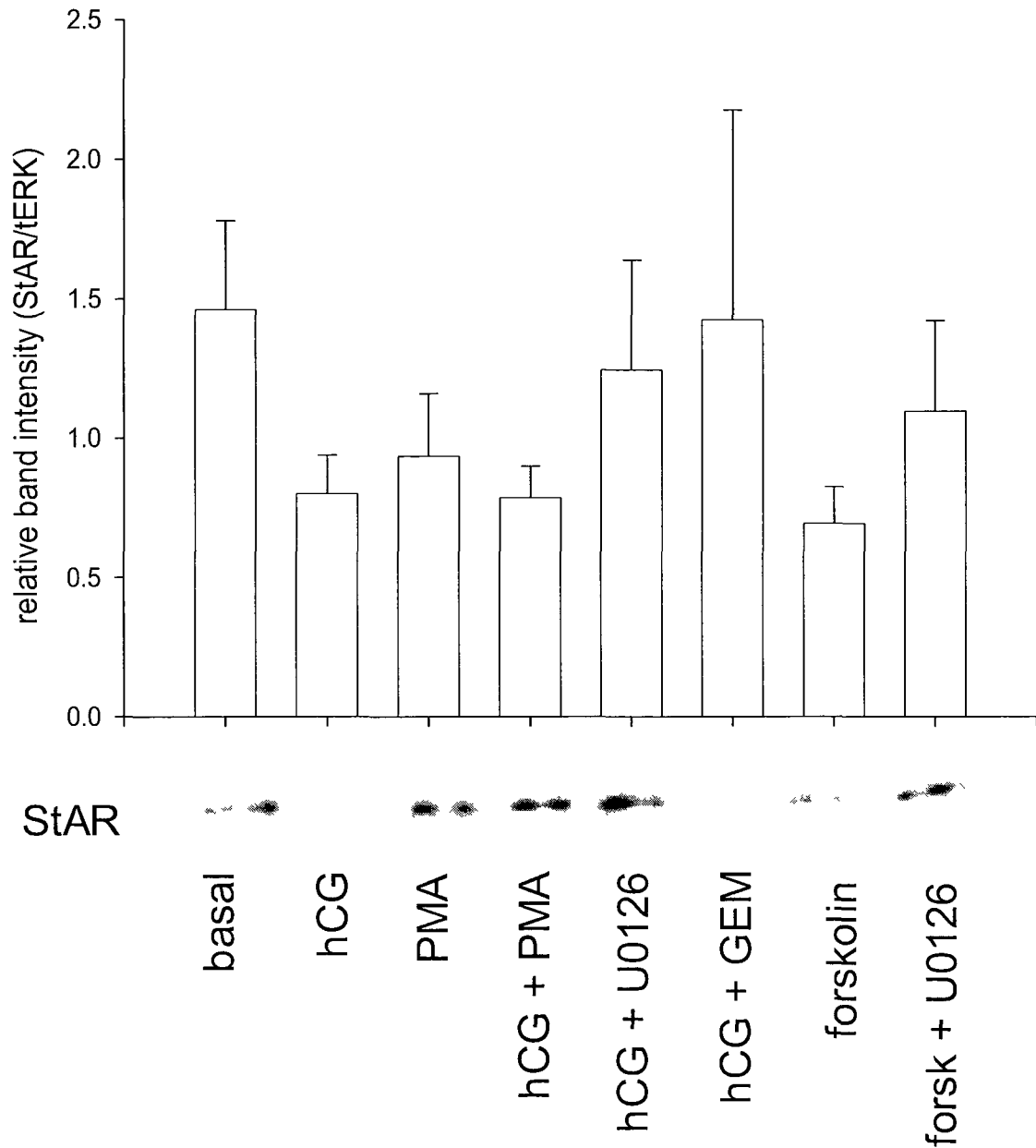


Figure 4.11. StAR protein in mitochondrial fractions of goldfish testis fragments treated 18 h with GEM (100 μ M) followed by 2 h treatment with hCG (1 IU/mL), GEM, phorbol 12-myristate 13-acetate (PMA; 500 nM), forskolin (1 μ M) and U0126 (10 μ M). U0126 treatment applied 1 h prior to hCG, PMA or forskolin. Representative western blot shown. Different letters indicate statistical significance (mean \pm SEM, n = 3).

4.4 DISCUSSION

The current study demonstrated that P-ERK 1/2 is required for maximal hCG-stimulated steroidogenesis in goldfish testis fragments. The inhibitory effect of GEM on testosterone production is not dependent upon the PKC pathway. In addition, GEM treatment in the presence of hCG reduced levels of mitochondrial P-ERK 1/2 in goldfish testis fragments. Although the function of mitochondrial P-ERK 1/2 in the goldfish testis is unknown it may be involved in the activation of StAR protein as has been demonstrated in mouse Leydig cells (Poderoso et al., 2008). Such a decline in P-ERK 1/2 could result in reduced activation of StAR protein and mitochondrial cholesterol transport.

Stimulation of the PKC pathway with PMA inhibited hCG-stimulated steroidogenesis in goldfish testis fragments and this is in agreement with other studies (Wade and Van Der Kraak, 1991). Treatment with PMA or BIS did not influence the effect of GEM on hCG-stimulated steroidogenesis, suggesting that the PKC pathway is not involved in the testis response to GEM. Stimulation of PKC increased P-ERK 1/2 in goldfish testis fragments but this is likely not the mechanism by which it inhibits steroidogenesis as P-ERK 1/2 was required for hCG-stimulated steroidogenesis. Other studies on goldfish testis demonstrated that PKC activation increased the release of polyunsaturated fatty acids (eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) that impair steroidogenesis by inhibiting the production of prostaglandins and reducing hCG-stimulated cAMP production (Wade et al., 1994; Wade et al., 1994). These responses may involve PPAR activation by

EPA or DHA, both of which activate fish PPARs in transactivation assays (Leaver et al., 2005; Kondo et al., 2007).

Treatment with the MAPK inhibitor U0126 decreased testosterone synthesis by approximately 50%, suggesting that P-ERK 1/2 is required for hCG-stimulated steroidogenesis in goldfish testis fragments. Although not previously investigated in fish testes, this finding is in agreement with studies in Atlantic croaker and common carp granulosa and thecal cells (Benninghoff and Thomas, 2006; Paul et al., 2010) but not with studies in isolated brown trout ovarian thecal layers (Méndez et al., 2005). Phosphorylated ERK 1/2 is also required for hormone-stimulated steroidogenesis in mammalian Leydig cells (Martinelle et al., 2004; Martinat et al., 2005).

