MECHANISMS OF ACTION OF PHYTOSTEROLS ON GOLDFISH (*CARASSIUS AURATUS*) GONADAL STEROIDOGENESIS.

by

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An unpublished story of Sherlock Holmes, or “How we sometimes omit the obvious”

Author unknown

Sherlock Holmes and Dr. John Watson were on a camping trip. After a good meal and a bottle of wine, they settled down for the night and soon fell into a deep sleep. It was some hours later that Holmes awoke and nudged his faithful friend.

"Watson, look up and tell me what you see."

Watson replied, "I see thousands and thousands of stars."

"And what does that tell you?"

Watson pondered for a little while. He knew, and one day even hoped to emulate Holmes’ penetrating logic and detailed observation. "Well Holmes, astronomically it tells me that there are billions of stars, all suns like our own, and therefore potentially millions of planets. Astrologically, I observe that Saturn is in Leo. Horologically, I deduce that the time is approximately a quarter past three. Theologically, I can see that there is a strong argument in all this majesty that God is all-powerful and that we are small and insignificant. Meteorologically, I suspect from the stillness and clarity of the air that we will have a beautiful day tomorrow."

Watson allowed himself a little smile, for he had surely covered every eventuality. He continued, not a little smugly, "Tell me Holmes, what more could this possibly tell you?"

Holmes was silent for a minute, then spoke. "Watson, I really think the important point is that someone has stolen our tent."

ABSTRACT

β-Sitosterol, a phytosterol found in high concentrations in pulp mill effluents, has been proposed as one of the causative agents for the steroid depressions and reproductive dysfunctions observed in fish exposed to pulp mill effluents. In this study, goldfish (*Carassius auratus*) were exposed to a mixture of phytosterols rich in β-sitosterol to determine its effects on gonad steroidogenesis. In the first series of experiments, β-sitosterol (75% pure) (150 μg/g, silastic pellet implants) caused significant depressions in the steroidogenic pathway downstream of pregnenolone in both male and female goldfish. The second series of experiments confirmed previous work in brook trout (*Salvelinus fontinalis*) demonstrating a mixture of phytosterols rich in β-sitosterol (55%) does not affect the ability of cytochrome P450scc to convert cholesterol to pregnenolone in male goldfish. In the final set of experiments, phytosterols (150 μg/g, silastic pellet implants, 55% β-sitosterol) and β-sitosterol (95% pure) decreased the reactive pool of cholesterol in male goldfish, indicating that β-sitosterol (and other phytosterols) may be affecting the rate of cholesterol transfer across the mitochondrial membrane. These results support the hypothesis that β-sitosterol's key effects on plasma steroid depression are via the steroidogenic pathway.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

11KT	=	11-Ketotestosterone (17β-Hydroxy-4-androstene-3,17-dione)
17P4	=	17α-Hydroxyprogesterone (17-Hydroxy-4-pregnene-3,20-dione)
17P5	=	17α-Hydroxypregnenolone (3β,17-Dihydroxy-5-pregnen-20-one)
AD	=	Androstenedione (4-Androstene-3,17-dione)
ANOVA	=	Analysis of Variance
3β-HSD	=	3β-Hydroxysteroid dehydrogenase
17β-HSD	=	17β-Hydroxysteroid dehydrogense
β-Sit	=	β-Sitosterol
BKME	=	Bleached Kraft Mill Effluent
Chol	=	Cholesterol
CPM	=	Count Per Minute
DDT	=	Dichlorodiphenyltrichloroethane
DDE	=	Dichlorodiphenylethane
DES	=	Diethylstilbestrol
DHEA	=	Dehydroepiandrosterone (3β-Hydroxy-5-androsten-17-one)
E_2	=	17β-Estradiol
EDS	=	Endocrine Disrupting Substance
EE_2	=	Ethinyl Estradiol
ELISA	=	Enzyme-Linked Immuno Sorbent Assay

EtOH	=	Ethanol
FSH	=	Follicle Stimulating Hormone
GC	=	Gas Chromatography
GC-MS	=	Gas Chromatography – Mass Spectroscopy
GnRH	=	Gonadotropin Releasing Hormone
GSI	=	Gonadosomatic Index
GtH	=	Gonadotropic Hormone
hCG	=	Human Chorionic Gonadotropin
HMG-CoA	=	3-Hydroxy-3-Methylglutaryl Coenzyme A reductase
HPLC	=	High Performance Liquid Chromatography
HSD	=	Hydrosteroid Dehydrogenase
iV	=	*In Vitro*
LH	=	Luteinizing Hormone
MFO	=	Mixed Function Oxygenase
NADPH	=	β-Nicotinamide Adenine Dinucleotide Phosphate
NP	=	Nonylphenol
NSB	=	Non-Specific Binding
P4	=	Progesterone (4-Pregnene-3,20-dione)
P5	=	Pregnenolone (3β-Hydroxy-5-pregnen-20-one)
P450c17	=	Cytochrome P450 (17α-hydroxylase amd 17,20-lyase)
P450scc	=	Cytochrome P450 side chain cleavage

PAH	=	Polynuclear aromatic hydrocarbon
PCB	=	Polychlorinated biphenyls
PME	=	Pulp Mill Effluent
prep	=	Preparation
prod	=	Production
RIA	=	Radioimmunoassay
StAR	=	Steroidogenic Acute Regulatory Protein
STWE	=	Sewage Treatment Works Effluent
SU-10603	=	3-(7-Chloro-1,2,3,4-tetrahydro-1-oxo-2-naphtyl) pyridine
T	=	Testosterone
TCDD	=	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TCR	=	Total Counts Reference
TMS	=	Tricaine Methane Sulfonate
Trilostane	=	4,5-Epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile
Vtg	=	Vitellogenin

I. INTRODUCTION

1. Executive summary

Chemicals released into the environment can affect the endocrine systems of organisms, thereby affecting their survival. Endocrine disruption has been reported in many different species, from invertebrates to mammals, including humans. In this study, goldfish (*Carassius* auratus) were used to study the effect of β-sitosterol, a phytosterol high in concentration in pulp mill effluents and known to affect fish steroidogenesis. β-Sitosterol has been shown to inhibit steroidogenesis at its first step: the conversion of cholesterol to pregnenolone.

In this study, the effects of β-sitosterol on enzyme activity in the steroidogenic pathway in male goldfish were examined. A number of enzymes downstream of cholesterol conversion to pregnenolone were observed to be affected by β-sitosterol exposure. The critical effect of β-sitosterol on steroidogenesis, however, appears to be its interference with cholesterol movement from the outside to the inside of the mitochondria, where cholesterol to pregnenolone conversion occurs by action of cytochrome P450scc. This movement is the rate limiting step in steroidogenesis.

Future studies should examine the effect of β-sitosterol on StAR protein and the mechanism by which cholesterol is transported across the mitochondrial membrane.

2. Fish reproductive endocrinology

Reproduction in fish is controlled by the hypothalamic-pituitary-gonadal (H-P-G) axis (Figure 1). Environmental cues, such as photoperiod or temperature, are decoded by sensory organs and integrated by the brain, a process called transduction. The central nervous system then synthesizes the information. If a positive cue for reproduction is recorded (*e.g.* increased temperature and photoperiod), the hypothalamus is stimulated to produce gonadotropic-releasing hormone (GnRH), which in turn stimulates gonadotropic hormone (GtH) release from the pituitary gland (Figure 1) (Eckert, 1988; Bond, 1996). In goldfish, the neurotransmitter dopamine can inhibit GtH release by the pituitary (Chang *et al.*, 1990).

There are two forms of GtHs, corresponding to the two forms of GtHs found in other vertebrates. GtH I is similar to mammalian follicle-stimulating hormone (FSH) and is thought to induce active gonadal growth and gametogenesis. GtH II is analogous to mammalian luteinizing hormone (LH), and is thought to be responsible for gonadal maturation and spawning (Kawauchi *et al.*, 1989).

In general terms, GtH II acts on the gonads and stimulates the production of sex steroids (Figure 1): estrogens, androgens and progesterone. Androgens, such as testosterone (T) and 11-ketotestosterone (11KT) (Figure 2), are the dominant male sex hormones; estrogens, such as 17β-estradiol (E_2) (Figure 2), are the dominant female sex hormones. Unlike other vertebrates, female fish produce a large amount of T that is not converted to E_2; T is thus found in high quantities in the blood of both sexes.

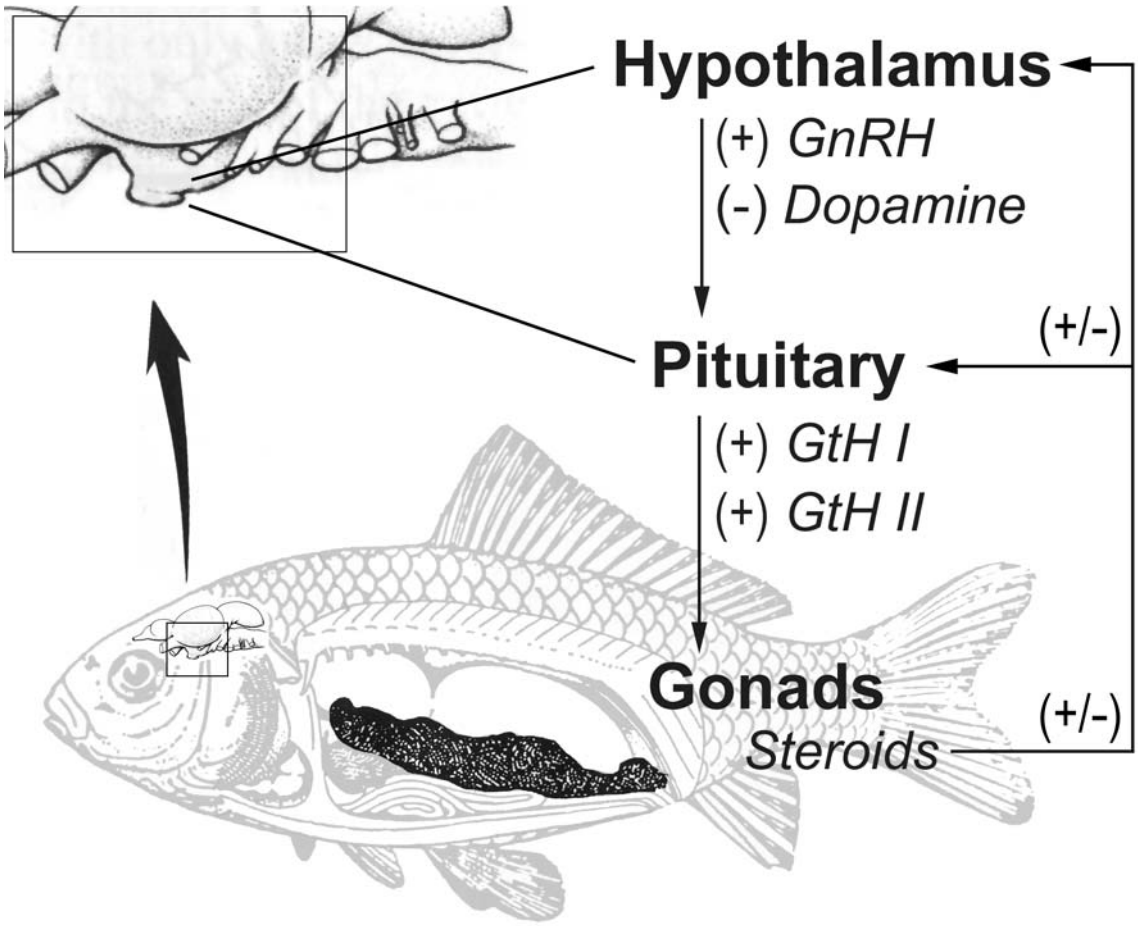

Figure 1: The hypothalamic-pituitary-gonadal axis. Composite picture based on Hervey and Hems (1968) and Bond (1996).

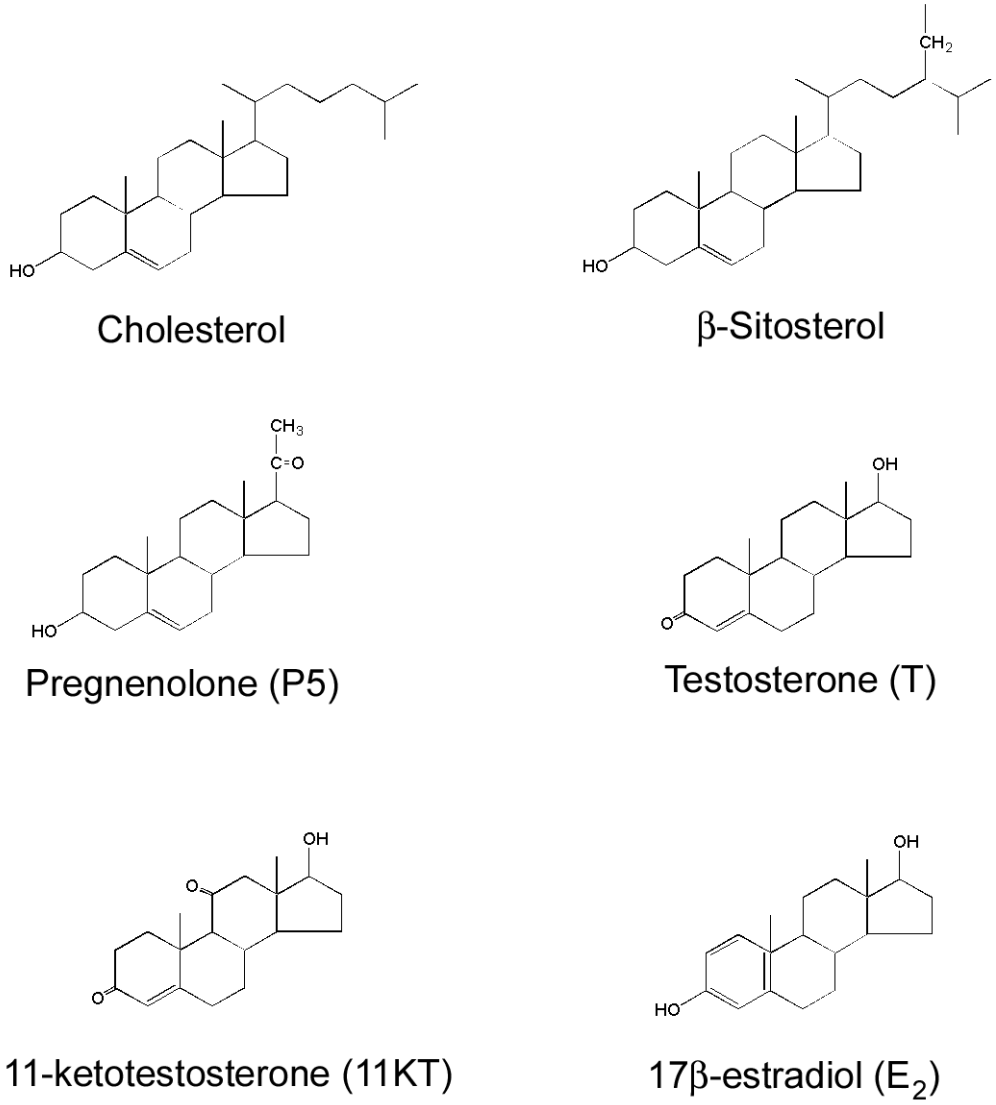

Figure 2: Structures of cholesterol, β-sitosterol, and various steroids.

Steroids regulate key metabolic processes, the promotion of secondary sexual characteristics and reproductive behaviour. Estrogens play a crucial role in the development of eggs in females, and E_2 stimulates synthesis of vitellogenin (Vtg), a yolk precursor protein produced by the liver in mature female fish (Redding and Patiño, 1993).

All sex steroids are derived from cholesterol (Figure 3), and are produced via numerous enzymatic conversions in gonadal cells (Leydig and Sertoli cells in males and follicles in females). Both 11KT and E_2 are derived from T (although 11KT can also be derived from androstenedione) (Figure 3).

Cholesterol itself can be either supplied to the cell from plasma cholesterol, or synthesized *de novo* from acetate by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is activated by GtH (Pedersen, 1988).

GtH and the final steroids can also act on the upper levels of the hypothalamic-pituitary-gonadal axis by either positive or negative feedback (Trudeau *et al.*, 1993; Habibi and Huggard, 1998) (Figure 1). The whole system is very sensitive to extremely minute quantities of steroids, and an artificial change in one of them, even if very small, can have a tremendous effect (Colborn *et al.*, 1993).

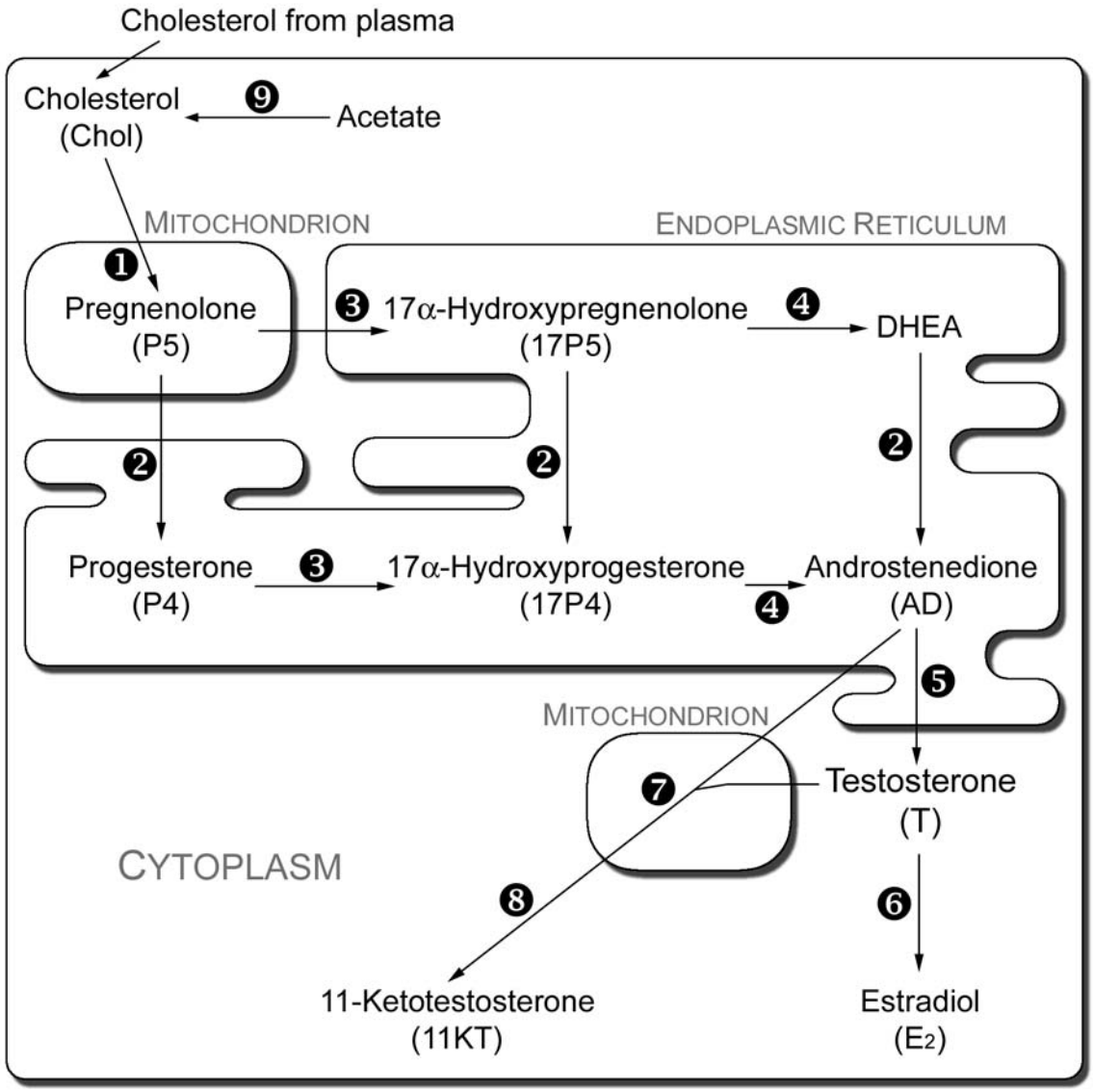

Figure 3: The primary steroid biosynthetic pathway in a gonadal cell. Enzymes are: (1) cytochrome P450 side-chain cleavage - P450scc; (2) 3β-hydroxysteroid dehydrogenase - 3β-HSD; (3) P450c17 (17α-hydroxylase); (4) P450c17 (C17,20-lyase); (5) 17β- HSD; (6) cytochrome P450 aromatase; (7) 11β-hydroxylase; (8) 11β-HSD; (9) HMG-CoA reductase. Based on Matty (1985), McMaster *et al.* (1995a) and Hall (1998).