The function of ERK 1/2 in steroidogenesis is complex and in some cases may also have an inhibitory component. Blocking the activation of ERK1/2 in MA-10 mouse Leydig cells decreased PKC-mediated increases in StAR protein and increased PKA-mediated increases in StAR protein and its phosphorylation but with decreases in steroid production (Manna et al., 2007). In human granulosa cells ERK 1/2 inhibition enhances gonadotropin-stimulated steroid production and StAR transcript levels (Seger et al., 2001; Tajima et al., 2003). Despite increasing LH-induced StAR protein, activation of ERK 1/2 may inhibit steroidogenesis by affecting downstream steroidogenic enzymes in mouse Leydig cells (Martinat et al., 2005; Manna et al., 2006; Manna et al., 2007) and bovine thecal cells (Tajima et al., 2005). PKA activation involves a specific mitochondrial ERK1/2 activation in Leydig cells

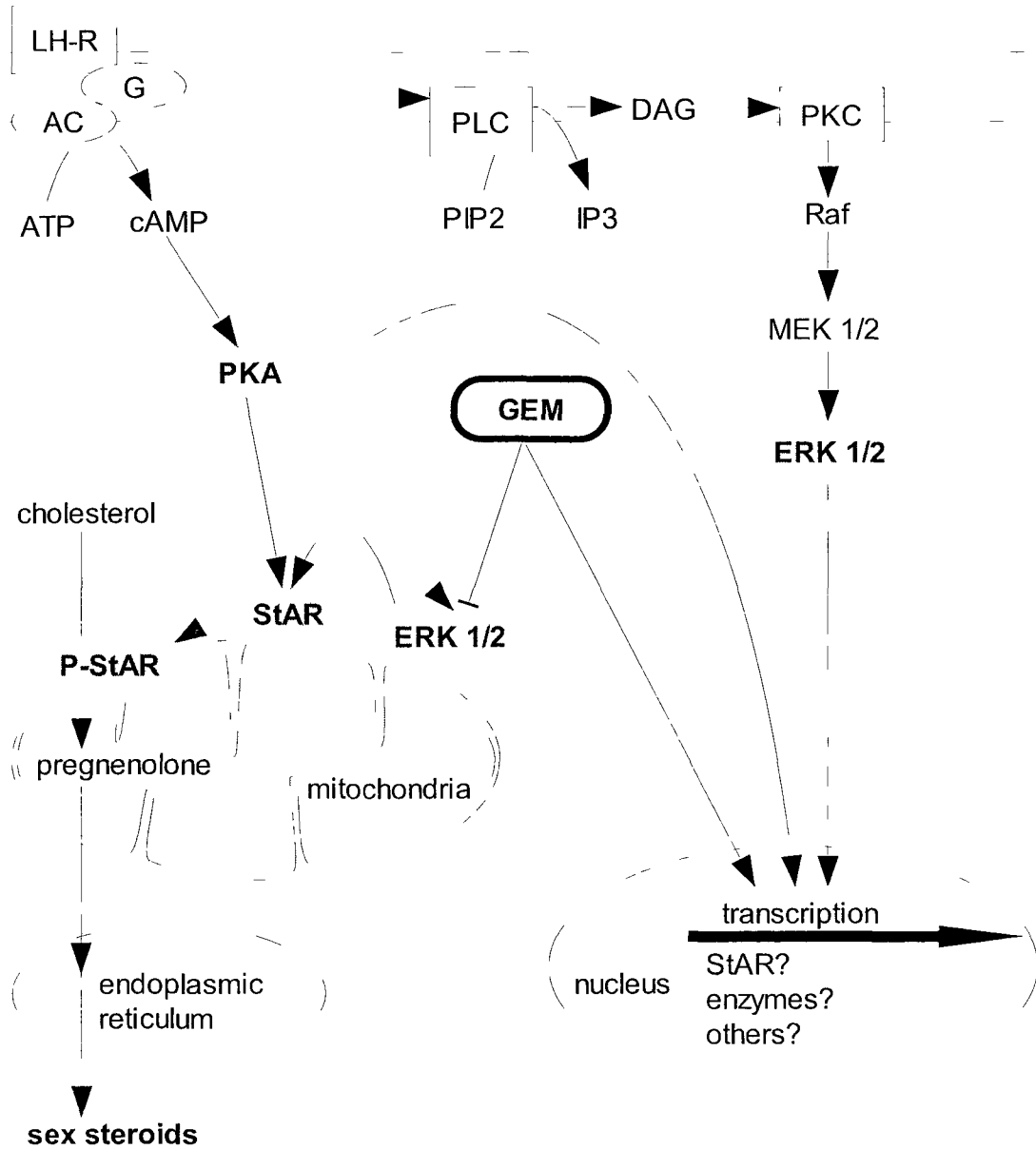


Figure 4.12. Proposed model of the involvement of gemfibrozil (GEM) in the signaling pathways controlling hormone-stimulated steroidogenesis in the goldfish testis. Hormonal stimulation of the luteinizing hormone receptor (LH-R) leads to activation of protein kinase A (PKA), mitochondrial extracellular signal-regulated kinase 1/2 (ERK 1/2), and steroidogenic acute regulatory (StAR) protein. This stimulates mitochondrial cholesterol transport and synthesis of sex steroids. GEM treatment decreases mitochondrial P-ERK 1/2 thereby inhibiting testosterone synthesis. Phospholipase C (PLC) induced by LH-R may stimulate protein kinase C (PKC) activity leading to ERK 1/2 activation which does not participate in acute steroidogenesis but may be involved in the control of gene transcription.

that is required for StAR phosphorylation and cholesterol transport, while the PKC pathway leads instead to increased cytosolic and nuclear P-ERK 1/2 (Poderoso et al., 2008). This suggests that the stimulating pathway (PKA or PKC) and the cellular localization of P-ERK 1/2 determines the effect on the steroidogenic pathway. The acute regulation of steroidogenesis may be stimulated by PKA and ERK 1/2, while PKC leads to increased nuclear P-ERK 1/2 and effects on the expression of steroidogenic genes. The expression of StAR in goldfish testis fragments was not influenced by either PKC activation or reductions in P-ERK 1/2. The absence of change in the rate of transcription of the StAR protein gene suggests that the results of the current study reflect changes in the regulation of acute steroidogenesis by these treatments.

Forskolin-stimulated testosterone synthesis in goldfish testis fragments was also inhibited by U0126 suggesting that LH receptor activation of PKC, through increased phospholipase C activity, is not required for this effect. Treatment of MA-10 Leydig cells with cAMP analogues increased P-ERK 1/2, demonstrating PKA-dependent ERK 1/2 phosphorylation (Manna et al., 2007; Poderoso et al., 2008). In the goldfish, total tissue P-ERK 1/2 was dramatically increased by PKC stimulation but not by hCG treatment. In contrast, mitochondrial fraction P-ERK 1/2 was increased by hCG but was unaffected by PMA treatment, suggesting that mitochondrial ERK 1/2 phosphorylation is regulated by the PKA pathway in the goldfish testis. In cytosolic extracts P-ERK 1/2 was not significantly affected by PMA or hCG treatment. This lack of effect of PKC may be explained by increased nuclear

P-ERK 1/2. Increases in nuclear localization of P-ERK 1/2 were observed in MA-10 Leydig cells treated with epidermal growth factor, a known stimulator of PKC (Poderoso et al., 2008). As a result of using tissue fragments rather than cell cultures, the fractionation methods used in the current study did not allow the recovery of highly enriched nuclear fractions. In mouse Leydig cells, PKA-dependent cholesterol transfer is dependent upon StAR protein phosphorylation by P-ERK 1/2 in the presence of cholesterol (Poderoso et al., 2008; Poderoso et al., 2009). Mitochondrial P-ERK 1/2 may be required for mitochondrial cholesterol transport in the goldfish testis for the phosphorylation of StAR protein or perhaps another protein required cholesterol transport. It is not possible to confirm this phosphorylation of StAR protein because antibodies specific for the phosphorylated form in fish are not available.

Treatment of goldfish testis fragments with GEM reduced hCG-stimulated testosterone synthesis and mitochondrial P-ERK 1/2. This decrease in mitochondrial P-ERK 1/2 may cause a reduction in mitochondrial cholesterol transport and represent the mechanism by which GEM impairs steroidogenesis. A reduction in P-ERK 1/2 with GEM treatment has not previously been reported. Decreased P-ERK 1/2 with PPAR ligand treatment was previously reported in the hearts of mice treated with the fibrate drug fenofibrate (Duhaney et al., 2007) and human microvascular endothelial cells treated with rosiglitazone (Lombardi et al., 2008). Treatment with the fibrate agonist WY-14,643 results in enhanced ERK 1/2 activation in C2C12 myoblasts (Nakai et al., 2008), HepG2 cells (Banfi et al., 2003), primary mouse

hepatocytes (Mounho and Thrall, 1999) and immortalized mouse liver cells (Rokos and Ledwith, 1997). Treatment with dibutyl phthalate and di(2-ethylhexyl) phthalate, two phthalates with demonstrated PPAR-binding activities, increased P-ERK 1/2 in the testis of rats (Ryu et al., 2007; Ryu et al., 2008). Although the inhibition of P-ERK 1/2 in the testis of fish with fibrate treatment has not been previously reported, there is sufficient evidence to suggest a direct cross-talk between these pathways. In the goldfish testis GEM may inhibit the PKA induction of ERK 1/2 activation at the mitochondria. This inhibition could involve a PPAR α -dependent effect of GEM directly on ERK 1/2 at the mitochondria or on the upstream PKA pathway required for ERK 1/2 phosphorylation.

In the current study, cytosolic and mitochondrial fractions were prepared by differential centrifugation and the purity of these cellular fractions was not assessed. The assessment of subcellular fraction purity is typically not reported in other studies where differential centrifugation is employed for the purpose of isolating mitochondrial protein for western blots. These studies include those using methods identical to the current study (Clark et al., 1994; Manna et al., 1999; Manna et al., 2002) and also those where commercial kits involving similar procedures are used (Lyssimachou and Arukwe, 2007). In a study in which mitochondrial extract purity following differential centrifugation of mouse MA-10 Leydig cell lysates was assessed, estimates of extract purity were >90% for all samples (Poderoso et al., 2008). Detection of StAR protein in western blots using cytosolic fractions from goldfish testis fragments cultured *in vitro* was not successful due to very weak banding (data

not shown). In contrast, StAR protein western blots using mitochondrial fractions were much clearer. Although not a quantitative measure of extract purity, this observation is consistent with the expected localization of StAR protein in the mitochondria.

As in several other species the ERK 1/2 pathway is important for hCG-stimulated steroidogenesis in the goldfish testis. Activated mitochondrial ERK 1/2 may be required for the PKA-dependent phosphorylation of StAR protein, which is an important part of the rate-limiting step of cholesterol transport during steroidogenesis. Treatment with GEM inhibited hormone-stimulated testosterone synthesis in goldfish testis fragments and this inhibition was associated with a decrease in P-ERK 1/2 at the mitochondria. A reduction in mitochondrial P-ERK 1/2 presents a novel mechanism for GEM disruption of the steroidogenic pathway.

Chapter 5: General Discussion

The main objective of this work was to investigate the effects of PPAR ligands on the hypothalamic-pituitary-gonadal (HPG) axis *in vitro* in the male goldfish that could lead to the decreased plasma testosterone concentrations previously reported by Mimeault et al. (2005) in goldfish exposed to gemfibrozil (GEM) *in vivo*. In mammals, fibrate pharmaceuticals function through a PPAR-nuclear receptor mechanism, so the mechanisms by which these effects originated was also of interest to further understand the functions of PPARs in the goldfish. To achieve these objectives, the effects of GEM and other known PPAR inducing chemicals were studied *in vitro* on two components of the HPG axis in the male goldfish, the pituitary gland and the testis. The endocrine function of dispersed pituitary cells or testis fragments from the goldfish was assessed following incubation with GEM. Hormone-stimulated testosterone synthesis in the goldfish testes was inhibited by GEM treatment suggesting that decreased testosterone concentrations in goldfish exposed to waterborne GEM (Mimeault et al., 2005) may result from a direct effect of this drug on the testis. The inhibition of steroidogenesis in the goldfish testis was attributed to a decrease in mitochondrial cholesterol transport in the absence of changes in StAR protein transcript or total protein abundance. Treatment with GEM was also associated with decreases in P-ERK 1/2 at the mitochondria, highlighting the importance of P-ERK 1/2 in hCG-stimulated steroidogenesis in the goldfish and suggesting that P-ERK 1/2 is required for mitochondrial cholesterol transport and is

potentially involved in StAR protein phosphorylation.

5.1 Hypotheses tested

Hypothesis 1: Fibrates affect the goldfish pituitary by decreasing the secretion of hormones important for the stimulation of testicular steroidogenesis, leading to a decrease in sex steroid synthesis and secretion, and circulating concentrations of testosterone.

Prediction 1: Fibrate treatment of dispersed pituitary cells will suppress either basal or stimulated LH or GH secretion from goldfish pituitary cells *in vitro*.

The function of isolated goldfish pituitary cells was assessed through measurements of LH and GH secretion when stimulated by sGnRH and in the non-stimulated state (Chapter 2). The results of the pituitary cell culture experiments were generally not supportive of the hypothesis that GEM had a direct effect on the pituitary gland. Treatment with GEM did not affect pituitary hormone secretion in a manner that would explain decreased plasma testosterone concentrations *in vivo*. Rosiglitazone, a mammalian PPAR γ antagonist, decreased GH and LH secretion in the pituitary cultures which is consistent with studies in rodent and human pituitary cells (Heaney, 2003; Heaney et al., 2003; Ambrosi et al., 2004; Bogazzi et al., 2004). However, conclusions regarding the role of PPAR γ in fishes are difficult as this receptor is considerably divergent from that in mammals in structure and possibly function (Leaver et al., 2005; Kondo et al., 2007). Data supporting the alternative hypothesis that GEM inhibits the steroidogenic pathway in the testis resulted in a

switch in the focus of further experiments to this tissue.

Hypothesis 2: Fibrates directly impair the steroidogenic pathway in the goldfish testis leading to decreases in sex steroid synthesis and secretion, and circulating concentrations of testosterone.

Prediction 2: Testosterone secretion from goldfish testis fragments cultured *in vitro* will be significantly impaired by fibrate treatment. This impairment may include basal rates of hormone secretion, but will be most evident during hormone-stimulated steroidogenesis (i.e., hCG treatment).