3. Endocrine disruption

(a) Action of endocrine disrupting substances (EDSs)

The endocrine system can be thought of as a complex system of biochemical communication that coordinates the activities of the different cell systems of an organism by secreting hormones. Hormones bind to specific receptors on target cells, which in turn activate a cascade of effects (*e.g.* synthesis of a specific protein, adjustment of blood circulation and chemistry, regulation of reproductive cycles, development of a certain tissue type, etc). Exposure to even low levels of hormonally active agents (termed endocrine disrupting substances, or EDSs) results in significant dysfunctions in endocrine systems (Colborn *et al.*, 1996, Solomon and Schettler, 2000) in a wide range of species from invertebrates to humans (Colborn *et al.*, 1993; Cooper and Kavlock, 1997; Crisp *et al.*, 1998; Sumpter, 1998; Van Der Kraak, 1998; Depledge and Billinghurst, 1999; IEH, 1999; Taylor and Harrison, 1999; Bowerman *et al.*, 2000; Juberg, 2000; Matthiessen, 2000).

A wide range of synthetic chemicals have been reported to have reproductive and endocrine-disrupting effects, from insecticides to industrial chemicals (Colborn *et al.*, 1993; Cheek *et al.*, 1998), but only a fraction of all synthetic chemicals released in the environment have been tested for endocrine disruption (Jobling *et al.*, 1995). Organisms in aquatic environments are especially vulnerable to pollutants, since contaminants can be accumulated via both waterborne (through the gills) and food web routes of exposure.

(b) Use of fish to study EDSs in the aquatic environment

Fish reproduction is sensitive to environmental factors, and is particularly vulnerable to EDSs in the aquatic environment (Kime, 1999; Jalabert *et al*., 2000). Fish exposed to industrial effluents exhibit a wide array of abnormalities of the reproductive or thyroidal systems (Van Der Kraak *et al.*, 1992a; Hontela *et al.*, 1994; McMaster *et al.*, 1995b; Knudsen *et al.*, 1997; Black *et al.*, 1998b; Zhou *et al.*, 2000; Pacheco and Santos, 2001). There are many industrial chemicals currently known to affect the endocrine system of fish (Colborn *et al.*, 1993; Jobling *et al.*, 1995), such as nonylphenol (NP) (Le Gac *et al.*, 2001; Tabata *et al.*, 2001), tetrachloro dibenzodioxin (TCDD) (Wu *et al.*, 2001), polychlorinated biphenyls (PCBs) (Black *et al.*, 1998a), polynuclear aromatic hydrocarbon (PAHs) (Johnson *et al.*, 1997), dichlorodiphenyltrichloroethane (DDT) and its metabolite dichlorodiphenylethane (DDE) (Zaroogian *et al.*, 2001).

Fish exposed to domestic sewage or fish from polluted rivers in the UK (Purdom *et al.*, 1994; Harries *et al.*, 1996; Lye *et al.*, 1997; Hall *et al.*, 1997; Sumpter, 1997; McArdle *et al.*, 2000), Belgium (Witters *et al.*, 2001), Italy (Viganò *et al.*, 2001), Sweden (Noaksson *et al.*, 2001) and Japan (Nasu *et al*., 2001) exhibit reproductive dysfunctions, often associated with xenoestrogenic stimulation (*e.g.* production of vitellogenin in males). A study by the UK Environment Agency (Desbrow *et al.*, 1996) identified three hormones in sewage treatment works effluents (STWEs) that were responsible for the majority of the estrogenic effect: 17β-estradiol (E_2), oestrone and the synthetic hormone ethinyl estradiol (EE_2). The presence of these hormones in high concentrations in

STWEs is associated with higher population densities (which concentrate human wastes in a smaller area).

4. Pulp mill effluents

Among the many types of industrial effluents associated with endocrine disruption, pulp mill effluents (PME), and in particular bleached kraft mill effluents (BKME), have been best studied. The pulp and paper industry is the third largest user of freshwater (after the heavy metal and chemical industries); water consumption can be as high as 60 m^3 per ton of paper produced (Thompson *et al.*, 2001).

Along with acute toxic effects (reviewed in Ali and Sreekrishnan, 2001), BKME exposure induces several reproductive dysfunctions in wild fish: decreased plasma steroid levels, decreased gonadal biosynthetic capacity, increased age to sexual maturation and reduced expression of secondary sexual characteristics (McMaster *et al.*, 1991; Adams *et al.*, 1992; Munkittrick *et al.*, 1992; Van Der Kraak *et* al., 1992a; McMaster *et al.*, 1995b; Kovacs *et al.*, 1995; Munkittrick *et al.*, 1998; Kukkonen *et al.*, 1999; Mellanen *et al.*, 1999; Munkittrick, 2001). Exposure to bleached kraft pulp mill effluents in the lab induces similar responses (McMaster *et al.*, 1996; Tremblay and Van Der Kraak, 1999).

Initially, dioxins and furans were the suspected causative agents of endocrine disruption in BKME (Peck and Daley, 1994), but technological changes in the pulping process that removed dioxins and furans did not resolve the negative effects of BKME on reproductive endpoints (Van Der Kraak *et al.*, 1998). While partial or complete removal of chlorine from the pulping process resulted in much lower acute toxicity of the final

effluent, it did not solve the reproductive problems (Munkittrick *et al.*, 1994; Munkittrick *et al.*, 1997; Karels *et al.*, 1999).

Also present in BKME are natural plant compounds, such as resin acids, genistein and phytosterols, particularly β-sitosterol (Folke *et al.*, 1993; Cook *et al.*, 1997; Kiparissis *et al.*, 2001). Several studies showed that natural plant compounds can have endocrine-disrupting effects (Labov, 1977; Kaldas and Hughes, 1989; Wynne-Edwards, 2001), and it has been suggested that wood-derived compounds, and in particular β-sitosterol, might account for some of the reproductive effects of BKMEs (MacLatchy and Van Der Kraak, 1995; Mellanen *et al.*, 1996; MacLatchy *et al.*, 1997; Van Der Kraak *et al.*, 1998; Kukkonen *et al.*, 1999; Lehtinen *et al.*, 1999, Tremblay and Van Der Kraak, 1999).

5. β-Sitosterol

β-Sitosterol (Figure 2) is a plant estrogen (phytoestrogen), and it has cell membrane functions similar to those of cholesterol in animal systems (Hartmann, 1998). It is the major phytosterol found in pulp mill effluents, and has been reported at concentrations as high as 220 μg/L in the final effluent (Cook *et al.*, 1997).

Hughes (in Chapin *et al.*, 1996) argues that phytoestrogens have been selected for throughout evolution by plants precisely because of this chemical mimicry of mammalian hormones. This “antifertility strategy” is used by plants against herbivory, a subtle antipredatory strategy which ultimately reduces the reproductive potential of herbivores

(Wynne-Edwards, 2001). If this is true, then phytoestrogens present in pulp mill effluents could be affecting exposed fish populations in a similar way, *i.e.* reducing the reproductive potential by altering the function of reproductive endocrine systems.

β-Sitosterol has been shown to have estrogen-like effects in fish (MacLatchy *et al.*, 1995; Mellanen *et al.*, 1996; Tremblay and Van Der Kraak, 1998; Latonelle *et al.*, 2000). β-Sitosterol, which differs from cholesterol by the addition of an ethyl group on C24 (Figure 2), causes a decrease in plasma T and 11KT in males and E_2 in females (MacLatchy and Van Der Kraak, 1995; MacLatchy *et al.*, 1997; Gilman, 2000), binds to rainbow trout hepatic estrogen receptors (Tremblay and Van Der Kraak, 1998) and induces the expression of vitellogenin (Vtg) in males (MacLatchy et al., 1995; Tremblay and Van Der Kraak, 1998; Tremblay and Van Der Kraak, 1999). Vitellogenin induction in males has been used as a biomarker for estrogen-mediated endocrine disruption (Hansen *et al.*, 1998).

Czech *et al.* (2001) showed that exposure to 100 ng/L of β-sitosterol in *Lymnaea stagnalis* (a hermaphroditic pulmonate gastropod very abundant in European freshwater systems) caused a distinct atrophy of the albumen gland. The albumen gland is an accessory sexual organ (Ruppert and Barnes, 1994) under direct endocrine control of the central nervous system or the dorsal bodies (Bride and Gomot, 1995). It is responsible for the production of albumen, which provides energy and some protection to the eggs (Audesirk and Audesirk, 1993), a function similar to that of Vtg in fish. While it is not clear whether exposure for a longer period and at higher concentrations of β-sitosterol

would affect reproductive success (Czech *et al.*, 2001), the fact that β-sitosterol affects the size of the albumen gland (thereby presumably reducing its function) in gastropods suggests that it has the potential to act as an endocrine disruptor in invertebrates as well as in vertebrates.

β-Sitosterol is a biologically active compound in mammals: it stimulates uterine growth (an estrogen-mediated effect) in several species of mammals (El Samannoudy *et al.*, 1980; Malini and Vanithakumari, 1993), indicating its estrogenicity. A diet rich in β-sitosterol reduces total liver cholesterol in rats (Kritchevsky *et al.*, 1999). Likewise, in humans, phytosterols (particularly β-sitosterol, campesterol and stigmasterol) have long been known to be hypocholesterolemic (Heinemann *et al.*, 1991; Phillips *et al.*, 1999) and a diet rich in phytosterols has been used to treat the disease hypercholesterolemia. It has also been suggested that such a diet could also decrease rates of cancer and heart disease (Raicht *et al.*, 1980; Knight and Eden, 1995; Adlercreutz, 1999). A diet rich in β-sitosterol has been advocated as a non-invasive treatment for benign prostatic hyperplasia (Berges *et al.*, 1995; Wilt *et al.*, 1999). Recently, scientists have been warning that the negative effects of such abnormally high dietary exposures to phytosterols may outweigh the positive effects (Goldstein, 2000; Moghadasian, 2000), and there is special concern for pregnant women because the developing foetus may be highly susceptible to phytoestrogens (Jefferson and Newbold, 2000).

6. Mechanisms of action of β-sitosterol in fish

Investigations into the effects of β-sitosterol revealed that it affects the steroidogenic pathway at its first step, the conversion of cholesterol to pregnenolone (P5) by cytochrome P450 side-chain cleavage (P450scc) (MacLatchy *et al.*, 1995; MacLatchy *et al.*, 1997). This has long been known to be the rate-limiting step of steroidogenesis (Stone and Hechter, 1954, cited in Jefcoate *et al.*, 1992). Four different sites of action of β-sitosterol have been suggested (Figure 4): (1) on the supply of cholesterol to the cell (either from plasma or *de novo* synthesis by HMG-CoA reductase), (2) on the transfer of cholesterol from the outside to the inside of mitochondria, where it can be converted to P5 by cytochrome P450scc, (3) on the rate of metabolism of cholesterol to P5 or (4) downstream of P5, on the enzymatic conversions from P5 to the final steroid (Figure 3).

While there is a slight decrease of plasma cholesterol observed in goldfish exposed to β-sitosterol (Gilman and MacLatchy, 1996; Gilman *et al.*, 1997), this decrease does not seem to have a substantial effect on gonadal cholesterol (MacLatchy *et al.*, 1997), hence supply of cholesterol to the cell does not seem to be affected by β-sitosterol, possibly because of an increased *de novo* synthesis of cholesterol from acetate.

β-Sitosterol could be interfering with the rate of transfer of cholesterol to the inner membrane of the mitochondria (cholesterol translocation), thereby reducing the amount of substrate available for P450scc. This would ultimately reduce the production of P5, and is the mechanism by which other contaminants (*e.g.* TCDD in rats; Moore *et al.*, 1991) affect P5 production at the rate-limiting step.

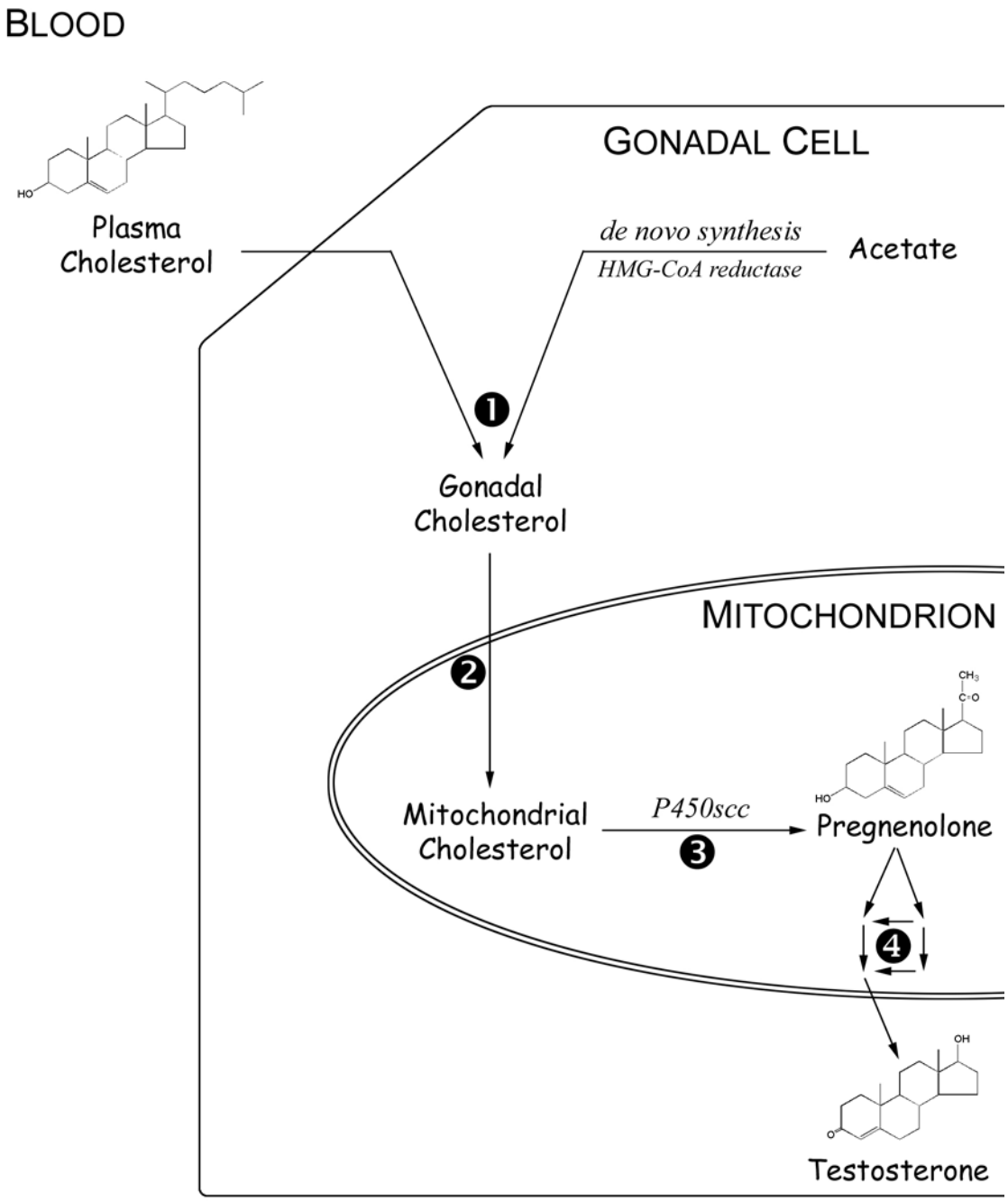

Figure 4: Possible sites of action of β-sitosterol. Numbers refer to the following actions: (1) supply of cholesterol to the cell; (2) transfer of cholesterol to the inside of the mitochondrion; (3) rate of metabolism of cholesterol to P5; (4) downstream of P5.

Gilman (2000) reported that β-sitosterol had no significant effect on P450scc activity in male brook trout (*Salvelinus fontinalis*) during gonadal regression, indicating that the activity of P450scc is not affected by β-sitosterol exposure.

MacLatchy *et al.* (1997) also suggested that β-sitosterol might have an effect downstream of P5, on the enzymatic conversions between P5 and T (Figure 3). *In vitro* gonadal incubations from β-sitosterol-treated fish, when supplied with 25-hydroxycholesterol (a substrate that will readily cross the mitochondrial membrane), had increased P5 production but not T production. This suggests that other enzymes downstream of P450scc may also be affected by β-sitosterol. This hypothesis also requires further investigation.

7. Objectives and hypotheses

In this research project, common goldfish (*C. auratus*) were used to study the effect of β-sitosterol on teleost steroidogenesis. Goldfish were chosen as the test species because they have often been used in lab experiments and a great deal is known about their biology, physiology and endocrinology (Peter *et al.*, 1986; Chang *et al.*, 1992; Van Der Kraak *et al.*, 1992b; Stoskopf, 1993; Trudeau *et al.*, 1993; Habibi and Huggard, 1998; Pati and Habibi, 2000; Klausen *et al.*, 2001).