Basal and stimulated testosterone synthesis in goldfish testis fragments was used as an endpoint for the study of the effects of GEM on steroidogenesis (Chapter 3). In goldfish testis fragments incubated overnight with GEM, hCG-stimulated testosterone synthesis was significantly inhibited by 40-50%. This decline is similar to that observed during a 14 day *in vivo* exposure of male goldfish to environmental concentrations of GEM (Mimeault et al., 2005) and is evidence that the effect of GEM may be directly at the testis. The mechanism by which GEM impairs steroidogenesis in the goldfish testis appears to be through a reduction in mitochondrial cholesterol transport. Testosterone synthesis was inhibited in goldfish testis fragments pre-treated with GEM in the presence of hCG and forskolin (an adenylyl cyclase activator). This inhibition of steroidogenesis was restored with 25-hydroxycholesterol or pregnenolone treatment. The synthesis of cAMP was not impaired by GEM treatment, and PBR transcript and StAR protein transcript and total protein remained unchanged. However, mitochondrial P-ERK 1/2 levels were

significantly reduced (Chapter 4), possibly leading to decreased phosphorylation and activation of StAR protein (P-StAR could not be estimated). Inhibition of steroidogenesis was observed with GEM and fenofibrate (FEN), and in some experiments with the mammalian PPAR α agonist WY-14,643. Other fibrates including bezafibrate (BEZ) and clofibrate (CLO) were without effect. A somewhat different complement of fibrates (CLO and FEN) inhibited ACTH-stimulated cortisol synthesis in rainbow trout head kidney cells (Mimeault et al., in preparation; discussed earlier). Few studies have directly investigated fibrate binding to fish PPARs but several fibrates have been confirmed as ligands for fish PPARs in transactivation experiments. In rainbow trout gill cells transfected with a construct containing the rat thiolase B peroxisome proliferator response element GEM, BEZ and WY-14,543 increased reporter gene transcriptional activity (Liu et al., 2005). In all other fish species studied to date, WY-14,643 also increased transcriptional activity in similar assays (Boukouvala et al., 2004; Leaver et al., 2005; Liu et al., 2005; Kondo et al., 2007).

5.2 Extracellular signal-regulated kinase (ERK) 1/2 and steroidogenesis in the goldfish

An important result of these studies was the positive role for P-ERK 1/2 in hCG-stimulated testosterone synthesis in goldfish testis fragments (Chapter 4). This finding is consistent with recent studies in mouse MA-10 Leydig cells in which the hCG/cAMP/PKA pathway increases mitochondrial P-ERK 1/2 content, P-ERK 1/2

participates directly in the phosphorylation of StAR protein at the mitochondria, and P-ERK 1/2 is required for maximal stimulation of steroid synthesis (Martinelle et al., 2004; Manna et al., 2007; Poderoso et al., 2008; Poderoso et al., 2009). Previous studies of the influence of P-ERK 1/2 on fish ovarian tissue steroidogenesis are mixed, with P-ERK 1/2 being reported as both stimulatory and inhibitory (Méndez et al., 2005; Benninghoff and Thomas, 2006; Paul et al., 2010). Similar apparently conflicting reports exist in rodent models where P-ERK 1/2 activity is associated with decreased StAR protein expression and steroid production (Seger et al., 2001; Tajima et al., 2003; Martinat et al., 2005; Manna et al., 2006; Renlund et al., 2006; Manna et al., 2007). This may reflect different functions for P-ERK 1/2 localized to different parts of the cell. For example, P-ERK 1/2 may be stimulatory in acute steroidogenesis (mitochondrial StAR phosphorylation) but inhibitory in the chronic regulation of steroidogenesis (down-regulation of StAR protein expression). Many intracellular signaling pathways may increase ERK 1/2 phosphorylation, however each may differentially regulate mitochondrial and nuclear P-ERK 1/2 resulting in different cellular responses. For example, in mouse MA-10 Leydig cells the PKA pathway increases mitochondrial P-ERK 1/2 and acute steroidogenesis while EGF treatment only increases cytosolic and nuclear P-ERK 1/2 (Poderoso et al., 2008).

5.3 Effects of phthalates and perfluorinated alkyl acids on goldfish testis

Several phthalates (PHs) and perfluorinated alkyl acids (PFAAs) tested in this study inhibit hCG-stimulated steroidogenesis in goldfish testis fragments at

concentrations similar to those observed for fibrates. The objective of these experiments was to compare these results with the effects of GEM and FEN. Phthalates and PFAAs function as PPAR ligands and have diverse effects on reproductive development, function and success in rodents (Lovekamp and Davis, 2001; Corton and Lapinskas, 2005; Ryu et al., 2007; Shi et al., 2007; Boberg et al., 2008; Shi et al., 2009). It is not known whether PHs or PFAAs are ligands for PPARs in fish; however PH and PFAA exposure is also associated with disrupted reproductive function in fish (Kim et al., 2002; Oakes et al., 2004; Norman et al., 2007; Carnevali et al., 2010). The current studies suggest that many of the PPAR ligands detected in the aquatic environment have the potential to inhibit steroid synthesis in wild fishes. Further research should investigate the mechanisms behind PH and PFAA inhibition of steroidogenesis in the goldfish to determine whether they are similar to those responsible for fibrate inhibition.

5.4 Are the effects of PPAR-agonists dependent upon PPARs?

It is not clear whether the responses to GEM, FEN, PHs or PFAAs in goldfish testis fragments are a result of direct PPAR activation or if the responses are PPAR-independent. Several PPAR-independent effects of PPAR ligands have been documented, particularly for PPAR γ ligands (Weng et al., 2006; Scatena et al., 2008), but also for fibrates (Cunard et al., 2002; Pahan et al., 2002; Cunard et al., 2002; Ropero et al., 2009). In the current studies, there is only indirect evidence for a PPAR-dependent mechanism. Fibrates including WY-14,643, BEZ, and GEM,

stimulate known PPAR response elements in PPAR transcriptional assays using fish cells and receptors (Leaver et al., 2005; Liu et al., 2005; Kondo et al., 2007). Transcripts for all 3 PPAR subtypes are detected in goldfish testes (Mimeault et al., 2006; C. Cameron, unpublished results). The decrease in stimulated testosterone synthesis in goldfish testis fragments was observed with GEM and FEN, and in both cases was restored by 25-hydroxycholesterol treatment, suggesting a similar mechanism for both fibrates. The inhibition of testicular steroidogenesis in the goldfish by PHs and PFAAs may also require PPAR α , as these compounds are known PPAR α ligands in rodents. Despite the lack of direct evidence, the simplest explanation for similar effects with a wide variety of compounds with known PPAR α binding ability is through a PPAR-dependent mechanism.

5.5 The effect of GEM on mitochondrial P-ERK 1/2

The effects of GEM on mitochondrial P-ERK 1/2 may suggest an effect that is not dependent on PPRE-binding and transcriptional control. Ligands for PPAR γ are known to both increase (Kim et al., 2009; Yamada et al., 2010) and decrease (Kim et al., 2009; Zang et al., 2009; Park et al., 2009) ERK 1/2 phosphorylation. These effects may be either PPAR-dependent or PPAR-independent depending upon the ligand and the experimental model (Yamada et al., 2010; Luconi et al., 2010). Few studies have investigated the effects of PPAR α ligands on ERK 1/2 phosphorylation, although WY-14,643 treatment is associated with increased P-ERK 1/2 (Banfi et al., 2003; Nakai et al., 2008). In contrast, GEM treatment of goldfish testis fragments

resulted in a decrease in mitochondrial fraction P-ERK 1/2. Decreased P-ERK 1/2 has been observed following treatment with GW501516 (a PPAR β ligand) which suppresses lipopolysaccharide induction of P-ERK 1/2 in differentiated 3T3-L1 adipocytes (Rodríguez-Calvo et al., 2008). The mechanism of the decrease in mitochondrial P-ERK 1/2 by GEM may be indirect without a direct interaction between GEM/PPAR α and ERK 1/2. The effect of GEM may involve the regulation of a protein regulating the MAPK/ERK signaling cascade in the goldfish testis. As an example, GEM inhibition of the MAPK/ERK pathway could involve another protein such as the Raf kinase inhibitor protein (RKIP) which interferes with Raf-1 activation of MEK, a kinase upstream of ERK 1/2 (Yeung et al., 2000; Trakul and Rosner, 2005; Zeng et al., 2008).