The overall objectives of this research were: (1) to evaluate the effects of β-sitosterol downstream of P450scc in the steroidogenic pathway; (2) to investigate the

effects of β-sitosterol on P450scc activity; and (3) to study the effects of β-sitosterol on cholesterol mobilization to the inside of the mitochondria.

The following hypotheses were tested:

(a) Effect of β-sitosterol on steroidogenesis downstream of P5.

H_0: There is no difference in *in vitro* T production between incubations from control and β-sitosterol groups when supplied with any of the steroids downstream of P5 (*i.e.* P4, 17P5, 17P4, DHEA or AD) (Figure 3).

H_A: β-Sitosterol decreases steroidogenesis downstream of P5.

(b) – Effect of β-sitosterol on P450scc activity.

H_0: There is no difference in mitochondrial P5 production between control and β-sitosterol groups (especially in preps with sonicated mitochondria).

H_A: β-Sitosterol decreases the activity of P450scc.

(c) – Effect of β-sitosterol on cholesterol transfer.

H_0: There is no difference in mitochondrial P5 production between control and β-sitosterol groups (in preps with intact mitochondria and no exogenous substrate added).

H_A: β-Sitosterol reduces the reactive pools of endogenous cholesterol in the mitochondria of β-sitosterol-treated fish, a good indication that β-sitosterol interferes with cholesterol translocation across the mitochondrial membrane.

II. MATERIALS AND METHODS

1. General Information

(a) Supplies

Chemicals and lab supplies

Unless otherwise indicated in the text, all chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON) and all lab supplies from Fisher Scientific Ltd. (Nepean, ON).

Fish

Goldfish (*C. auratus*) were purchased from DAP International (Etobicoke, ON) and acclimated in stock tanks (620 and 1,350 L flow-through systems with a turnover rate of 24h; 18-20ºC dechlorinated City of Saint John water) for at least one month prior to experiments. All fish were fed commercial trout chow (Corey Feed Mills, Fredericton, NB) at a rate of approximately 2% of their body weight every second day. Once transferred to experimental tanks (62-L flow-through systems with a turnover rate of 12h; 18-20ºC dechlorinated City of Saint John water), the fish were fed commercial trout chow at a rate of 1.5% of their body weight daily. A 14:10 light:dark photoperiod was maintained throughout the year.

(b) Data analysis and statistical software

Data were tabulated using Microsoft Excel 2000 (Microsoft Corp., Seattle, WA). Unless otherwise indicated, a significance level of α=0.05 was used. Data were always tested for normal distribution and equality of variance first. If the data were not normally distributed, they were log-transformed, and re-tested for normality. Data that could not be made normal through log-transformation (or data where variances were not equal) were analysed using non-parametric tests.

All simple statistical analyses (t-tests, paired t-tests, one-way ANOVA and their non-parametric equivalents) were done using SigmaStat 2.03 for Windows (SPSS Inc., Chicago, IL). More complex statistical analyses (multiple-way ANOVA, repeated-measures ANOVA) were done using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL). Nested ANOVAs were done "by hand" with Microsoft Excel 2000 using formulae in Sokal and Rohlf (1995) and Zar (1999).

2. β-Sitosterol and the steroidogenic pathway

(a) Experiment 1: Effect of β-sitosterol on the steroidogenic pathway in goldfish

Objective

MacLatchy *et al.* (1995) suggested that β-sitosterol not only affects the conversion of cholesterol to P5, but could also have effects downstream of P5 in the steroidogenic cascade. The purpose of this experiment was to test which parts of the steroidogenic pathway are affected by β-sitosterol exposure.

β-Sitosterol

β-Sitosterol for this experiment was a gift from Dr. Glen Van Der Kraak (University of Guelph, Guelph, ON). It was analysed by gas chromatography-mass spectrometry (GC-MS) by Dr. Mark Hewitt (National Water Research Institute, Environment Canada, Burlington, ON) and found to be 72.6% pure. Impurities in the mixture are other common plant sterols such a campesterol, stigmasterol, stigmastanol and dihydrobrassicasterol.

Silastic® pellets

Silastic® pellets were used to expose goldfish to β-sitosterol. They have been used in the past as an alternative to water-borne exposure (Habibi *et al.*, 1989; Trudeau *et al.*, 1993; Gilman, 2000) and have been shown to be an effective method for β-sitosterol exposure (Gilman, 2000). The pellets were prepared by combining 1 g of an

unpolymerized Silastic® elastomer with 0.1 g of curing agent (Silastic® medical grade MDX4-4210, Dow Corning Corp., Midland, MI; gift from Dr. Kevin Halcrow, University of New Brunswick, Saint John, NB). The mixture was then spread in a 2 x 2 x 250 mm mold (food-grade plastic) and cured for 24h at room temperature to a semi-transparent silicone rubber strip. For β-sitosterol pellets, 250 mg of β-sitosterol were added during mixing, for a final concentration of 1 mg/mm (nothing was added for control pellets).

Fish

One week prior to the experiment, 48 fish of approximately the same size were transferred from their stock tank to four experimental tanks (12 fish per tank). Tanks were randomly assigned to experimental groups: two β-sitosterol tanks and two control tanks.

Exposure

On November 10th 1999, fish were anaesthetized by immersion in 0.05% tricaine methane sulfonate (TMS; Syndel Laboratories, Vancouver, BC), weighed and implanted with Silastic® pellets for final concentrations of 0 μg/g (control) or 150 μg/g of β-sitosterol; pellet length was calculated for each individual fish based on its weight. The pellets were implanted in the intra-peritoneal cavity using a 12G insertion needle, which was disinfected with ethanol and rinsed with water before each fish. The fish were

returned to their tanks and left undisturbed for the next 21 days, except for daily feeding in the morning.

Sampling

On December 1st 1999, the fish were anaesthetized by immersion in 0.05% TMS and bled by caudal puncture using 25G5/8 heparinized needles and 1 mL syringes (heparin solution: 150 IU of heparin per mL of double-distilled water). At least 500 μL of blood per fish were taken. After bleeding, fish were killed by spinal severance, weighed and sexed (by visual inspection of the gonad). Gonads were excised, weighed, placed in 1 mL Cortland's saline (App. I–S1 for composition) on ice and gonadosomatic index (GSI = gonad weight x 100 / total weight) was computed for each fish.

Based on GSI, four males and four females of similar reproductive state out of the 12 fish in each tank were selected and their gonads kept and used in the remainder of the experiment. The purpose of the GSI selection was to decrease the amount of within group variation.

Blood samples

The blood sampled from each fish was spun in a refrigerated centrifuge at 4ºC for 5 min at 1,470 *g*. The supernatant plasma was kept and frozen at -20ºC for later measurement of plasma T.

In vitro incubations

Two pieces of gonad (total of 20-25 mg) were incubated for 18h at 18ºC (App. I-S1 for detailed protocol). There were nine different incubations per gonad, which differed in the steroidogenic precursors added (Table 1). At the end of the 18h incubation period, 900 μL of the incubation medium was sampled and frozen at –20ºC for later analysis of T concentration.

Table 1: Nine different test solutions for incubations in experiment 1. All chemicals were diluted in ethanol prior to addition of Cortland's saline (final ethanol concentration in the incubation medium < 1%). Amounts were based on McMaster *et al.* (1992).

Solution ID	Contents (in 1mL of Cortland's saline)
Basal	---
hCG	10 IU of hCG
Chol	5 μg of 25-hydroxycholesterol
P5	100 ng of pregnenolone
P4	100 ng of progesterone
17P5	100 ng of 17α-hydroxypregnenolone
17P4	100 ng of 17α-hydroxyprogesterone
DHEA	100 ng of DHEA
AD	100 ng of androstenedione

Sample processing

Plasma samples: Steroid molecules were separated from other plasma proteins using a dry-ice acetone bath extraction protocol (App. I–S2) and plasma T concentration was determined by radioimmunoassay (RIA) (App. I–S3). Intra-assay variability for plasma T was 3.6% and inter-assay variability was 4.8%.

Incubation samples: *In vitro* T production during the 18h incubation period was also measured by RIA. Intra-assay variability for *in vitro* T was 3.2% and inter-assay variability was 5.4%.

Data analysis

The Dixon test for outliers was used to determine if there were any outliers in the data set (significance set at α=0.02) (Kanji, 1993).

Weight, GSI and plasma T levels: Data were analysed using a two-way nested ANOVA to determine if there were any tank effects (nested factor) and treatment effects (fixed-effects factor) (Sokal and Rohlf, 1995; Zar, 1999). If there was no tank effect, data were pooled and t-tests were used to determine if there were overall treatment effects.

In vitro T production: Data were analysed using a two-way nested ANOVA to determine if there were any tank effects (nested factor) and treatment effects (fixed-effects factor). If there was no tank effect, data were pooled and multiple t-tests were used to determine if there were differences in *in vitro* T production between treatment groups (control vs. β-sitosterol) for each of the nine incubations separately.

3. β-Sitosterol and P450scc activity

(a) Preliminary experiment: Finding a reducing precursor to NADPH in goldfish

Objective

Cytochrome P450scc requires β-nicotinamide adenine dinucleotide phosphate (NADPH) as an energy source (Hall, 1998). It is impossible, however, to supply NADPH directly to intact mitochondrial preparations (NADPH does not enter the mitochondria by simple diffusion; Moran *et al.*, 1994), and a reducing precursor has to be supplied. In rats, isocitrate was the most effective precursor (McNamara and Jefcoate, 1990) but it may not be optimal in fish (Gilman, 2000). Other potentially effective precursors are other intermediates of the Krebs cycle, such as succinate or malate (Xu *et al.*, 1989; Moran *et al.*, 1994; Kim *et al.*, 1997).

The purpose of this experiment was to identify the best reducing precursor to use in intact fish mitochondrial preparations.

Sampling

On May 17th 2000, fish of approximately the same size were taken from their stock tank, anaesthetized by immersion in 0.05% TMS, killed by spinal severance, weighed and sexed (by visual inspection of the gonads). The gonads were excised, weighed, placed in 1 mL isolation buffer (App. I–S4 for composition) on ice, and GSIs were computed.

Four males of similar reproductive states with large testes (more than 800 mg) were sampled. Their gonads were kept and used for the remainder of the experiment.

Mitochondrial isolations

Gonadal mitochondria were isolated for each of the selected gonads. The gonads were chopped into small, 1-mm cubes, homogenized and serially centrifuged to isolate the mitochondria. All manipulations took place in refrigerated equipment or on ice (App. I-S4 for detailed protocol).

Trilostane and SU-10603

In mitochondrial incubations where P5 production was measured, the conversion of P5 down the steroidogenic pathway had to be inhibited. Trilostane (WIN24540; gift from Sanofi Recherche, Paris, France) and SU-10603 (gift from Novartis Pharmaceuticals Corp., Summit, NJ) inhibit the steroidogenic enzymes responsible for converting P5 to P4 (3β-HSD; Figure 3) or to 17P5 (P450c17, 17α-hydroxylase; Figure 3), respectively (Bakker *et al.*, 1978; Naville *et al.*, 1991).

Incubation protocol

One-hundred microliters of the final mitochondrial preparation was added to 1.785 mL of incubation medium in a shaking water bath at 18ºC (temperature to which fish were acclimated). Fifteen microliters of ethanol were added, containing trilostane

(final concentration of 10 μM; dissolved in ethanol on the day of use), SU-10603 (final concentration of 200 nM; dissolved in ethanol on the day of use) and 25-hydroxycholesterol (final concentration of 10 μM; stored dissolved in ethanol at -20ºC). 25-Hydroxycholesterol is a form of cholesterol that can freely diffuse across the mitochondrial membrane and enter the mitochondria, where it can then be converted to P5.

The final incubation medium (1.9 mL) contained less than 1% ethanol to avoid ethanol toxicity. A sample of 0.475 mL was removed and placed in 3 mL of ethyl acetate in a glass scintillation vial on ice. Each vial was immediately capped, vortexed and stored at –20ºC for later analysis of P5 concentration.

The reaction was then initiated by adding 75 μL of the reducing precursor solution to each incubation vial at precisely timed intervals. There were seven different reducing precursor solutions (Table 2).

Table 2: Different reducing precursor solution.

Solution ID	Contents (dissolved in incubation medium on day of use)
Basal	Only incubation medium, no precursors added
ISO 10	10 mM isocitrate ($_{DL}$-isocitric acid, trisodium salt)
ISO 50	50 mM isocitrate
MAL 10	10 mM malate (maleic acid, monosodium salt)
MAL 50	50 mM malate
SUC 10	10 mM succinate (succinic acid)
SUC 50	50 mM succinate

After 15 min, 0.5 mL of incubation medium was removed and placed into 3 mL of ethyl acetate in a glass scintillation vial on ice (at precisely timed intervals, so that each vial incubated exactly 15 min). Each vial was immediately capped and vortexed to stop the reaction, and then stored at –20ºC for later analysis of P5 concentration.

Protein quantification

The Bio-Rad Protein Assay (Bio-Rad Laboratories Canada Ltd., Mississauga, ON), a colorimetric assay based on Bradford (1976), was used to determine the amount of protein in the mitochondrial preparations (App. I–S5 for detailed protocol).

Sample processing

Pregnenolone content at 0 and 15 min was measured by RIA. Intra-assay variability for P5 was 4.5% and inter-assay variability was 3.0%. P5 production was calculated by subtracting initial levels at 0 min from those at 15 min.

Data analysis

The Dixon test for outliers was used to determine if there were any outliers in the data set (significance set at α=0.02) (Kanji, 1993).

Mitochondrial P5 production: Six paired t-tests were used to determine if there were any differences in P5 production between basal and each reducing precursor incubation separately (Sokal and Rohlf, 1995).

(b) Experiment 2: Effect of β-sitosterol on P450scc activity in male goldfish

Objective

Gilman (2000) showed that there was no significant effect of β-sitosterol on P450scc activity in regressed male brook trout (*Salvelinus fontinalis*). This experiment was designed to test if the same conclusions hold in male common goldfish.

β-Sitosterol

β-Sitosterol for this experiment was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Technically, instead of a β-sitosterol mixture, it was more a "phytosterol mixture rich in β-sitosterol". A proton and ^{13}C NMR spectra and a gas chromatography (GC) analysis done by Sigma showed the mixture was 55% β-sitosterol, 22% campesterol and 13% dihydrobrassicasterol, with other common plant sterols (such as stigmasterol or stigmastanol) making up the remaining balance.

General note

Because of restrictions imposed by the mitochondrial isolations (mitochondrial isolations are a lengthy process and there is a limit to the number of gonads that can be processed in a day), the experiment was done on two separate days. The first trial spanned July 15th 2000 to August 15th 2000, while the second spanned July 16th 2000 to August 16th 2000. The exposure period for both trials was 31 days. As much as possible, all manipulations were kept the same.

Fish

On July 8th 2000 (and again on July 9th 2000 for trial 2), 32 large fish of approximately the same size were transferred from their stock tanks to two experimental tanks (16 fish per tank). Tanks were randomly assigned to either experimental group: β-sitosterol or control.

Exposure

On July 15th 2000 (or July 16th for trial 2), the fish were anaesthetized by immersion in 0.05% TMS, weighed and implanted with Silastic® pellets for a concentration of either 0 μg/g (control) or 150 μg/g of β-sitosterol (pellet length was calculated for each fish individually based on its weight). The pellets were implanted in the intra-peritoneal cavity using a 12G insertion needle. The needle was disinfected with ethanol and rinsed with water before each use. The fish were returned to their tanks and left undisturbed for the next 31 days and were fed daily in the mornings.

Sampling

On August 15th 2000 (or August 16th for trial 2), the fish were anaesthetized by immersion in 0.05% TMS and bled by caudal puncture using 25G3/8 heparinized needles with 1 mL syringes (heparin solution: 150 IU of heparin per mL of double-distilled water). At least 500 μL of blood per fish was taken (the plasma was then separated and stored at –20ºC for later plasma T analysis, as per previous experiment). After bleeding,

the fish were killed by spinal severance, weighed and sexed (by visual inspection of the gonads). Their gonads were excised, weighed, placed in 1 mL of isolation buffer (App. I-S4 for composition) on ice and GSIs were computed.

Based on GSI, three males of similar reproductive states out of the 16 fish in each tank were selected and their gonads used for the remainder of the experiment.

Mitochondrial isolation

Gonadal mitochondria were isolated from each of the selected gonads as previously described. At the end of the isolation protocol, one quarter (0.5 mL) of each mitochondrial preparation was sonicated in a microcentrifuge tube on ice for three 10-sec intervals at 200kHz (Branson Cell Disruptor Model 200, Branson Ultrasonic Corp., Danbury, CT).

Incubations

There were four different incubation treatments, each with two sub-treatments (cholesterol addition) (Table 3). Each mitochondrial preparation was incubated in duplicate in each of eight possible combinations (Table 3).

Table 3: Eight different incubation treatments for mitochondrial preparations in experiment 2.

Condition of mitochondria	Initiators added	Condition of mitochondria where P450scc is tested	25-OH cholesterol
Broken + intact	Malate + NADPH	Intact AND broken	Yes
Broken + intact	Malate + NADPH	Intact AND broken	No
Broken + intact	Malate only	Intact mitochondria only	Yes
Broken + intact	Malate only	Intact mitochondria only	No
Only broken (*)	NADPH only	Broken mitochondria only	Yes
Only broken (*)	NADPH only	Broken mitochondria only	No
Broken + intact	None (buffer only)	None	Yes
Broken + intact	None (buffer only)	None	No

(*) Sonicated mitochondrial preparations

The malate + NADPH incubations used non-sonicated preparations, which contained both broken and intact mitochondria. P450scc in both types of mitochondria was activated by adding 75 μL of isolation buffer containing malate (maleic acid, monosodium salt; dissolved in isolation buffer on day of use; final concentration of 10 mM) and NADPH (tetrasodium salt; dissolved in isolation buffer on day of use; final concentration of 1 mM).

The malate only incubations also used non-sonicated preparations (containing both intact and broken mitochondria), but only intact mitochondria were activated by adding 75 μL of isolation buffer containing malate only (10 mM final concentration), without NADPH.

The NADPH only incubations used sonicated mitochondrial preparations, and hence contained only broken mitochondria. Only NADPH was added to these incubations, dissolved in 75 μL of isolation buffer for a final concentration of 1 mM. Because the mitochondrial membrane had been disrupted, NADPH could now freely reach P450scc.

The last set of incubations used the non-sonicated preparation (containing both intact and broken mitochondria) but neither malate nor NADPH was added. Instead, 75 μL of simple isolation buffer was added. This incubation will show basal, unstimulated production of P5.

Incubation protocol

The incubation protocol was very similar to that previously described in the preliminary experiment, with only minor changes. One hundred microliters of the final mitochondrial preparation (or of the sonicated preparation for incubations with NADPH only) were added to 1.785 mL of incubation medium in a shaking water bath at 18ºC. Fifteen microliters of ethanol were added, containing trilostane (final concentration of 10 μM; dissolved in ethanol on the day of use) and SU-10603 (final concentration of 200 nM; dissolved in ethanol on the day of use). Half of the incubations also received 25-hydroxycholesterol (final concentration of 10 μM; stored dissolved in ethanol at -20ºC). The final incubation medium (1.9mL) contained less than 1% ethanol to prevent ethanol toxicity. A sample of 0.475mL was removed and placed in 3mL of ethyl acetate

in a glass scintillation vial on ice. Each vial was immediately capped, vortexed and stored at –20ºC for later analysis of P5 concentration.

The reaction was initiated by adding (at precisely timed intervals) 75 μL of isolation solution containing either both malate and NADPH, only malate, only NADPH, or just isolation buffer (see Table 2).

After 15 min of incubation (and again after 30 min), a sample of 0.5 mL was removed and placed into 3 mL of ethyl acetate in a glass scintillation vial on ice (at precisely timed intervals, exactly 15 or 30 min after the time of reaction initiation). Each vial was immediately capped and vortexed to stop the reaction, and then stored at –20ºC for later determination of P5 concentration.

Sample processing

Protein content of the mitochondrial preparations, plasma T of each fish and P5 content of the incubation medium were then measured as previously described.

Data analysis

The Dixon test for outliers was used to determine if there were any outliers in the data set (significance set at α=0.02) (Kanji, 1993).

Weight, GSI and plasma T levels: Two-way ANOVAs were used (factors: treatment and trial) to determine if there were any effects of treatment or trial.

Mitochondrial P5 levels: A between-subjects repeated-measures ANOVA incorporating all factors (within-subjects factor: time; between-subjects factors: treatment, incubation, substrate addition and trial) was used to determine if there were any differences due to trial (Girden, 1992; Zar, 1999). Data from each trial were then separated and analyzed in the same way.

For each incubation, between-subjects repeated-measures ANOVAs (within-subject factor: time; between-subjects factors: treatment and substrate addition) were run to determine if treatment and/or substrate addition (or a combination thereof) had any effect on P5 levels.

The sonicated preparation incubations show the effects of β-sitosterol on P450scc activity alone (other incubations show the effects of β-sitosterol on P450scc along with other factors, because some mitochondria are intact). Results from the sonicated preparations were therefore of particular interest, and more thoroughly analyzed than the other incubations. A repeated-measures ANOVA (within-subjects factor: time; between-subjects factor: treatment) was used to determine if there were differences between treatment groups at each substrate condition (with or without 25-hydroxycholesterol).

4. β-Sitosterol and cholesterol transfer

(a) Preliminary experiment: Effect of Ovaprim on gonadal biosynthetic capacity in goldfish

Objective

Ovaprim (Syndel Laboratories, Vancouver, BC) contains a combination of a salmon gonadotropin-releasing hormone analogue (sGnRH-A) and the dopamine antagonist domperidone dissolved in propylene glycol (MacLatchy and Van Der Kraak, 1995; Leu and Chou, 1996). Ovaprim is thus a powerful stimulator of GtH release in teleosts. The purpose of this experiment was to test if Ovaprim could also increase gonadal biosynthetic capacity within 24h of injection, which would serve as biochemical "magnifying glass" to study reactive pools of cholesterol in the final experiment.

Fish

One week prior to the experiment, 48 fish of approximately the same size were transferred to four experimental tanks (12 fish per tank). Tanks were randomly assigned to experimental groups: two Ovaprim treatment tanks and two control tanks.

Exposure

On January 14th 2000, the fish were anaesthetized by immersion in 0.05% TMS, weighed and bled by caudal puncture using 25G3/8 heparinized needles with 1 mL syringes (heparin solution: 150 IU of heparin per mL of double-distilled water). At least

400 μL of blood was sampled per fish (the plasma was separated and stored at –20ºC for later plasma T analysis, as per previous experiments). The fish were then tagged by clipping the tips of the fins (pectoral and pelvic fins were clipped in alternating combinations, so as to allow later identification of each individual). They were injected with 0.5 μL/g of fish saline (0.9% NaCl saline) (control) or 0.5 μL/g of Ovaprim intra-peritoneally using a 25G3/8 needle and a graduated glass syringe. The needle was replaced after each injection, and the syringe was rinsed with fish saline between groups. After injection, the fish were released back in the tanks to recover.

Sampling

Exactly 24h later, the fish were again anaesthetized by immersion in 0.05% TMS, identified using their fin markings and bled by caudal puncture using 25G3/8 heparinized needles with 1 mL syringes. At least 500 μL of blood was sampled per fish (the plasma was then separated and stored at –20ºC for later plasma T analysis, as per previous experiments). After bleeding, the fish were killed by spinal severance and sexed (by visual inspection of the gonads). The gonads were excised, weighed, placed in 1 mL of Cortland's buffer (App. I-S1 for composition) on ice and GSIs were computed.

Based on GSI, four males and four females of similar reproductive states out of the twelve fish in each tank were selected. Their gonads were kept and used for the remainder of the experiment.

In vitro incubations

Two pieces of gonads (total of 20-25 mg) were incubated for 18h at 18ºC in duplicate (App. I–S1 for detailed protocol). There were two possible incubations: a basal incubation with only Cortland's buffer and an hCG-stimulated incubation with 10 IU of hCG per mL of incubation medium. At the end of the 18h incubation period, 900 μL of the incubation medium was sampled and frozen at –20ºC for later analysis of T concentration.

Sample processing

Plasma T and *in vitro* T production were measured as previously described in experiment 1.

Data analysis

The Dixon test for outliers was used to determine if there were any outliers in the data set (significance set at α=0.02) (Kanji, 1993).

Weight and GSI: Data were analysed using two-way nested ANOVAs to determine if there were any tank effects (nested factor) and treatment effects (fixed-effects factor) (Sokal and Rohlf, 1995; Zar, 1999). If there were no tank effects, data were pooled and t-tests were used to determine if there were any significant differences between treatment groups.

Plasma T levels: Data for before and after injection were analysed using two-way nested ANOVAs to determine if there were any tank effects (nested factor) and treatment effects (fixed-effects factor). If there were no tank effects, data were pooled and paired t-tests were used to determine if there were any differences in levels before vs. after injection of vehicle (control) or Ovaprim.

In vitro T production: Data were analysed using a two-way nested ANOVA to determine if there were any tank effects (nested factor) and treatment effects (fixed-effects factor). If there were no tank effects, data were pooled and *in vitro* T production data for each incubation (basal and hCG) were analyzed using simple t-tests to determine if there were any differences between treatment groups (control vs. Ovaprim).

(b) Preliminary experiment: Effect of $_{DL}$-aminoglutethimide (AMG) on mitochondrial cholesterol pool in male goldfish

Reactive pool of cholesterol – theory

Reactive cholesterol is cholesterol inside the mitochondria, readily available to P450scc for conversion to P5 (McNamara and Jefcoate, 1990). This experiment was based on the idea that if β-sitosterol interferes with the rate of cholesterol transfer across the mitochondrial membrane, then the reactive pool of cholesterol in mitochondria from β-sitosterol exposed fish will be smaller than in control fish. If β-sitosterol does not affect cholesterol transfer, then the reactive pools of cholesterol would be of equal sizes. This

same logic was used to show that TCDD interfered with cholesterol translocation in rats (Moore *et al.*, 1991).

$_{DL}$-Aminoglutethimide (AMG)

A mechanism to boost the build-up of reactive pools of cholesterol was required to enhance potential differences between treatment groups (control vs. β-sitosterol).

Aminoglutethimide inhibits testicular steroidogenesis *in vivo* (El Safoury and Bartke, 1974) by inhibiting P450scc activity, but too little is retained within the mitochondria *in vitro* to continue blocking P450scc (Moore *et al.*, 1991). Privalle *et al.* (1983) successfully doubled the reactive pool of cholesterol in rat adrenal mitochondria by injecting 10 mg of AMG 20 min prior to death.

Aminoglutethimide has been used in teleosts: Deb and Bhattacharya (1986) successfully used AMG to block P450scc in freshwater perch (*Anabas testudinous*) by injecting 30 μg of AMG per g of fish every second day for 15 days.

Objective

The purpose of this experiment was to identify an appropriate dosage and interval between AMG injection and death to successfully build up the reactive pool of cholesterol inside gonadal mitochondria. This experiment was a necessary step in acquiring knowledge about AMG effects in goldfish and some technical expertise for the following experiment.

Preparation of AMG solutions

The solution was prepared on the day of the injection (August 3rd 2000) by dissolving AMG in a minimal volume of 1 M hydrochloric acid (HCl) and diluting with fish saline (0.9% NaCl saline) to a pH of 3 (Moore *et al.*, 1991). This is the optimal pH for AMG stabilization (Dr. Colin Jefcoate, University of Wisconsin, Madison, WI; pers. comm.).

Three different dosages were prepared by diluting different amounts of AMG (Table 4). Because larger amount of AMG require larger volumes of HCl to dilute, fish in different AMG treatment groups received different injection volumes (Table 4).

Table 4: Four different protocols used to reach target concentrations of AMG.

ID	Target dose of AMG	Amount of AMG	Volume of HCl used to dissolve	Volume of fish saline to dilute	Volume to inject for target dose
Blank	0μg/g	0	250μL	25mL	40.6μL/g
AMG50	50μg/g	35.5mg	100μL	10mL	14.1μL/g
AMG100	100μg/g	74.5mg	150μL	15mL	20.1μL/g
AMG200	200μg/g	123.1mg	250μL	25mL	40.6μL/g

Fish

One week prior to the experiment, 32 fish of approximately the same size were transferred from stock tanks into four experimental tanks (eight fish per tank).

Exposure

On August 2nd 2000, the fish were anaesthetized by immersion in 0.05% TMS, weighed and injected intra-peritoneally with 0.5 μL/g of Ovaprim using a 25G3/8 needle and a graduated glass syringe. The needle was replaced after each injection. The fish were then returned to their tanks. Fish were injected with Ovaprim to stimulate steroidogenesis (see previous preliminary experiment).

Exactly 24h later, the fish were anaesthetized by immersion in 0.05% TMS, weighed and injected with either 0 (blank), 50, 100 or 200 μg/g of AMG (Table 4) using a 25G3/8 needle and a graduated glass syringe. The needle was disinfected with ethanol and rinsed with distilled water before each injection. Between AMG groups, the needle was replaced and the syringe was rinsed with fish saline solution. After AMG injection, the fish were returned to their tanks to recover.

One hour later, the fish were again anaesthetized by immersion in 0.05% TMS and bled by caudal puncture using 25G3/8 heparinized needles and syringes (heparin solution: 150 IU of heparin per mL of ddH2O). At least 500 μL of blood was sampled per fish (the plasma was separated and stored at –20ºC for later plasma T analysis, as per previous experiments). The fish were killed by spinal severance, weighed, and sexed (by visual inspection of the gonads). The gonad were excised, weighed, placed in 1 mL of isolation buffer (App. I–S4 for composition) on ice, and GSIs were calculated.

Based on GSI, the gonads of two males in similar reproductive states out of the eight fish in each tank were selected. Gonadal mitochondria were isolated for each of the selected gonads as previously discussed (App. I-S4 for detailed protocol).

Incubation protocol

Two-hundred microliters of the final mitochondrial preparation was added to 1.690 mL of incubation medium in a shaking water bath at 18ºC (temperature to which the fish were acclimated). Ten microliters of ethanol were added, containing trilostane (dissolved on day of use; final concentration of 10 μM) and SU-10603 (dissolved in ethanol on day of use; final concentration of 200 nM). The incubation medium (1.9 mL) contained less than 1% ethanol to avoid ethanol toxicity.

A sample of 190 μL was removed and placed in 1.5 mL of ethyl acetate in a glass scintillation vial on ice. Each vial was immediately capped, vortexed and stored at –20ºC for later analysis of P5 concentration.

The reaction was initiated by adding 90 μL of isolation buffer containing malate (maleic acid, monosodium salt; dissolved in isolation buffer on the day of use; final concentration of 10mM) to each incubation tube at carefully timed intervals. Note that no exogenous substrate (*e.g.* 25-hydroxycholesterol) was added, so that pregnenolone production is entirely dependent on the reactive pool of cholesterol inside the mitochondria.

At 5, 10, 15, 20, 30, 45, 60 and 90 min, 200 μL were removed from each incubation tube and placed in 1.5 mL of ethyl acetate in a glass scintillation vial on ice. Each vial was immediately capped and vortexed to terminate the reaction. At the end of each set, the samples were taken to the freezer and stored at –20ºC for later analysis of P5 concentration.

Sample processing

Protein content of the mitochondrial preparation, plasma T for each fish and P5 content of the incubation medium were determined as previously described.

Data analysis

Weight, GSI and plasma T levels: Data were analysed using a one-way ANOVA to determine if there were any differences among treatment groups.

Mitochondrial P5 production: P5 production for each time interval was plotted for each treatment (control, AMG50, AMG100 and AMG200), and the data were graphically analyzed. No statistical tests were run on these data because of the low sample size (n=2).

(c) Experiment 3: Effect of β-sitosterol on the rate of cholesterol transfer across the mitochondrial membrane in male goldfish

Objective

The objective of this experiment was to determine if β-sitosterol exposure *in vivo* had any effect on the size of the reactive pool of cholesterol inside gonadal mitochondria. An increase would suggest that β-sitosterol is interfering with cholesterol transfer across the mitochondrial membranes.

Two trials

Two different trials of this experiment were carried out. The first trial spanned August 5th 2000 to September 2nd 2000 (exposure period of 28 days), while the second spanned May 21st 2001 to June 14th 2001 (exposure period of 24 days). While both trials aimed to illustrate the effects of β-sitosterol on mitochondrial pools of cholesterol, there are important differences in the two which will be pointed out in the text.

β-Sitosterol

β-Sitosterol for the first trial was the 55% pure β-sitosterol used in experiment 2. The large percentage of other phytosterols in the mixture (22% campesterol, 13% dihydrobrassicasterol, and 10% other common phytosterols) prompted a second trial with a purer β-sitosterol preparation.

β-Sitosterol for the second trial was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). High performance liquid chromatography (HPLC) analysis done by the supplier showed it to be 95.7% pure β-sitosterol. Impurities in the mixture are other common plant sterols.

Fish

One week prior to the start of the experiment, fish of approximately the same length and weight were transferred from their stock tanks to four 62-L experimental tanks (10 fish per tank in trial 1, 12 fish per tank in trial 2). Tanks were randomly assigned to experimental groups: two for β-sitosterol and two for control.

Exposure

On August 5th 2000 (or May 21st in trial 2), the fish were anaesthetized by immersion in 0.05% TMS, weighed and implanted with Silastic® pellets containing either 0 μg/g (control) or 150 μg/g of β-sitosterol preparation. The pellets were implanted in the intra-peritoneal cavity using a 12G insertion needle, disinfected with ethanol and rinsed with water after each implantation. The fish were then returned to their tanks, and left undisturbed (except for the daily feeding) for the remainder of the exposure period.

Ovaprim injection

On September 1st 2000 (or June 12th for trial 2), the fish were anaesthetized by immersion in 0.05% TMS, weighed and injected intra-peritoneally with 0.05 μL/g of Ovaprim using a 25G3/8 needle with a graduated syringe. A new needle was used for each fish. The fish were then returned to their tanks to recover.

Preparation of the AMG solution

The AMG solutions were prepared on the morning of the next day (September 2nd 2000 for trial 1, June 13th 2001 for trial 2).

In trial 1, a total of 40 mg of AMG was dissolved in 100μL HCl and then diluted in 10 mL fish saline (0.9% NaCl saline) for a pH of 3 (optimum pH for AMG). For a dose of 50 μg/g of AMG, each fish had to be injected with 12.5 μL/g of solution. The blank solution (vehicle, no AMG) was also prepared by adding 100 μL of HCl to 10 mL of fish saline (0.9% NaCl) for a pH of 3.

In trial 2, a slightly different method was used to minimize the volume of the injections. A total of 100 mg of AMG was dissolved in 800 μL of 1 M HCl to which 330 μL of 1 M NaOH and 4 mL fish saline (0.9% NaCl saline) were added for a final pH of 3. For a dose of 50 μg/g of AMG, each fish would have to be injected with only 2.56 μL/g of solution. The blank solution (vehicle, no AMG) was prepared by adding 400 μL of 1 M HCl to 330 μL of 1 M NaOH and 4.4mL of fish saline (0.9% NaCl) for a pH of 3.

Sampling

Exactly 24h after the Ovaprim injection, the fish were anaesthetized by immersion in 0.05% TMS and weighed. One of each of the control and β-sitosterol tanks was assigned at random to the AMG group, while the other (one control and one β-sitosterol) was assigned to the blank "no AMG" injection group. The AMG group was injected with AMG solution (for a dose of 50μg/g of AMG) using a 25G3/8 needle with a graduated syringe (the needle was replaced after each use), while the no-AMG group was injected with the same volume of blank solution. The fish were then returned to their tanks.

Sixty minutes later, the fish were again anaesthetized by immersion in 0.05% TMS, weighed and bled by caudal puncture using heparinized 25G3/8 needles and syringes (heparin solution: 150 IU of heparin per mL of ddH_2O). At least 500 μL of blood was sampled per fish (the plasma was separated and stored at –20ºC for later plasma T analysis, as per previous experiments).

The fish were killed by spinal severance and sexed (by visual inspection of the gonads). Gonads were excised, weighed, placed in 1 mL of isolation buffer (App. I–S4 for composition) on ice, and GSIs were calculated.

In trial 1, the gonads of three males in similar reproductive states out of each tank were selected based on GSI. In trial 2, an unbalanced M:F ratio of 1:3 in both no AMG tanks left only three males in similar reproductive states (instead of the intended four). In the AMG tanks, however, the gonads of four males were selected based on GSI.

Mitochondria isolation

In trial 1, gonadal mitochondria were isolated for each of the selected gonads as previously discussed (App. I-S4 for detailed protocol).

In trial 2, due to the limiting amount of gonad material in the no-AMG group, 400 mg of gonad tissue was used per preparation (instead of the usual 800 mg) for all groups (including the AMG group, to standardize the preparations as much as possible). To compensate, the isolation buffer volume used in the isolation procedure was lowered to 4 mL (instead of 8 mL) to maintain the same concentration of gonad material to isolation volume (as suggested in Ballantyne, 1994). All other aspects of the isolation protocol were kept the same as in trial 1.

Incubations

The incubation protocol for both trials was the same as previously described in the preliminary AMG experiment, with the exception of sampling intervals. In trial 1, the incubation medium was sampled at 5, 10, 15 and 20 min. In trial 2, these intervals were changed to 6, 12, 18 and 24 min to accommodate the larger sample size.