5.6 Future research

Future research should investigate the functions of ERK 1/2 and PPARs in testicular steroidogenesis in fish, as well as the potential for PPAR ligands in the environment to disrupt steroidogenesis in wild animals. In the goldfish, P-ERK 1/2 is required for hCG-stimulated steroidogenesis, as it is in rodent models where it is required for StAR protein phosphorylation at the mitochondria (Poderoso et al., 2008; Poderoso et al., 2009). It is unclear whether P-ERK 1/2 participates in the phosphorylation of StAR protein, or of another protein, in the goldfish testis. Very little is known about the activation of StAR protein in fishes and antibodies recognizing the phosphorylated form of this protein in fish are needed. Further

studies are also needed to explore the role of PPARs in the response to GEM and other peroxisome proliferators in goldfish testes in order to determine if the decrease in mitochondrial P-ERK 1/2 in response to GEM is PPAR-dependent or -independent. The involvement of PPARs in the responses to PPAR ligands in the environment will have implications in determining the risks associated with exposure to mixtures of these ligands.

Studies are also needed to confirm a similar mechanism for GEM *in vivo* and whether there is potential for such effects in wild fish. The dose of GEM found to be inhibitory in these *in vitro* studies (100 μM) is considerably higher than those detected in STP effluent (0.006 μM ; Metcalfe et al., 2003b), surface water (0.45 nM; Metcalfe et al., 2003b), and in plasma of goldfish exposed to 1.5 $\mu\text{g/L}$ (0.006 μM) GEM (an environmental concentration) in water for 2 weeks (0.68 μM ; (Mimeault et al., 2005). The various PPAR ligands found in surface water, including fibrates, PHs and PFAAs, are also detected at concentrations well below 100 μM (Fromme et al., 2002; Metcalfe et al., 2003a; Suja et al., 2009; Nakayama et al., 2010). However, accumulation of these PPAR ligands from water or food may occur, as reported for GEM (Mimeault et al., 2005) and PFAAs (Moody et al., 2002; Martin et al., 2004), increasing the effective concentrations of these compounds. In addition to the potential for PPAR ligand accumulation from water at environmental concentrations that appear to be below any NOAEL (no observable adverse effect level), the combination of a variety of compounds with a similar mechanism of action (e.g., PPAR α activation) would result in a much higher effective exposure concentration.

Receptors such as PPARs with multiple ligands (some which likely have yet to be identified) make estimations of acceptable levels of each of these in aquatic environments difficult. Another consideration in determining the effects of PPAR ligand exposure is the comparison of *in vitro* versus *in vivo* effects. Inhibition of steroidogenesis may occur in animals exposed to significantly lower doses for longer periods of time. For example, >10 µM GEM was required for inhibition of steroidogenesis in these *in vitro* studies with a total exposure time of 24 h, while *in vivo* plasma concentrations of 0.68 µM during a 2 week exposure were associated with significant decreases in plasma testosterone concentration (Mimeault et al., 2005).

5.7 Overall significance

The key significant findings of this thesis are summarized below:

(1) GEM inhibits hCG-stimulated steroidogenesis in goldfish testis fragments in vitro but does not affect hormone secretion from dispersed goldfish pituitary cells.

These findings suggest that the decreases in plasma testosterone concentrations in male goldfish exposed to waterborne GEM is the result of an effect on the testis.

(2) GEM treatment of goldfish testis fragments in vitro results in a decreased testosterone response to hCG stimulation that is restored by 25-

hydroxycholesterol.

The step in the steroidogenic pathway in goldfish testis fragments that is affected by GEM appears to be the transport of cholesterol into the mitochondria. This result is not explained by changes in transcript abundance of StAR protein or PBR, or by changes in total StAR protein.

(3) The hCG-stimulated increase in mitochondrial P-ERK 1/2 is inhibited in the presence of GEM.

Although the role of P-ERK 1/2 in mitochondrial cholesterol transport is not clear, this decrease in P-ERK 1/2 may be a mechanism by which GEM inhibits mitochondrial cholesterol transport.

(4) Phosphorylated ERK 1/2 is required for maximal hCG- and forskolin-stimulated steroidogenesis in the goldfish testis.

Treatment of goldfish testis fragments with hCG increases mitochondrial P-ERK 1/2 and this increase in P-ERK 1/2 is required for the acute regulation of steroidogenesis. Mitochondrial ERK 1/2 may participate in the activation of StAR protein.

Appendix: Effects of phthalates and perfluorinated compounds on *in vitro* testicular hCG-stimulated steroidogenesis in the goldfish, *Carassius auratus*

A.1 Introduction

Phthalates (PHs) are plasticizers used in the manufacture of soft plastics to increase their flexibility, most notably in polyvinylchloride plastics, and are detected in the environment (Staples et al., 2000; Fromme et al., 2002). A variety of PHs affect sexual development, circulating steroid concentrations and the steroidogenic pathway in male rodents (Akingbemi et al., 2001; Gazouli et al., 2002; Akingbemi et al., 2004; Borch et al., 2004; Corton and Lapinskas, 2005; Ryu et al., 2007; Ryu et al., 2007; Wang et al., 2007; Boberg et al., 2008). Decreased reproductive performance was observed in zebrafish (*Danio rerio*) exposed to di(2-ethylhexyl) phthalate (DEHP) (Carnevali et al., 2010).

Perfluorinated compounds (perfluoroalkyl acids, PFAAs), used as surfactants, non-stick and waterproof coatings in a variety of products, are also detected in the environment (Lau et al., 2007; Suja et al., 2009; Nakayama et al., 2010). Exposure to PFAAs alters gonadal gene expression patterns, rates of steroidogenesis and circulating steroids in rats (Shi et al., 2007; Shi et al., 2009; Shi et al., 2009). Treatment of fathead minnows (*Pimephales promelas*) with perfluorooctanoic acid (PFOA) resulted in decreased plasma testosterone concentrations in males and females (Oakes et al., 2004).

Fibrate pharmaceuticals including gemfibrozil (GEM) and fenofibrate inhibit *in vitro* hCG-stimulated steroidogenesis in goldfish testis fragments (Chapters 2, 3, 4).

Fibrates, PHs and PFAAs are ligands for peroxisome proliferator-activated receptors (PPARs). This study investigates whether PHs and PFAAs also affect *in vitro* steroidogenesis in goldfish testis fragments.