Sample processing

Protein content of each mitochondrial preparation, plasma T concentrations and P5 content of the incubation medium were determined as previously described.

In addition, an enzyme-linked immuno sorbent assay (ELISA) was used to determine plasma Vtg concentration in trial 2 only (App. I-S6 for detailed protocol). Intra-assay variability for plasma Vtg was 2.6%, and inter-assay variability was 5.1%.

Data analysis for trial 1

Because of the fundamental differences between trials (purity of the β-sitosterol, difference in amount of gonad material used), data for each trial were analysed separately.

The Dixon test for outliers was used to determine if there were any outliers in the data set (significance set at α=0.02) (Kanji, 1993).

Weight, GSI and plasma T levels: A two-way ANOVA (factors: treatment and AMG injection) was used to test for differences between treatment groups.

P5 content of the incubation medium: A between-subjects repeated-measures ANOVA incorporating all factors (within-subjects factor: time; between-subjects factors: treatment and AMG injection) was used to determine if there were any differences in P5 content of the medium between treatment and/or AMG injection groups (Girden, 1992; Zar, 1999).

Repeated-measures ANOVAs were then run for each AMG injection group separately (within-subjects factor: time; between-subjects factor: treatment) to determine if there were any differences between β-sitosterol and control within each AMG injection groups.

P5 production: Production for each AMG injection group was also plotted against time, and a repeated-measures ANOVA was run on P5 production (within-subjects factor: time; between-subjects factor: treatment) to test if there was a main treatment effect on P5 production. t-Tests were also used to determine if there were any differences between treatment groups at each production interval.

Data analysis for trial 2

Different AMG treatment groups (no AMG vs. AMG) were analyzed independently because of the differences in sample size (n=3 in the no AMG group and n=4 in the AMG group) and their initial differences in GSI (due in part to the limited choice imposed by the unbalanced sex ratio in the no AMG groups).

The Dixon test for outliers was used to determine if there were any outliers in the data set (significance set at α=0.02) (Kanji, 1993).

Weight, GSI and plasma T levels: t-tests were used to test for differences between β-sitosterol and control groups.

Plasma Vtg concentrations: t-tests were also used to test for differences between β-sitosterol and control groups.

P5 levels: Repeated-measures ANOVAs were run for each AMG injection group separately (within-subjects factor: time; between-subjects factor: β-sitosterol treatment) to determine if there were any differences between β-sitosterol and control groups.

P5 production: as in trial 1.

III. RESULTS

1. β-Sitosterol and the steroidogenic pathway

(a) Experiment 1: Effect of β-sitosterol on the steroidogenic pathway in goldfish

Overall effects using the entire data set (all fish)

Weight: There was no effect of tanks on weight in males or in females (two-way nested ANOVA, p=0.395 and p=0.776, respectively) (data not shown). Once the tanks were pooled, there was no difference in weight between β-sitosterol and control groups in males or in females (t-test, p=0.996 and p=0.873, respectively) (Table 5).

Table 5: Experiment 1 - Mean weight, GSI and plasma T for all fish in each treatment by sex. Numbers in parentheses are sample sizes. * denotes values that differ significantly from same sex control ($p<0.05$).

Group	Weight ± SE (g)	GSI ± SE	Plasma T (ng / mL) ± SE
Control %	47.07 ± 2.35 (14)	1.09 ± 0.21 (14)	1.26 ± 0.21 (13)
β-sit treated %	47.05 ± 4.11 (11)	0.99 ± 0.18 (11)	0.42 ± 0.11 (9)*
Control &	60.71 ± 6.06 (10)	1.61 ± 0.26 (10)	0.72 ± 0.08 (8)
β-sit treated &	59.56 ± 4.02 (12)	1.55 ± 0.17 (12)	0.62 ± 0.11 (12)

GSI: There was borderline significant effect of tank on GSI in males in males but not in females (two-way nested ANOVA, p=0.048 and p=0.284, respectively) (data not shown). There was no difference in GSI between β-sitosterol and control groups in males (tanks not pooled, two-way nested ANOVA, p=0.853) or in females (tanks pooled, t-test, p=0.833) (Table 5).

Plasma T: There was no effect of tank on plasma T in males or in females (two-way nested ANOVA, p=0.613 and p=0.264, respectively) (data not shown). Once the tanks were pooled, β-sitosterol exposure significantly decreased plasma T in males, but not in females (t-test, p=0.004 and p=0.450, respectively) (Table 5).

GSI and plasma T (only fish whose gonads were selected)

GSI: There was no statistical difference in GSI of selected fish between control and β-sitosterol in males or females (t-test, p=0.253 and p=0.125, respectively) (Table 6).

Table 6: Experiment 1 - GSI of fish selected for each treatment by sex. Numbers in parentheses are sample sizes.

Group	GSI ± SE
Control %	1.56 ± 0.24 (8)
β-sit treated %	1.18 ± 0.21 (8)
Control &	1.84 ± 0.26 (8)
β-sit treated &	1.36 ± 0.15 (8)

Plasma T: β-Sitosterol exposure significantly decreased plasma T in males, but not in females (t-test, p=0.011 and p=0.313, respectively) (Figure 5).

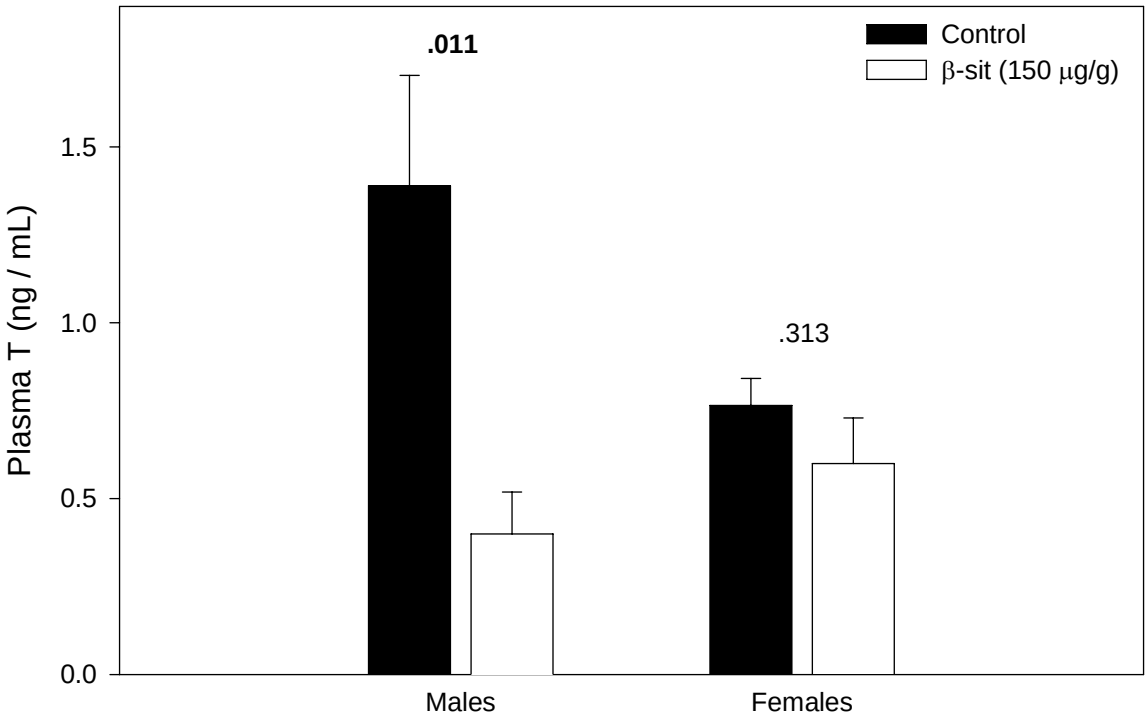

Figure 5: Experiment 1 - Mean plasma T in male and female goldfish. Error bars represent + 1SE. Numbers above pairs of bars are the p-values of simple t-tests between control and treatment groups within sex (n=8).

In vitro T production

There was no effect of tank on *in vitro* T production in males or in females (two-way nested ANOVA, p=0.491 and p=0.080, respectively) (data not shown). Data were thus pooled across tanks.

In males, *in vivo* β-sitosterol exposure significantly decreased *in vitro* T production in basal (t-test, p=0.001), hCG (t-test, p=0.003), 25-hydroxycholesterol (t-test, p=0.020), 17P5 (t-test, p=0.040) and DHEA incubations (t-test, p=0.015), while there was no significant difference between control and β-sitosterol in P5 (t-test, p=0.069), P4 (t-test, p=0.315), 17P4 (t-test, p=0.111) and AD incubations (Mann-Whitney Rank Sum Test, p=0.130) (Figure 6).

In females, *in vivo* β-sitosterol exposure significantly decreased *in vitro* T production in basal (Mann-Whitney Rank Sum Test, p=0.021), hCG (Mann-Whitney Rank Sum Test, p=0.038), 25-hydroxycholesterol (t-test, p=0.028), P5 (Mann-Whitney Rank Sum Test, p=0.007), 17P5 (t-test, p=0.006) and 17P4 incubations (t-test, p=0.033), while there was no significant difference between control and β-sitosterol in P4 (t-test, p=0.670), DHEA (t-test, p=0.848) or AD incubations (t-test, p=0.056) (Figure 7).

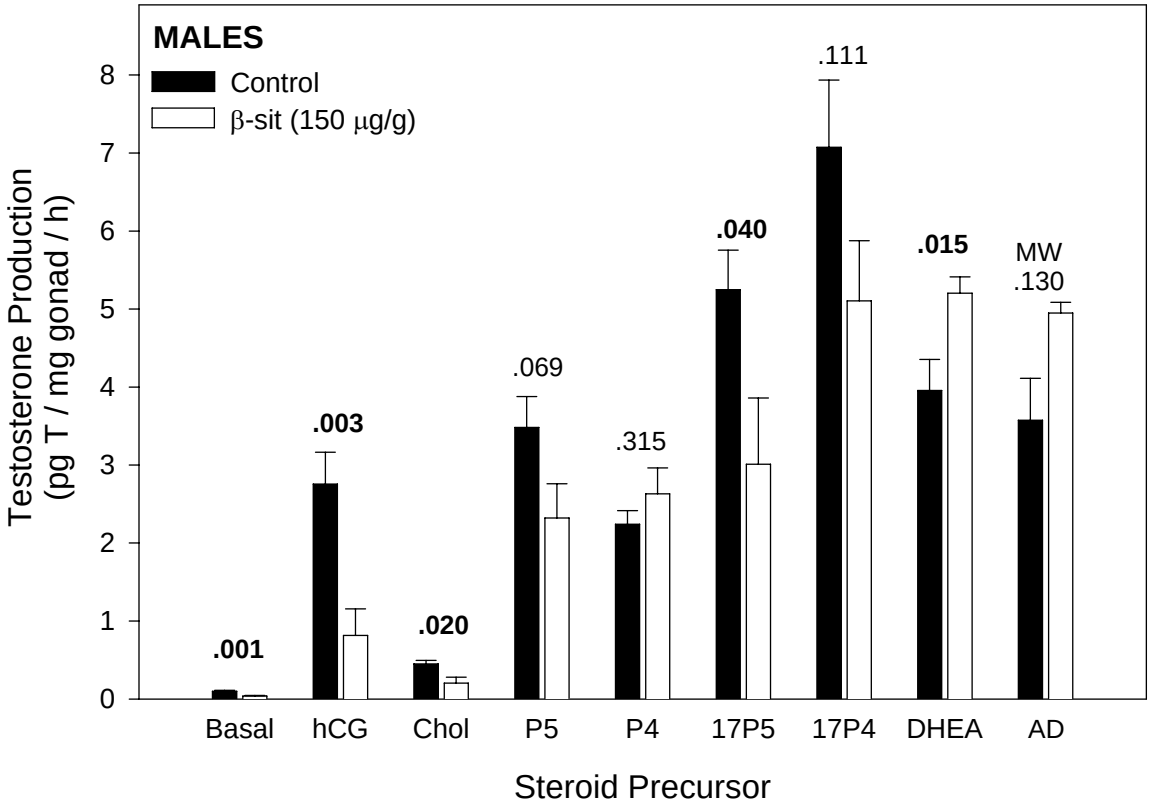

Figure 6: Experiment 1 - Mean *in vitro* T production of male gonads. Testes were incubated in saline solution alone (Basal), saline solution with 10 IU/mL of hCG (hCG), 5 μg/mL of 25-hydroxycholesterol (Chol), or 100 ng/mL of pregnenolone (P5), progesterone (P4), 17α-hydroxypregnenolone (17P5), 17α-hydroxyprogesterone (17P4), DHEA (DHEA) or androstenedione (AD). Error bars represent +1SE. Numbers above pairs of bars are p-values of simple t-tests between control and treatment, except in the final incubation (AD), where the number is the p-value resulting from a Mann-Whitney Rank Sum Test (MW) (n=8).

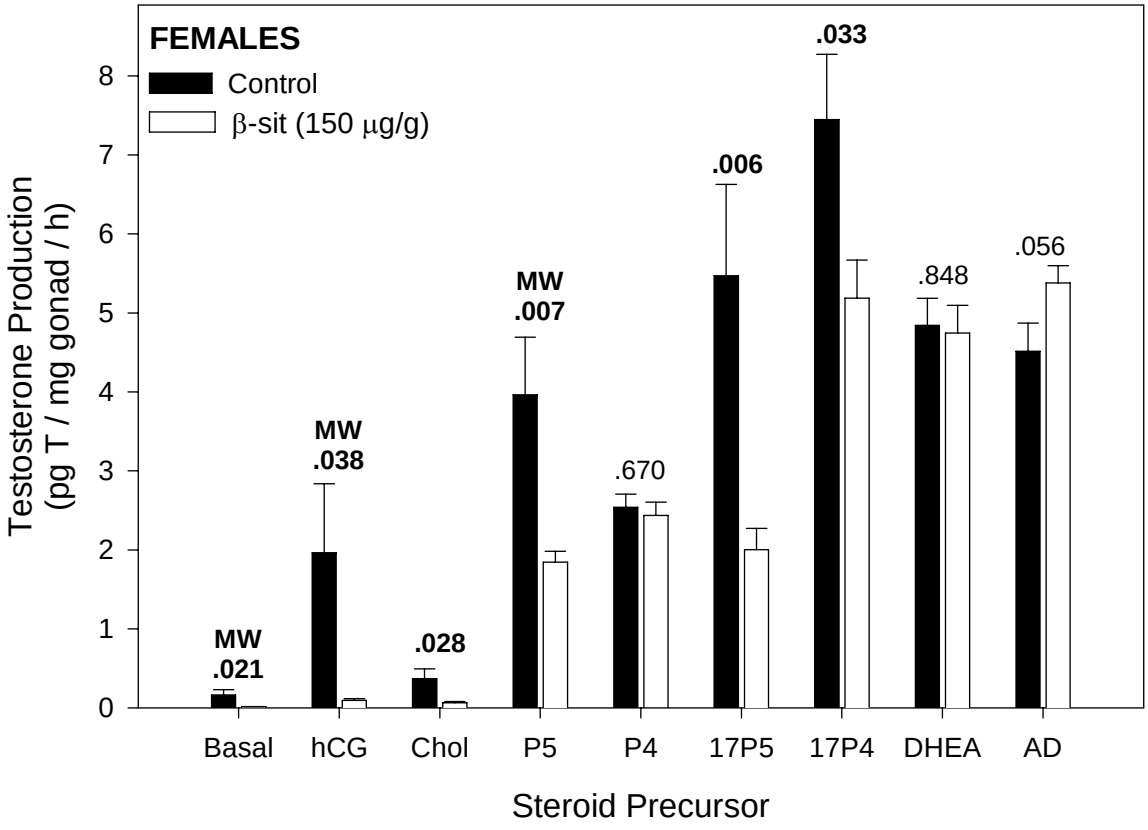

Figure 7: Experiment 1 - Mean *in vitro* T production of female gonads. Gonads were incubated in saline solution alone (Basal), saline solution with 10 IU/mL of hCG (hCG), 5 μg/mL of 25-hydroxycholesterol (Chol), or 100 ng/mL of pregnenolone (P5), progesterone (P4), 17α-hydroxypregnenolone (17P5), 17α-hydroxyprogesterone (17P4), DHEA (DHEA) or androstenedione (AD). Error bars represent +1SE. Numbers above pairs of bars are p-values of t-tests between control and treatment, except in the first two incubations (Basal and hCG), where the number is the p-value of a Mann-Whitney Rank Sum Test (MW) (n=8).

2. β-Sitosterol and P450scc activity

(a) Preliminary experiment: Finding a reducing precursor to NADPH in goldfish mitochondria

P5 production

The 10 mM isocitrate and malate incubations were the most efficient at inducing P5 production in intact mitochondrial preparations (Figure 8). Although the 50 mM malate incubation induced the highest mean P5 production, it was not statistically different from the basal P5 production because of its greater variance.

With isocitrate, mitochondrial P5 production in both the 10 mM and the 50 mM incubations was significantly higher than the basal incubation (paired t-tests, $p=0.018$ and $p=0.035$, respectively) (Figure 8).

With malate, only the 10 mM incubation was significantly higher than the basal incubation (paired t-test, $p=0.018$). The 50 mM incubation was not significantly different from the basal incubation (paired t-test, $p=0.139$) (Figure 8).

Neither the 10 mM nor the 50 mM succinate incubations were significantly different from the basal incubation (paired t-tests, $p=0.175$ and $p=0.236$, respectively) (Figure 8).

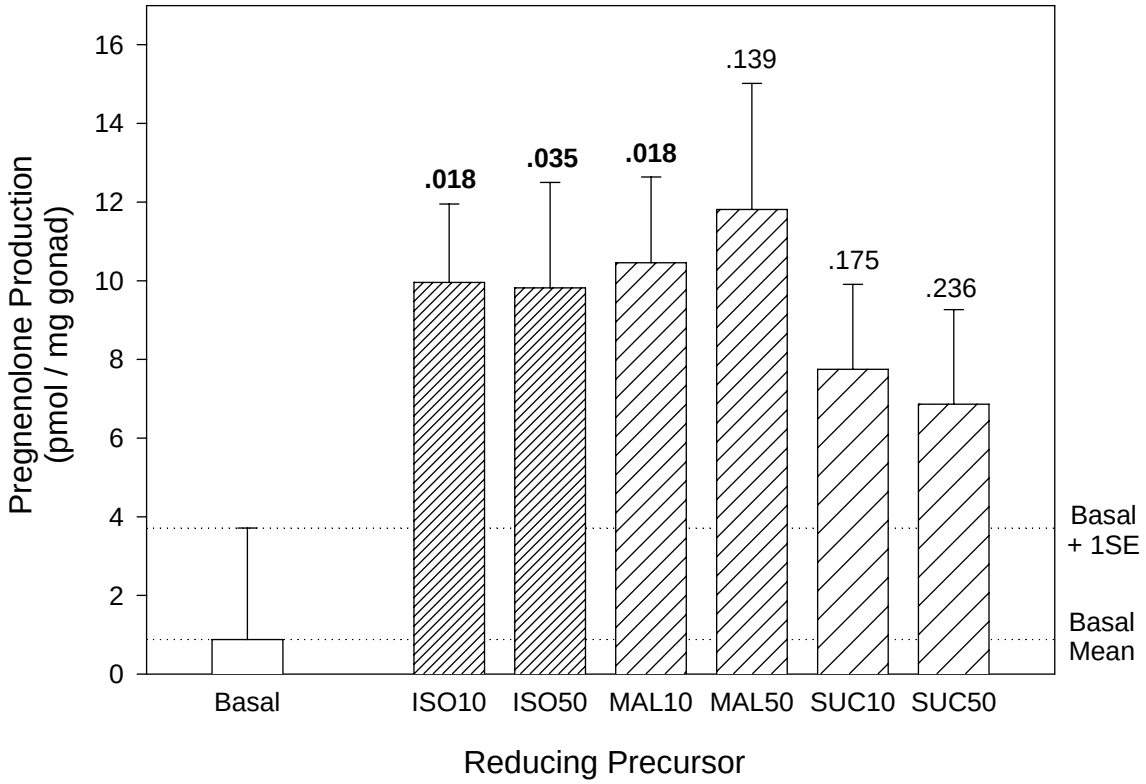

Figure 8: Mean *in vitro* P5 production of gonadal mitochondria isolated from male goldfish after 15 minutes in incubation media containing no reducing precursor (Basal), 10 or 50 mM of isocitrate (ISO10 and ISO50, respectively), 10 or 50 mM of malate (MAL10 and MAL50, respectively) and 10 or 50 mM of succinate (SUC10 and SUC50, respectively). Error bars represent +1SE. Numbers above bars represent p-values of paired t-tests between basal and each incubation (n=4).

(b) Experiment 2: Effect of β-sitosterol on P450scc activity in male goldfish

Overall effects using the entire data set (all fish)

A two-way ANOVA showed no significant interaction between trial and treatment on weight, GSI or plasma T ($p=0.751$, $p=0.254$ and $p=0.745$, respectively), and no difference in weight, GSI or plasma T between trials ($p=0.590$, $p=0.504$ and $p=0.227$, respectively) or treatment groups ($p=0.858$, $p=0.387$ and $p=0.149$, respectively) (Table 7).

Table 7: Experiment 2 – Mean weight, GSI and plasma T of all fish for each treatment group by trial. Numbers in parentheses are sample sizes.

β-Sit treatment	Weight (g) ± SE	GSI ± SE	Plasma T (ng/mL) ± SE
Trial 1 - Control	111.8 ± 27.7 (5)	0.743 ± 0.217 (5)	0.95 ± 0.29 (5)
Trial 1 - β-sit	102.0 ± 14.8 (4)	1.210 ± 0.320 (4)	0.59 ± 0.10 (4)
Trial 2 - Control	116.2 ± 15.0 (4)	0.856 ± 0.187 (4)	1.44 ± 0.53 (4)
Trial 2 - β-sit	119.0 ± 11.4 (5)	0.790 ± 0.164 (5)	0.87 ± 0.19 (5)

GSI and plasma T (only fish whose gonads were selected)

β-Sitosterol exposure decreased plasma T, but not significantly (Table 8). A two-way ANOVA revealed no significant interaction between trial and treatment on GSI or plasma T ($p=0.315$ and $p=0.959$, respectively), and no difference in GSI or plasma T between trials ($p=0.212$ and $p=0.421$, respectively) or treatment groups ($p=0.357$ and $p=0.081$, respectively) (Table 8).

Table 8: Experiment 2 - GSI and plasma T of selected fish by treatment and trial group. Numbers in parentheses are sample sizes.

Trial and β-sit group	GSI ± SE	Plasma T (ng/mL) ± SE
Trial 1 – Control	1.08 ± 0.10 (3)	1.38 ± 0.22 (3)
Trial 1 - β-sit exposed	1.45 ± 0.30 (3)	0.65 ± 0.11 (3)
Trial 2 – Control	1.03 ± 0.09 (3)	1.71 ± 0.64 (3)
Trial 2 - β-sit exposed	1.01 ± 0.13 (3)	0.95 ± 0.30 (3)
Control – trials pooled	1.06 ± 0.06 (6)	1.54 ± 0.31 (6)
β-sit exposed – trials pooled	1.23 ± 0.18 (6)	0.80 ± 0.16 (6)

P5 production

General effects: A between-subject repeated-measures ANOVA combining all factors (within-subject factor: time; between-subjects factors: treatment, incubation, substrate addition and trial) showed no significant 5^{th} or 4^{th} level interactions, but the presence of significant 3^{rd} level interactions (App. II-S1) required that the two trials be analyzed separately; between-subject repeated-measures ANOVAs (within-subjects factor: time; between-subject factors: treatment and substrate addition) were run for each of the four incubations.

Malate + NADPH (both broken and intact mitochondria): β-Sitosterol exposure had no significant effect on either changes of P5 over time or general P5 level. Addition of 25-hydroxycholesterol significantly increased P5 production (Figures 9 and 10, top left).

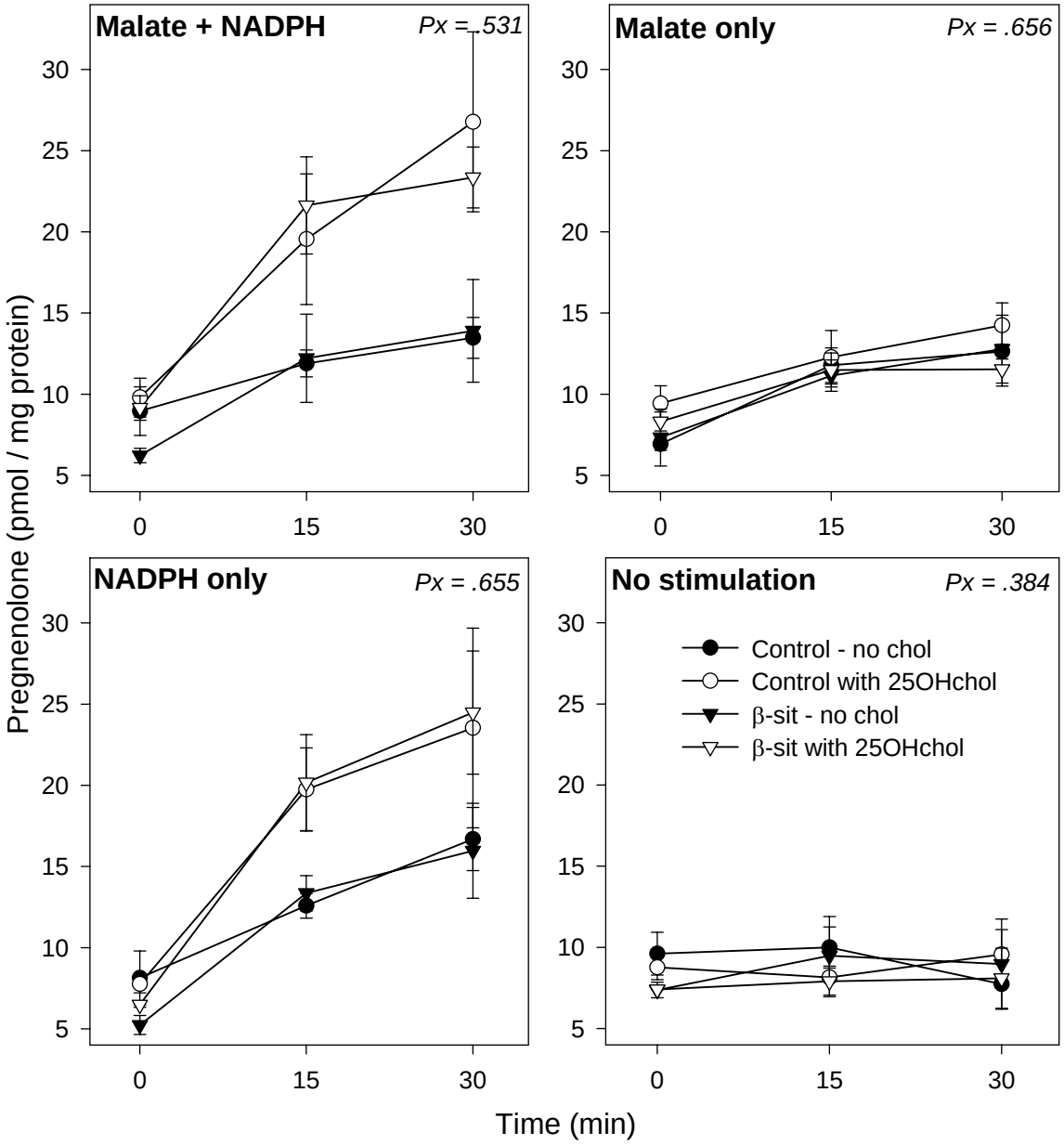

Figure 9: Experiment 2 - Mean P5 production of gonadal mitochondria in **trial 1**. Error bars represent ±1SE. The Px value represents the p value of the interaction between treatment and time (*i.e.* effect of treatment on changes in P5 over time) (n=3).

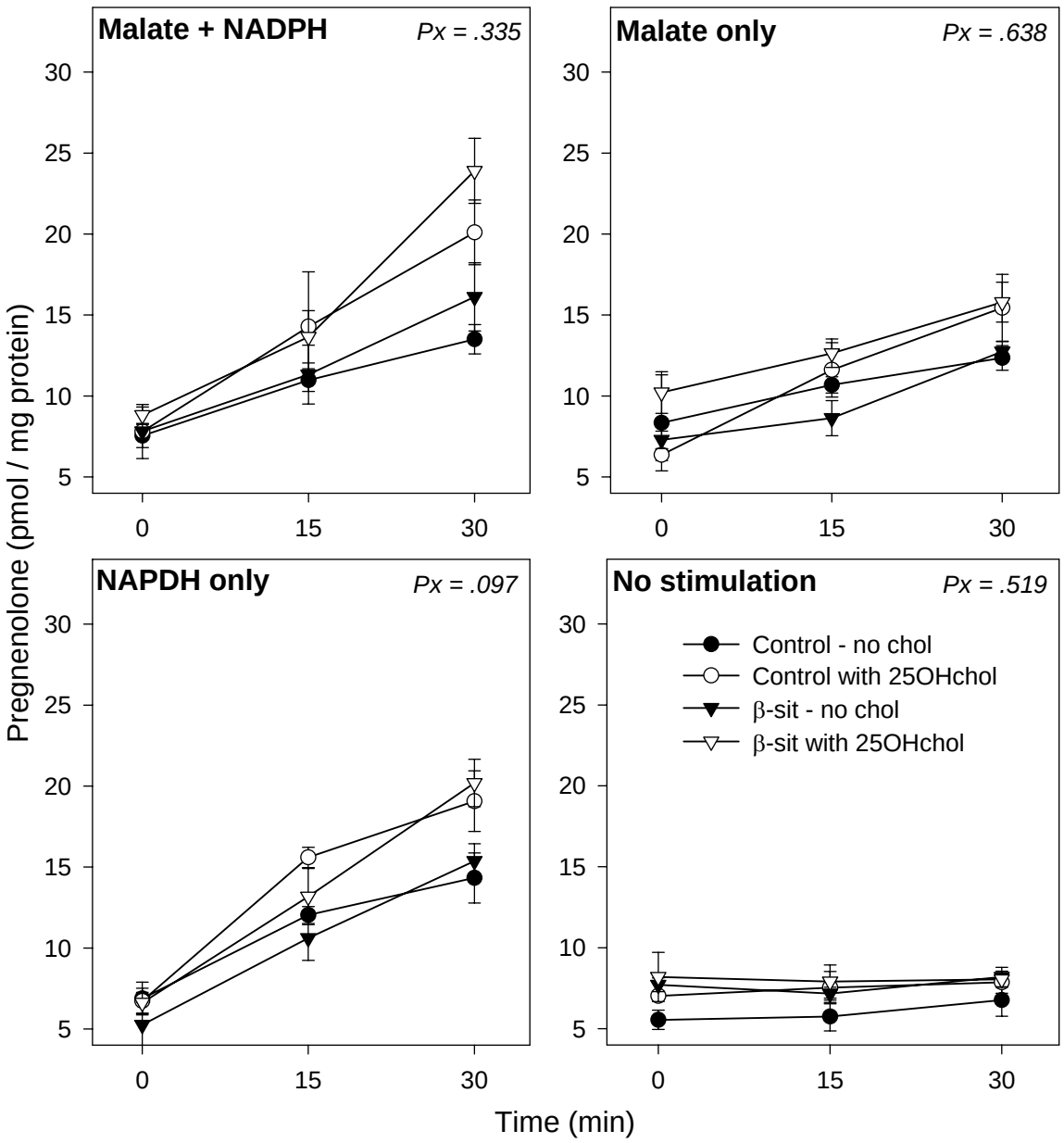

Figure 10: Experiment 2 - Mean P5 production of gonadal mitochondria in **trial 2**. Error bars represent ±1SE. The Px value represents the p value of the interaction between treatment and time (*i.e.* effect of treatment on changes in P5 over time) (n=3).

In both trials, the 3rd level interaction (time, treatment and substrate) was not significant (trial 1, p=0.510; trial 2, p=0.884) and the only significant 2nd level interaction was between time and substrate (trial 1, p=0.012; trial 2, p=0.033). More importantly, there was no interaction between time and treatment (trial 1, p=0.531; trial 2, p=0.335) and no main treatment effect (trial 1, p=0.758; trial 2, p=0.283). There was significant P5 production over time (p<0.001) (App. II-S2).

Malate only (intact mitochondria only): β-Sitosterol exposure had no significant effect on either changes of P5 over time or general P5 level. Addition of 25-hydroxycholesterol significantly increased P5 levels in trial 2 (Figure 9 and 10, top right).

In trial 1, the 3rd level interaction was not significant (p=0.422), and neither were any of the 2nd level interactions. Although there was significant P5 production over time (p<0.001), there was no main effect of either substrate (p=0.492) or treatment (p=0.493) (App. II-S3).

In trial 2, the 3rd level interaction was not significant (p=0.471), and none of the 2nd level interactions were significant. There was a significant main effect of substrate on P5 levels (p=0.029), but there was no main effect of treatment (p=0.591). There was significant P5 production over time (p<0.001) (App. II-S3).

NADPH only (broken mitochondria only): β-Sitosterol had no significant effect on either changes of P5 over time or general P5 level. Addition of 25-hydroxycholesterol significantly increased P5 levels (Figure 9 and 10, bottom left).

The responses in each trial differed slightly. In trial 1, the 3rd and 2nd level interactions were not significant; the only significant main effect was substrate addition ($p=0.035$) and a significant P5 production over time ($p<0.001$). There was no main effect of treatment ($p=0.823$) (App. II-S4).

In trial 2, the 3rd level interaction was not significant ($p=0.640$), and the only significant 2nd level interaction was between time and substrate ($p=0.019$). More importantly, there was no significant impact of treatment on P5 changes over time ($p=0.097$) and no main effect of treatment ($p=0.581$). There was significant P5 production over time ($p<0.001$) (App. II-S4).

No stimulation (incubations with no malate or NAPDH): In both trials, there was no significant interaction at any levels. There was no significant P5 production over time (trial 1, $p=0.657$; trial 2, $p=0.242$) (App. II-S5) (Figure 9 and 10, bottom right).

3. β-Sitosterol and cholesterol transfer

(a) Preliminary experiment: Effect of Ovaprim on gonadal biosynthetic capacity in goldfish

Overall effects using the entire data set (all fish)

Weight: There was no effect of tank on weight in males or in females (two-way nested ANOVA, p=0.468 and p=0.093, respectively) (data not shown). Data were pooled across tanks, and there was no difference between injection groups in males (Mann-Whitney Rank Sum Test, p=0.430) or in females (t-test, p=0.828) (Table 9).

Table 9: Mean weight and GSI for each injection group (control vs. Ovaprim) by sex. Numbers in parentheses are sample sizes.

Ovaprim treatment	Weight ± SE (g)	GSI ± SE
Control %	53.48 ± 3.34 (13)	1.73 ± 0.36 (12)
Ovaprim treated %	50.29 ± 3.16 (12)	1.80 ± 0.34 (12)
Control &	53.16 ± 5.25 (10)	1.38 ± 0.26 (10)
Ovaprim treated &	51.89 ± 2.97 (12)	1.09 ± 0.14 (12)

GSI: There was no effect of tank on GSI in males, but there was a significant difference among tanks in females (two-way nested ANOVA, p=0.661 and p=0.031, respectively) (data not shown). There was no difference between injection groups in males (tanks pooled, t-test, p=0.881) or in females (tanks not pooled, two-way nested ANOVA, p=0.624) (Table 9).

Effect of injection on plasma T levels

There was a significant increase in plasma T level after Ovaprim injection in males, but not in females (paired t-test, p=0.003 and p=0.068, respectively) (Figure 11). There was no change in plasma T levels after injection in the control (vehicle injections) in males or in females (paired t-test, p=0.090 and p=0.740, respectively) (Figure 11).

In vitro T production

There was no difference in *in vitro* T production among different tanks in males (two-way nested ANOVA, p=0.749) or in females (two-way nested ANOVA, p=0.431) (data not shown). Data was therefore pooled across tanks.

In males, gonads from Ovaprim treated fish produced significantly more T in 18h compared to those from control fish, both in basal and hCG-stimulated incubations (t-test, p=0.012 and p=0.010, respectively) (Figure 12). In females, on the other hand, gonads from Ovaprim treated fish produced the same amount of T in 18h as those from control fish, both in basal and hCG-stimulated incubations (t-test, p=0.596 and p=0.580, respectively) (Figure 12).