A.2 Materials & Methods

Di(2-ethylhexyl) phthalate (DEHP), 2-ethylhexanoic acid (2EHXA), perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) were purchased from TCI America (Portland, OR). Mono(2-ethylhexyl) phthalate (MEHP) was purchased from EQ Laboratories (Atlanta, GA). Dibutyl phthalate (DBP) was purchased from Fisher Scientific (Ottawa, ON). Methods for the culture of goldfish testis fragments, western blotting, cAMP assays and statistics were as described in Chapters 3 and 4. Tissues extracted for cAMP assays were not treated with IBMX during incubations. Real-time PCR was performed as described in chapter 4. Goldfish-specific luteinizing hormone receptor (LH-R) primers were provided by Jan Mennigen (F 5'-CCTCTGCATCGGTGTGTATC-3', R 5'-AGAGCGTGTGTGATCGTTGT-3').

A.3 Results

Incubation of goldfish testis fragments for 18 h with 100 μ M GEM, DEHP, MEHP, DBP, PFOA or PFOS inhibited hCG-stimulated testosterone synthesis over 6 h (Fig. A.1). This effect was not explained by decreases in transcript abundance for luteinizing hormone receptor (Fig. A.2), steroidogenic acute regulatory (StAR)

protein (Fig. A.3), or in changes in StAR protein as detected by western blot (Fig. A.4). Total tissue phosphorylated ERK 1/2 was not significantly affected by the various treatments (Fig. A.5). The dose-response for MEHP indicated that inhibition of steroidogenesis occurred at a dose (100 μ M) similar to that obtained with fibrates (Fig. A.6), and this dose did not reduce tissue cAMP concentrations (Fig. A.7).

A.4 Discussion

All compounds screened, with the exception of 2EHXA, inhibited hCG-stimulated steroidogenesis in goldfish testis fragments at a concentration of 100 μ M. The dose-response of MEHP demonstrated that this effect was not present at lower doses. This result is similar to that observed with fibrates (Chapter 3) in which lower doses did not inhibit steroidogenesis. None of the treatments significantly affected P-ERK 1/2 in goldfish testis fragments (total tissue). Mitochondrial P-ERK 1/2 was found to be decreased in GEM-treated goldfish testis fragments (Chapter 4) without significant changes in total tissue P-ERK 1/2. It was not possible to verify effects on mitochondrial P-ERK 1/2 in these experiments as the tissue was immediately frozen and tissue fractionation methods require fresh tissue. Further research should investigate the effect of PHs and PFAAs on mitochondrial P-ERK 1/2 in goldfish testis fragments. A demonstrated effect of fibrates, phthalates and perfluorinated compounds on P-ERK 1/2 in the goldfish testes would suggest a similar mechanism of action for these ligands in the inhibition of steroidogenesis.

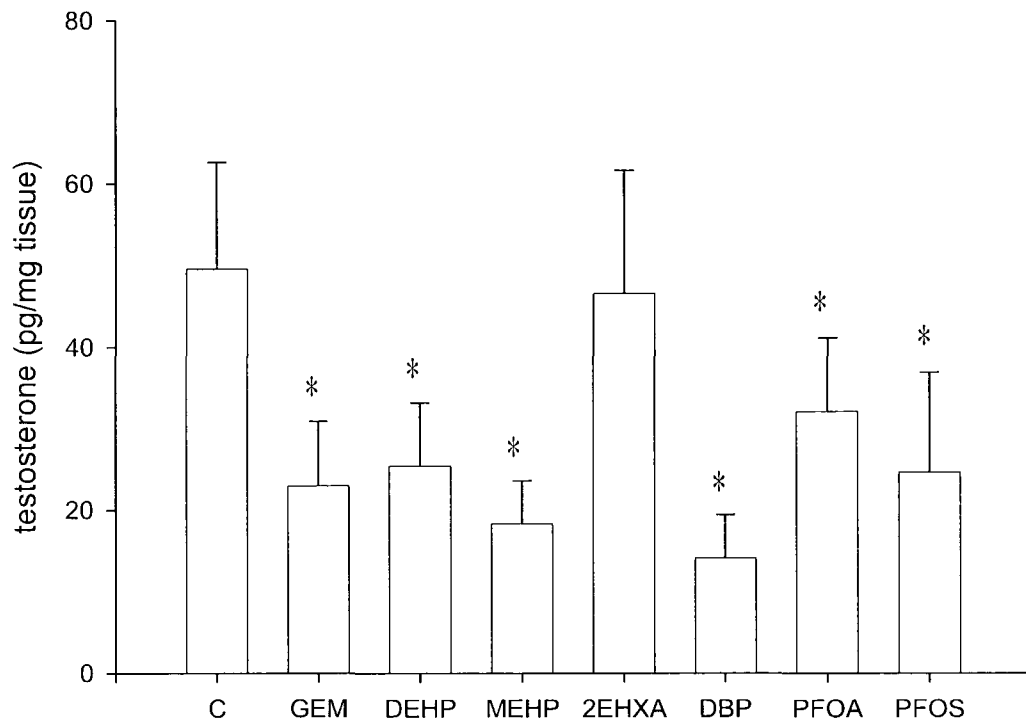


Figure A.1. hCG-stimulated (1 IU/mL) testosterone secretion from goldfish testis fragments over 6 h following 18 h incubation with gemfibrozil, phthalates, and perfluorinated compounds at 100 μ M (mean \pm SEM, n = 3, * p < 0.05 versus control) (C, control; GEM, gemfibrozil; DEHP, di-2-ethylhexyl phthalate; MEHP, mono-2-ethylhexyl phthalate; 2EHXA, 2-ethylhexanoic acid; DBP, dibutyl phthalate; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid).

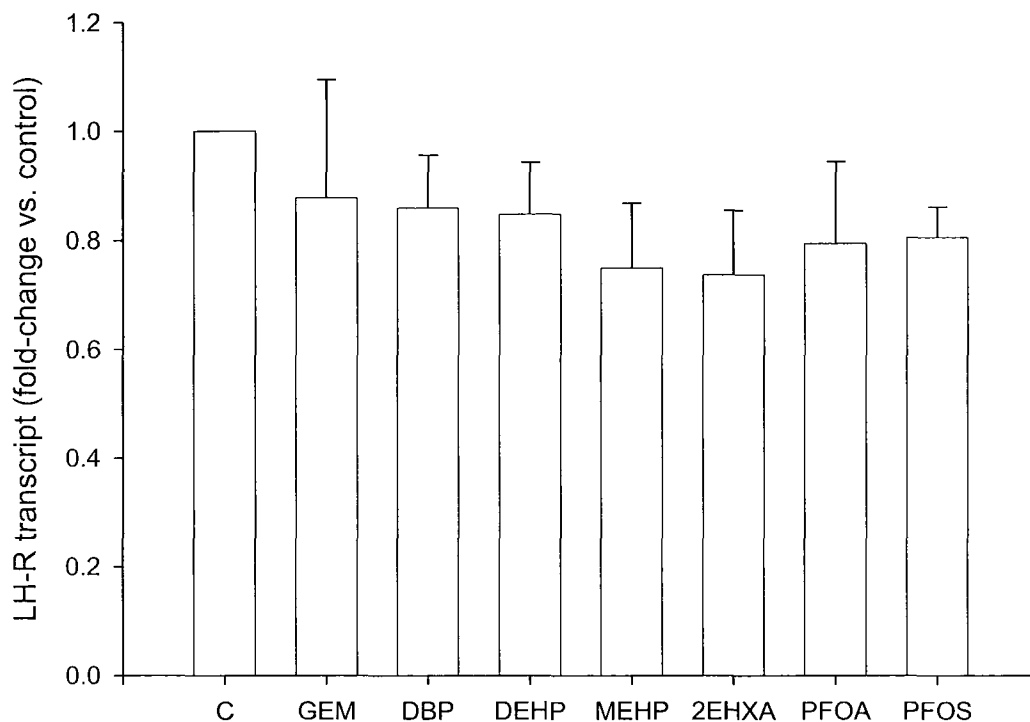


Figure A.2. LH-R transcript abundance (relative to EF-1 α control) in goldfish testis fragments incubated for 18 h with gemfibrozil, phthalates, and perfluorinated compounds at 100 μ M and treated for 6 h with 1 IU/mL hCG (mean \pm SEM, n = 3) (C, control; GEM, gemfibrozil; DEHP, di-2-ethylhexyl phthalate; MEHP, mono-2-ethylhexyl phthalate; 2EHXA, 2-ethylhexanoic acid; DBP, dibutyl phthalate; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid).

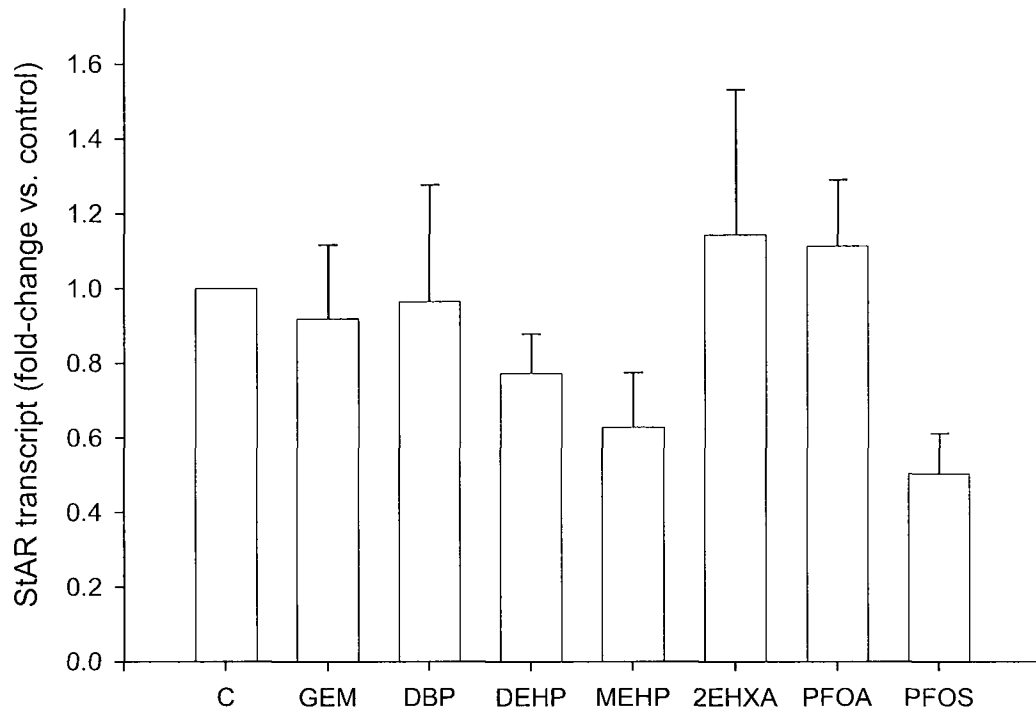


Figure A.3. StAR transcript abundance (relative to EF-1 α control) in goldfish testis fragments incubated for 18 h with gemfibrozil, phthalates and perfluorinated compounds at 100 μ M and treated for 6 h with 1 IU/mL hCG (mean \pm SEM, n = 3) (C, control; GEM, gemfibrozil; DEHP, di-2-ethylhexyl phthalate; MEHP, mono-2-ethylhexyl phthalate; 2EHXA, 2-ethylhexanoic acid; DBP, dibutyl phthalate; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid).

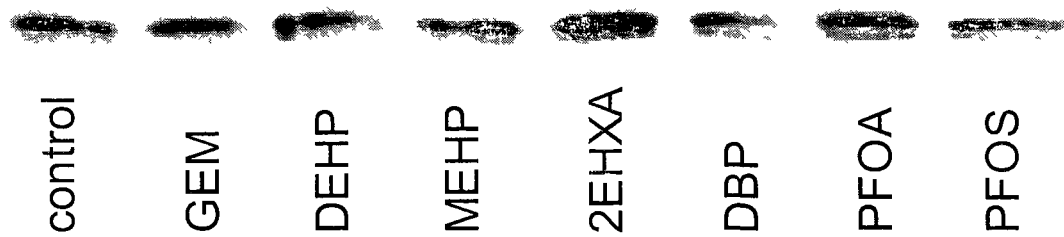


Figure A.4. Representative StAR western blot of goldfish testis fragments treated for 18h with gemfibrozil, phthalates and perflurorinated compounds at 100 μ M and stimulated for 6h with 1 IU/mL hCG (C, control; GEM, gemfibrozil; DEHP, di-2-ethylhexyl phthalate; MEHP, mono-2-ethylhexyl phthalate; 2EHXA, 2-ethylhexanoic acid; DBP, dibutyl phthalate; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid).