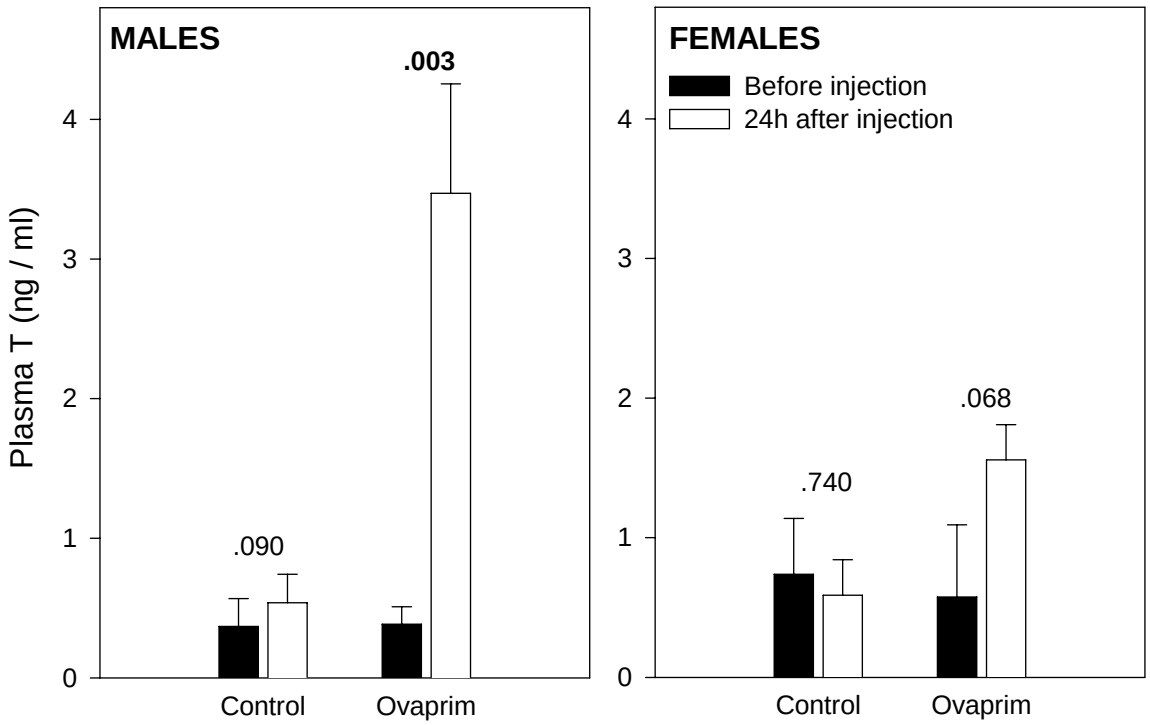

Figure 11: Mean plasma T levels in fish injected with vehicle (Control) or 0.05 μL/g Ovaprim. Error bars represent +1SE. Numbers above pairs of bars are p-values of paired t-tests testing for differences between plasma T levels before and after injections (n=8).

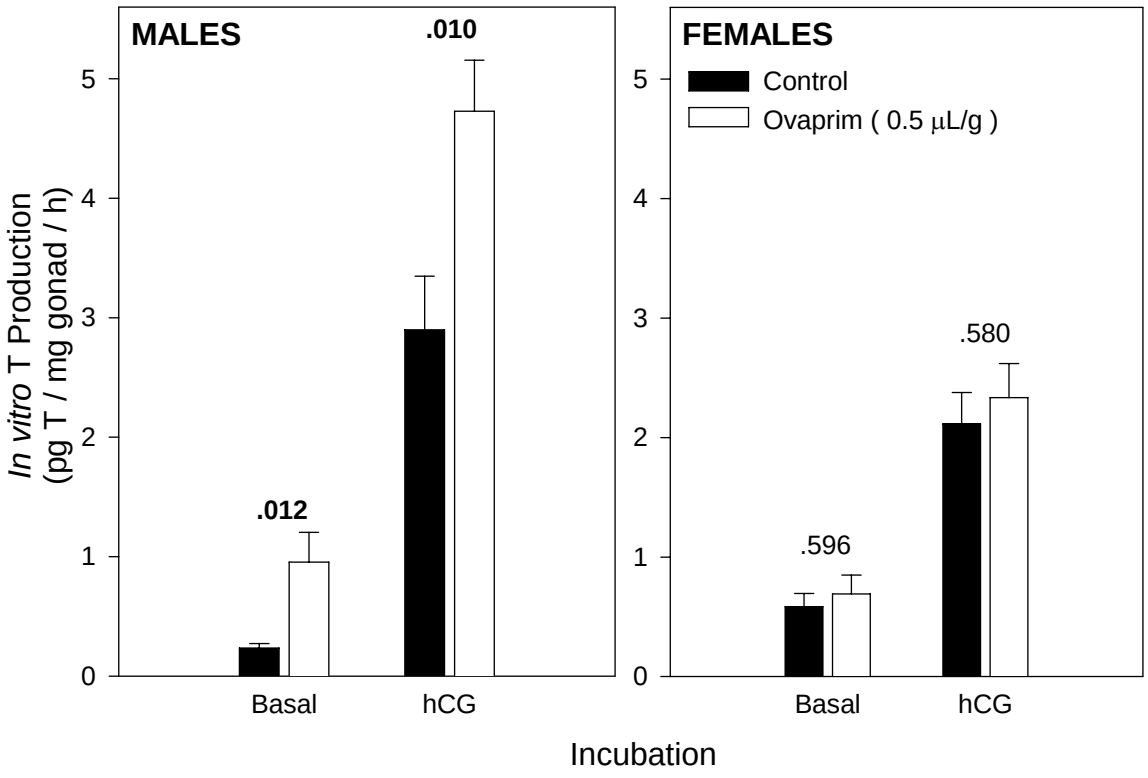

Figure 12: Mean *in vitro* T production. Error bars represent +1SE. Numbers above pairs of bars are p-values of simple t-tests comparing control vs. ovaprim treatment for each different incubation (Basal or hCG-stimulated) (n=8).

(b) Preliminary experiment: Effect of AMG on mitochondrial cholesterol pool in male goldfish

Overall effects

There was no significant difference in weight, GSI and plasma T (one-way ANOVA, p=0.707, p=0.968 and p=0.990, respectively) among the different AMG injection groups (Table 10).

Table 10: Mean weight, GSI and plasma T levels for each AMG injection group (control, AMG50, AMG100 and AMG200). Numbers in parentheses are sample sizes.

AMG group	Weight (g) ± SE	GSI ± SE	Plasma T (ng/mL) ± SE
Control	59.44 ± 5.24 (3)	0.98 ± 0.16 (3)	5.24 ± 1.60 (3)
AMG (50μg/g)	66.84 ± 5.61 (6)	0.88 ± 0.23 (6)	5.51 ± 2.37 (6)
AMG (100μg/g)	57.47 ± 6.29 (6)	1.02 ± 0.33 (6)	6.06 ± 1.37 (6)
AMG (200μg/g)	66.86 ± 13.94 (3)	0.82 ± 0.46 (3)	5.28 ± 1.71 (3)

P5 production

Figure 13 shows P5 production per interval (amount of P5 produced since the previous interval). Pregnenolone production was highest at 10 min with AMG50 and at 15 min with AMG100 and AMG200, while production in the control group remained at fairly low levels throughout.

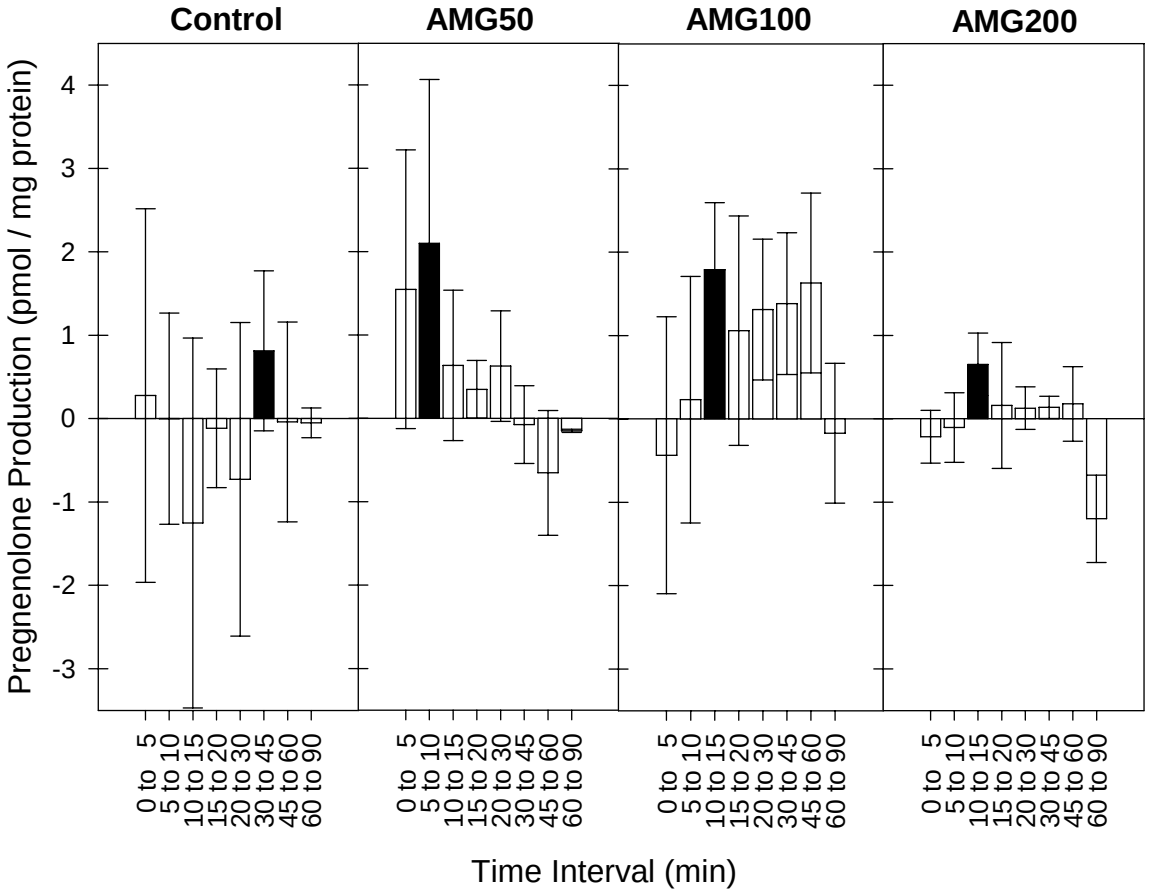

Figure 13: Mean P5 production per interval in each of the four different AMG injection groups (Control, 50, 100 and 200 μg of AMG per g). Error bars represent ±1SE. The black bar highlights the interval of highest P5 production for that group.

(c) Experiment 3 (trial 1): Effect of phytosterols on the rate of cholesterol transfer across the mitochondrial membrane in male goldfish

Overall effects using the entire data set (all male fish)

There was no significant interaction between treatment and AMG injection group on weight, GSI or plasma T (two-way ANOVA, p=0.235, p=0.493 and p=0.391, respectively), and there was no difference in weight, GSI or plasma T between treatment (p=0.334, p=0.612 and p=0.186, respectively) and AMG injection groups (p=0.504, p=0.895 and p=0.424, respectively) (Table 11).

Table 11: Experiment 3 (trial 1) – Mean weight, GSI and plasma T of all male fish for each combination of treatment and AMG injection group (C = control, no AMG; C+AMG = control, AMG injected; β = β-sitosterol, no AMG; β+AMG = β-sitosterol, AMG injected). Numbers in parentheses are sample sizes.

Group	Weight (g) ± SE	GSI ± SE	Plasma T (ng / mL) ± SE
C	33.66 ± 4.10 (4)	2.02 ± 0.39 (4)	7.52 ± 2.17 (4)
C+AMG	41.90 ± 5.24 (6)	1.63 ± 0.32 (6)	7.43 ± 1.20 (6)
β	34.68 ± 3.85 (7)	1.45 ± 0.51 (7)	4.37 ± 0.66 (7)
β+AMG	32.31 ± 2.40 (5)	1.71 ± 0.51 (5)	6.74 ± 1.83 (5)

GSI and plasma T (only fish whose gonads were selected)

There was no significant interaction between treatment and AMG injection on GSI or plasma T (two-way ANOVA, p=0.993 and p=0.321, respectively), and there was no significant difference in GSI or plasma T between treatment (t-test, p=0.200 and

p=0.443, respectively) and AMG injection groups (t-test, p=0.087 and p=0.768 respectively) (Table 12, Figure 14).

Table 12: Experiment 3 (trial 1) – Mean GSI of selected fish for each combination of β-sitosterol treatment and AMG injection group (C = control, no AMG; C+AMG = control, AMG injected; β = β-sitosterol, no AMG; β+AMG = β-sitosterol, AMG injected). Numbers in parentheses are sample sizes.

Group	GSI ± SE
C	2.32 ± 0.36 (3)
C+AMG	2.10 ± 0.22 (3)
β	2.88 ± 0.17 (3)
β+AMG	2.43 ± 0.35 (3)

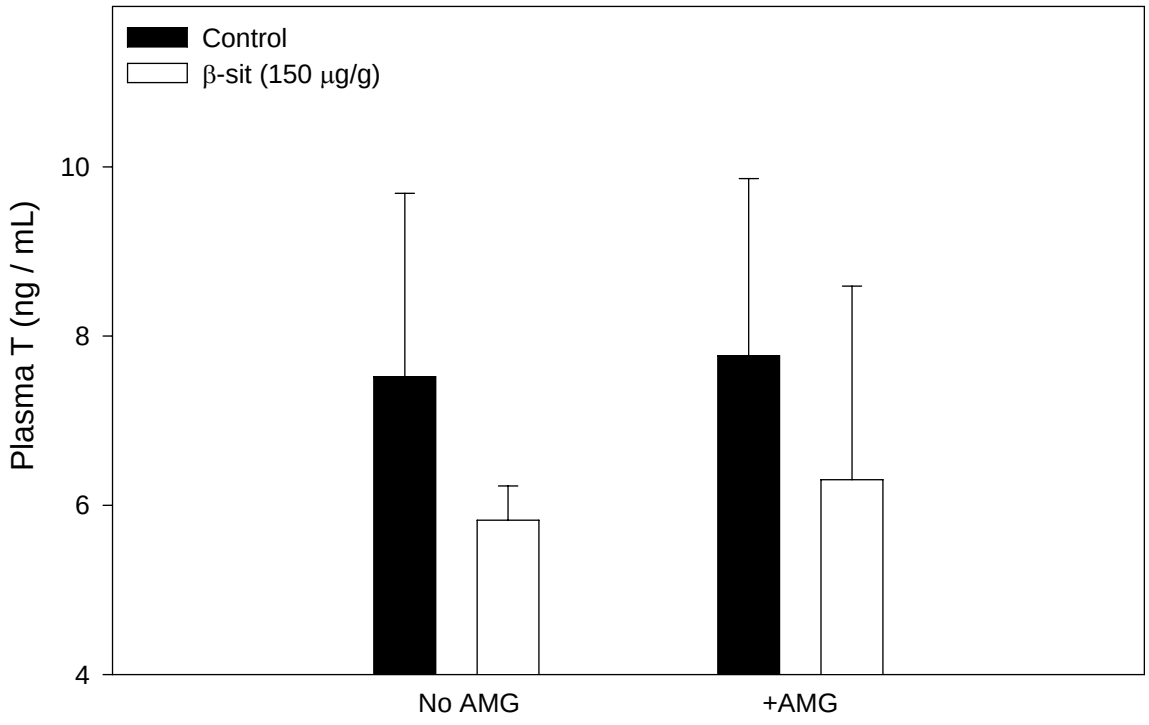

Figure 14: Experiment 3 **trial 1** – Mean plasma T levels for each β-sitosterol treatment by AMG injection groups. Error bars represent +1SE. There was no difference between any means (one-way ANOVA, p=0.621, n=3).

P5 production

A between-subject repeated-measures ANOVA (within-subject factor: time; between-subject factors: treatment and AMG injection) revealed a significant 3rd level interaction between time, treatment and AMG injection (p=0.009) (App. II-S6). To isolate the interaction, different AMG injection groups were separated and repeated-measures ANOVAs (within-subject factor: time; between-subject factor: treatment) were run on P5 levels and P5 production for each AMG injection group separately.

In the no-AMG incubations, β-sitosterol exposure significantly affected the changes in P5 levels over time (significant interaction between time and treatment, p=0.001, App. II-S7), and P5 levels diverged significantly with time (Figure 15, top). There was no significant main effect of treatment on P5 production (p=0.084, App. II-S7), although there was a clear trend of lower production in the β-sitosterol-treated groups (Figure 16, top).

In the AMG incubations, treatment did not significantly affect the changes in P5 levels over time (p=0.053, App. II-S8), but P5 levels still diverged significantly over time (Figure 15, bottom). As in the no-AMG group, there was no significant main effect of treatment on P5 production (p=0.132, App. II-S8), but there was a clear trend of lower production in the β-sitosterol-treated group (Figure 16, bottom).

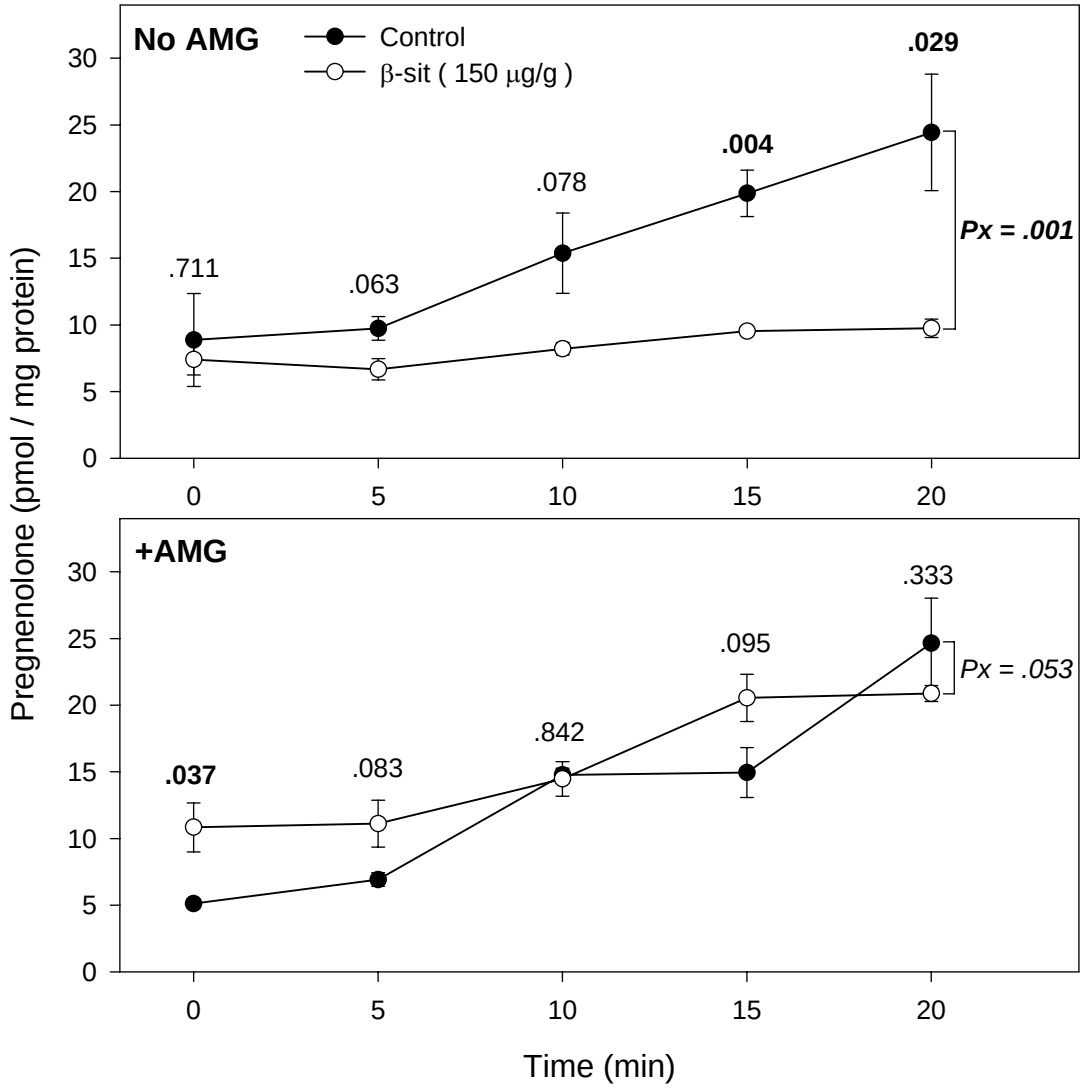

Figure 15: Experiment 3 **trial 1** – P5 changes over time. Error bars represent ±1SE. Numbers above set of means are p-values of simple t-tests between control and β-sitosterol at each time. Px (at the right) represents the p-value for the interaction between treatment and time (*i.e.* the effect of treatment on changes of P5 over time) (n=3).