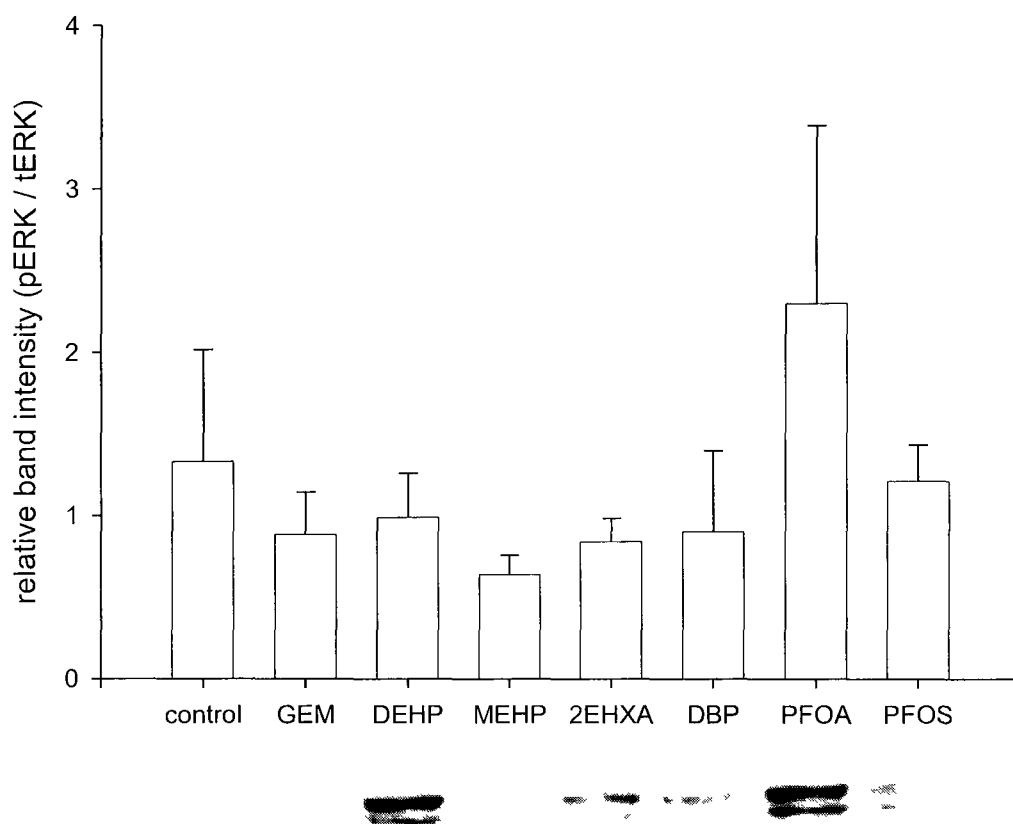


Figure A.5. Phospho-ERK 1/2 in goldfish testis fragments treated 18 h with GEM, phthalates, and perfluorinated compounds at 100 μ M followed by 6 h hCG (1 IU/mL) treatment. Representative western blot shown (mean \pm SEM, n = 3) (C, control; GEM, gemfibrozil; DEHP, di-2-ethylhexyl phthalate; MEHP, mono-2-ethylhexyl phthalate; 2EHXA, 2-ethylhexanoic acid; DBP, dibutyl phthalate; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid).

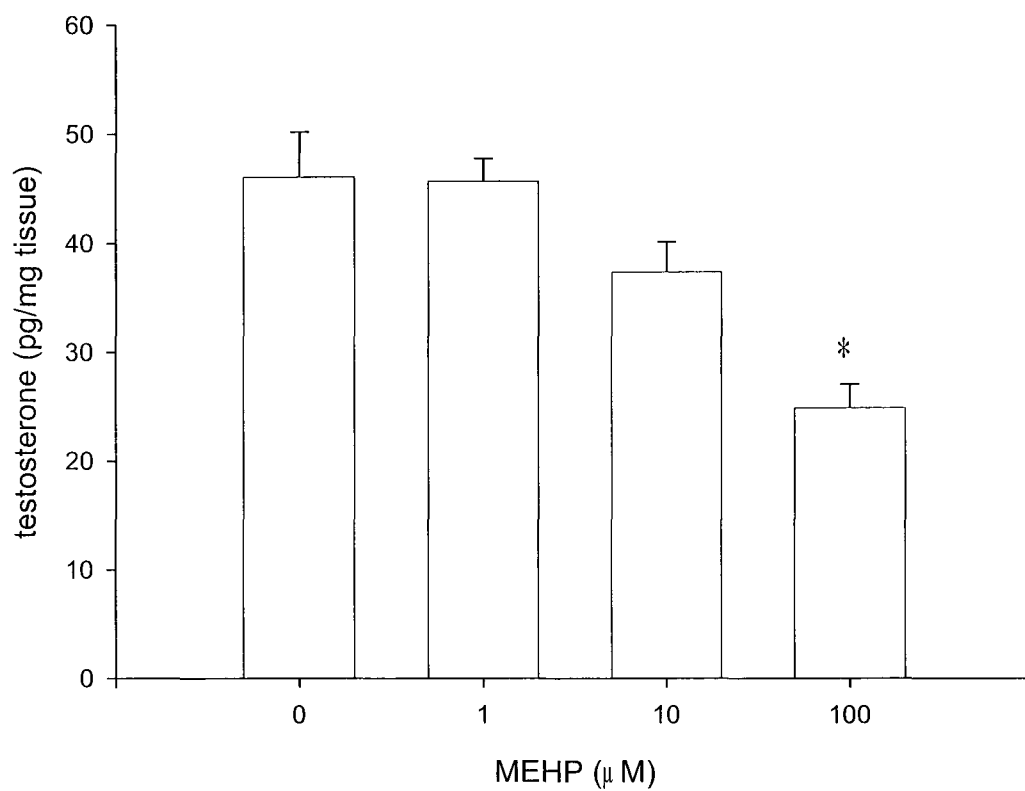


Figure A.6. hCG-stimulated (1 IU/mL) testosterone secretion from goldfish testis fragments over 6 h following 18 h incubation with mono-2-ethylhexyl phthalate (MEHP) (mean \pm SEM, n = 3, * p < 0.05 versus control).

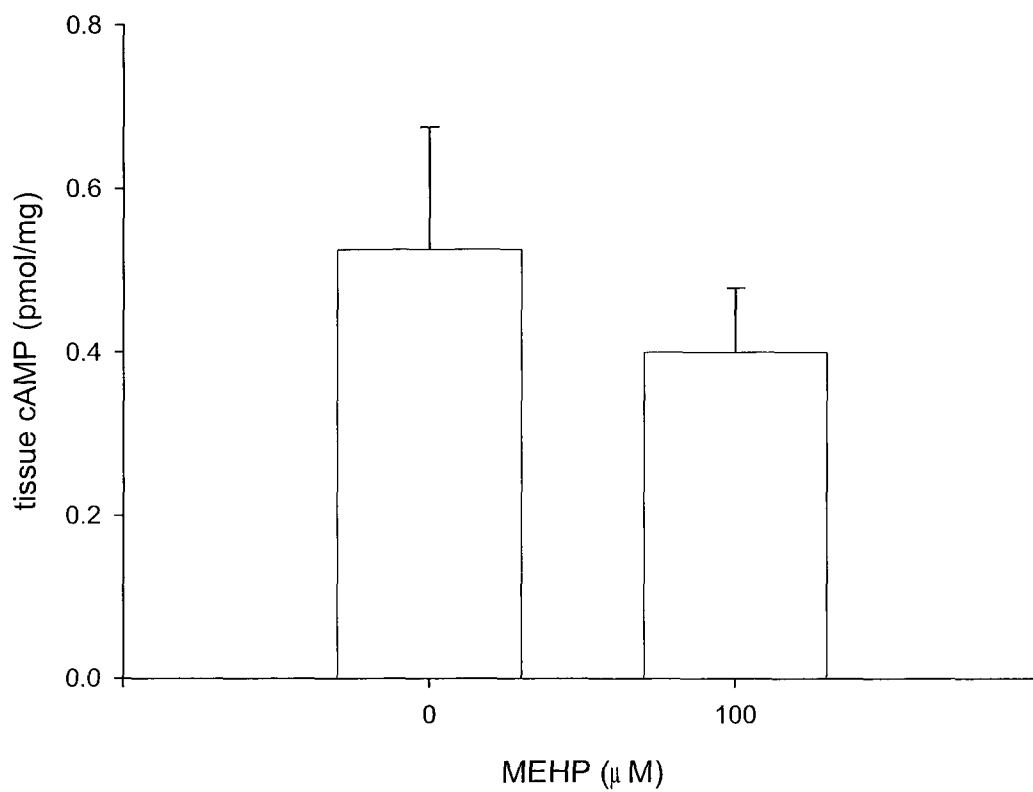


Figure A.7. Tissue cAMP concentrations in goldfish testis fragments incubated 18 h with mono-2-ethylhexyl phthalate (MEHP) and treated with 1 IU/mL hCG for 6 h (mean \pm SEM, n = 3).

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