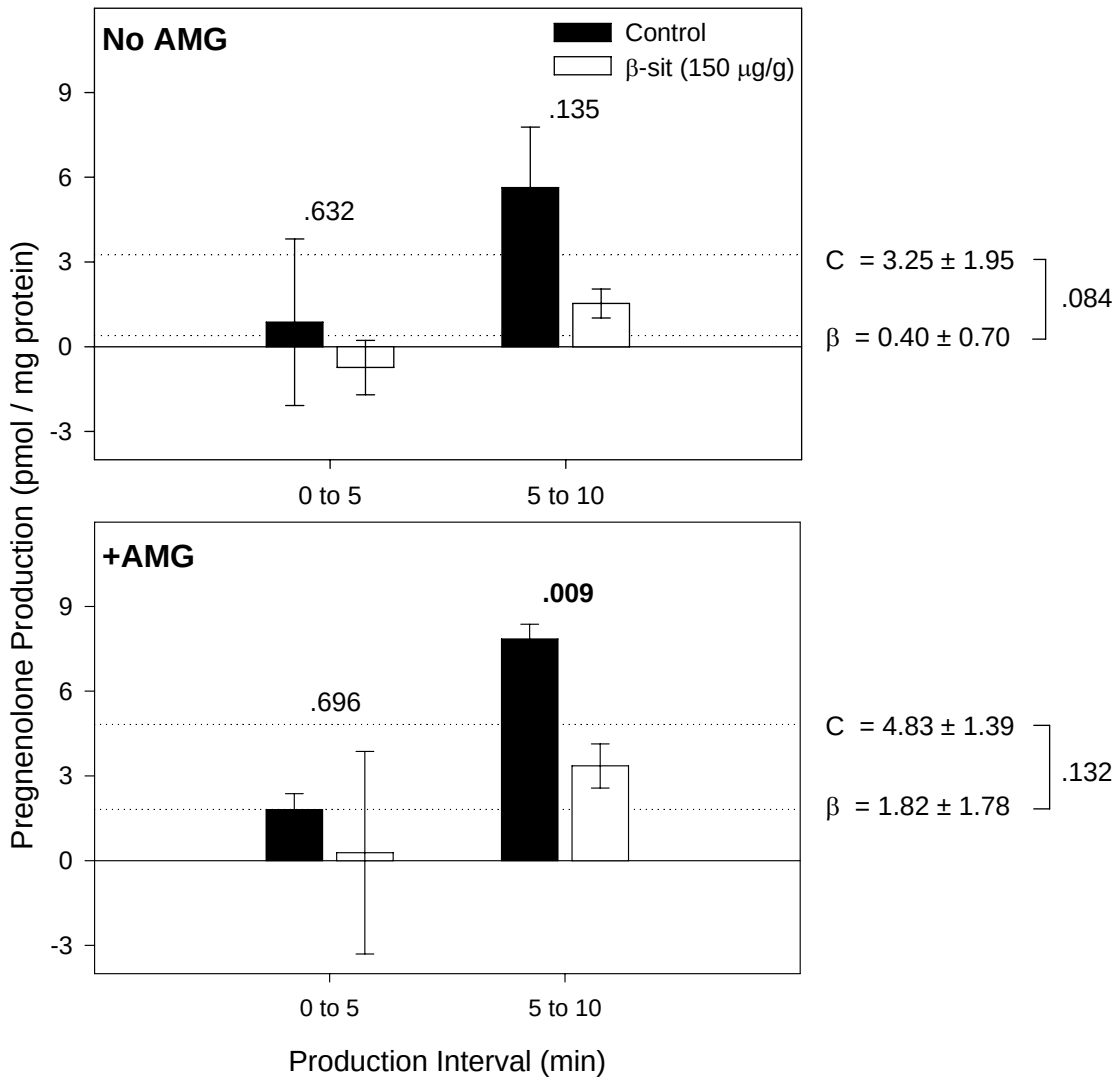

Figure 16: Experiment 3 **trial 1** – P5 production over time. Error bars represent ±1SE. Numbers above set of means are p-values of simple t-tests between control and β-sitosterol at each interval. Numbers at the right are means ± SE for control (C) and β-sitosterol (β) groups. Values at the right of the bracket are p-values for a main effect of treatment on P5 production (n=3).

(d) Experiment 3 (trial 2): Effect of β-sitosterol on the rate of cholesterol transfer across the mitochondrial membrane in male goldfish

Overall effects using the entire data set (all male fish)

There was no significant difference in weight, GSI or plasma T between treatments in the no-AMG group (t-test, p=0.179, p=0.366 and p=0.511, respectively) or in the AMG injection group (t-test, p=0.648, p=0.240 and p=0.181, respectively) (Table 13).

Table 13: Experiment 3 (trial 2) – Mean weight, GSI and plasma T of all male fish for each combination of treatment and AMG injection group (C = control, no AMG; C+AMG = control, AMG injected; β = β-sitosterol, no AMG; β+AMG = β-sitosterol, AMG injected). Numbers in parentheses are sample sizes.

Group	Weight (g) ± SE	GSI ± SE	Plasma T (ng / mL) ± SE
C	59.67 ± 5.83 (3)	1.20 ± 0.11 (3)	8.91 ± 0.21 (3)
C+AMG	53.18 ± 6.76 (6)	3.17 ± 0.71 (6)	16.48 ± 2.24 (6)
β	49.95 ± 1.32 (3)	1.56 ± 0.34 (3)	8.42 ± 0.66 (3)
β+AMG	56.90 ± 4.55 (7)	2.13 ± 0.49 (7)	13.81 ± 1.10 (7)

Plasma Vtg

Plasma Vtg was measured in both the no-AMG and the AMG injected groups. In the AMG injected group, exposure to β-sitosterol significantly increased plasma Vtg (p=0.024). In the no-AMG (vehicle injection) group, while there was a similar trend as in the AMG group, β-sitosterol exposure did not increase plasma Vtg significantly (p=0.225) (Figure 17).

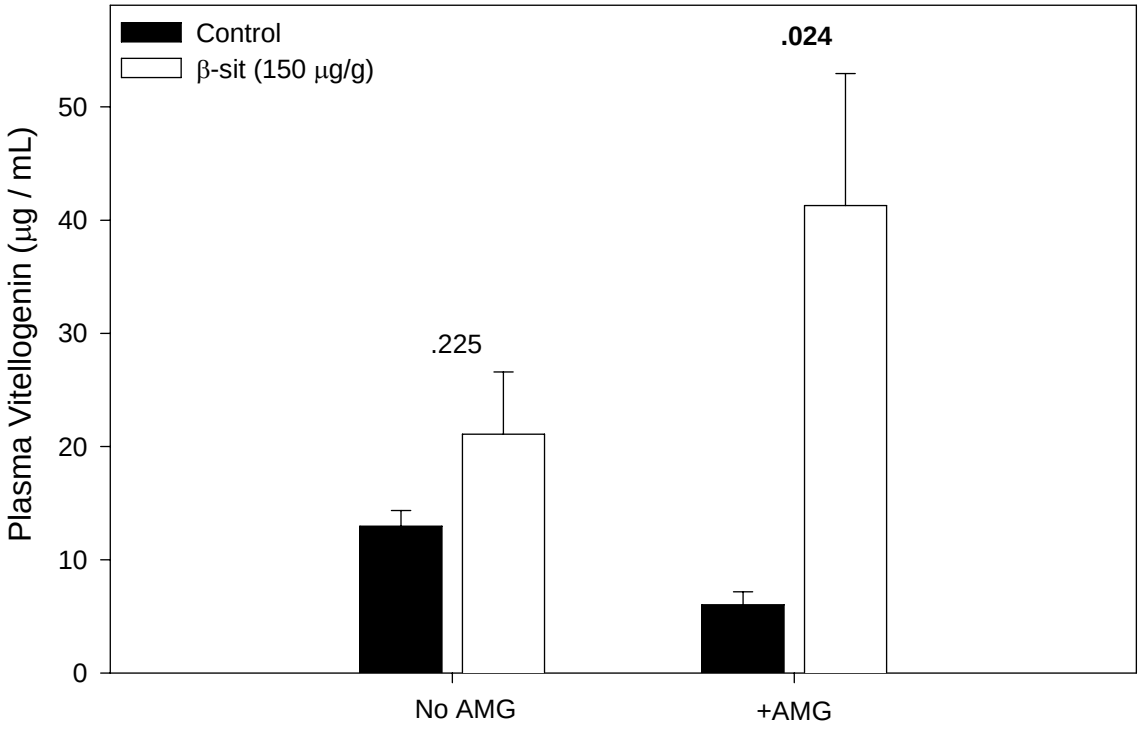

Figure 17: Experiment 3 **trial 2** – Mean plasma vitellogenin levels in male goldfish for each β-sitosterol treatment by AMG injection groups. Error bars represent +1SE. Number above bars is the p-value of a simple t-test between control and β-sitosterol group. (n=3 in the no-AMG group, n=4 in the +AMG group).

GSI and plasma T (only fish whose gonads were selected)

There was no significant difference in GSI or plasma T between β-sitosterol and control fish in the no-AMG group (t-test, p=0.366 and p=0.511, respectively) or in the AMG injection group (t-test, p=0.490 and p=0.120, respectively) (Table 14, Figure 18).

Table 14: Experiment 3 (trial 2) – Mean GSI of fish selected for each combination of treatment and AMG injection group (C = control, no AMG; C+AMG = control, AMG injected; β = β-sitosterol, no AMG; β+AMG = β-sitosterol, AMG injected). Numbers in parentheses are sample sizes.

Group	GSI ± SE
C	1.20 ± 0.11 (3)
C+AMG	2.25 ± 0.23 (4)
β	1.56 ± 0.34 (3)
β+AMG	2.71 ± 0.58 (4)

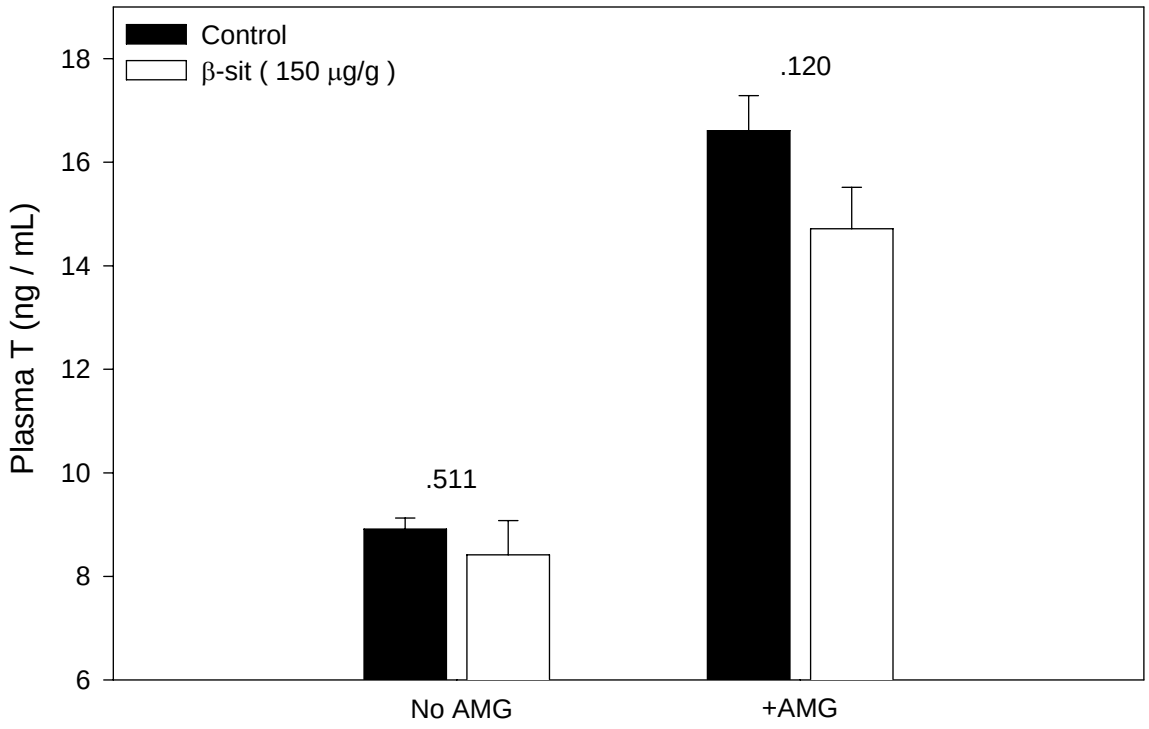

Figure 18: Experiment 3 **trial 2** – Mean plasma T levels for each β-sitosterol treatment by AMG injection groups. Error bars represent +1SE. Number above bars is the p-value of a simple t-test between control and β-sitosterol group (n=3 in the no AMG group, n=4 in the +AMG group).

P5 production

A repeated-measures ANOVA (within-subject factor: time; between-subject factor: treatment) showed a significant interaction between time and treatment in both AMG injection groups, *i.e.* β-sitosterol exposure significantly affected the changes in P5 levels over time (no-AMG, p=0.022; AMG, p=0.042) (App. II-S9 and S10) (Figure 19).

A similar repeated-measures ANOVA on actual P5 production in the first 12 min revealed that β-sitosterol exposure significantly decreased P5 production in the no-AMG injection group (p=0.021). While there was a similar trend in the AMG injection group, that decrease was not statistically significant (p=0.076) (App. II-S9 and S10) (Figure 20).

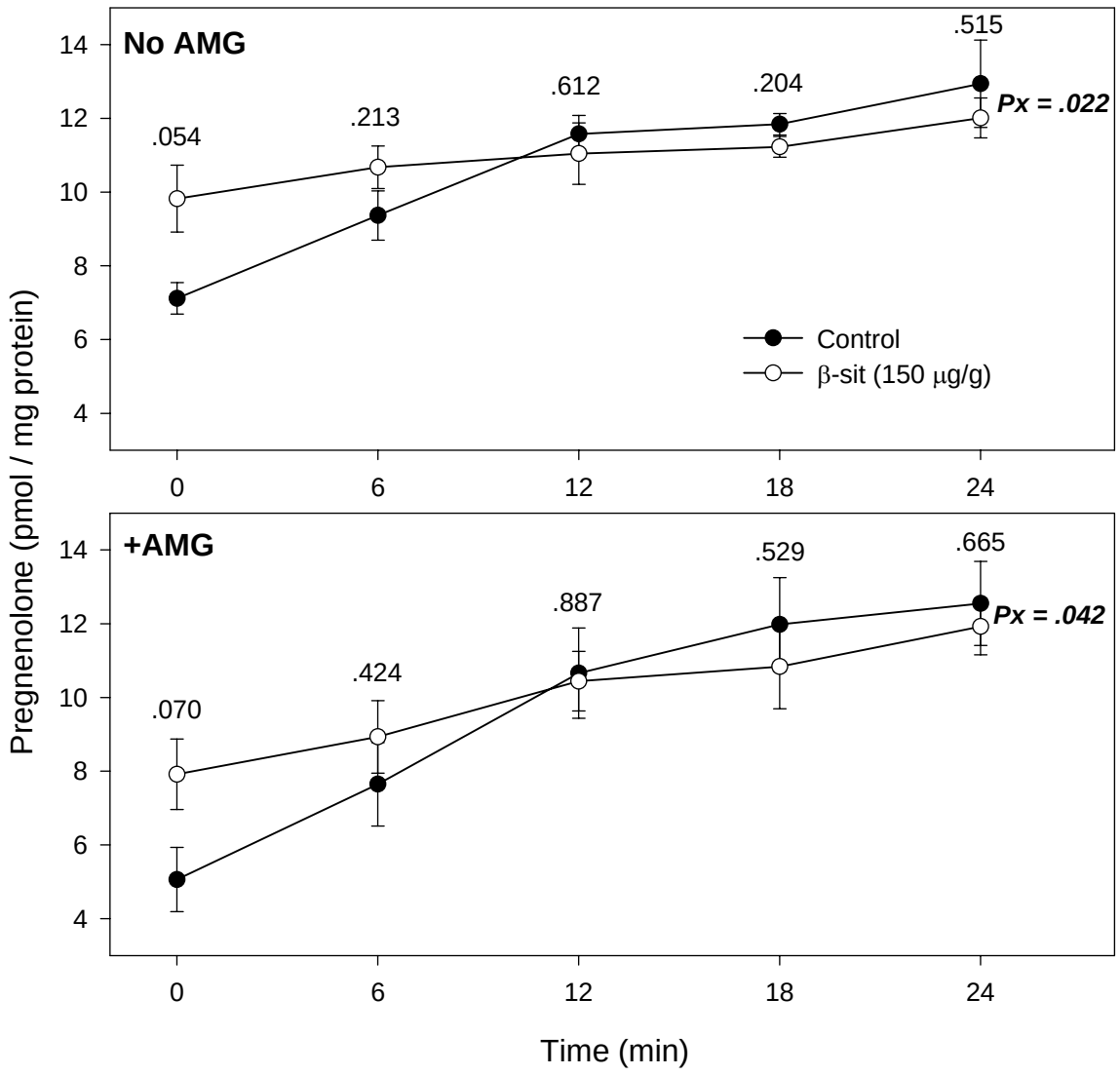

Figure 19: Experiment 3 **trial 2** – P5 changes over time. Error bars represent ±1SE. Numbers above set of means are p-values of simple t-tests between control and β-sitosterol for each time. Px (at the right) represent p-values of the interaction between treatment and time (*i.e.* effect of β-sitosterol on changes of P5 over time) (n=3 for the no AMG group, n=4 for the +AMG group).

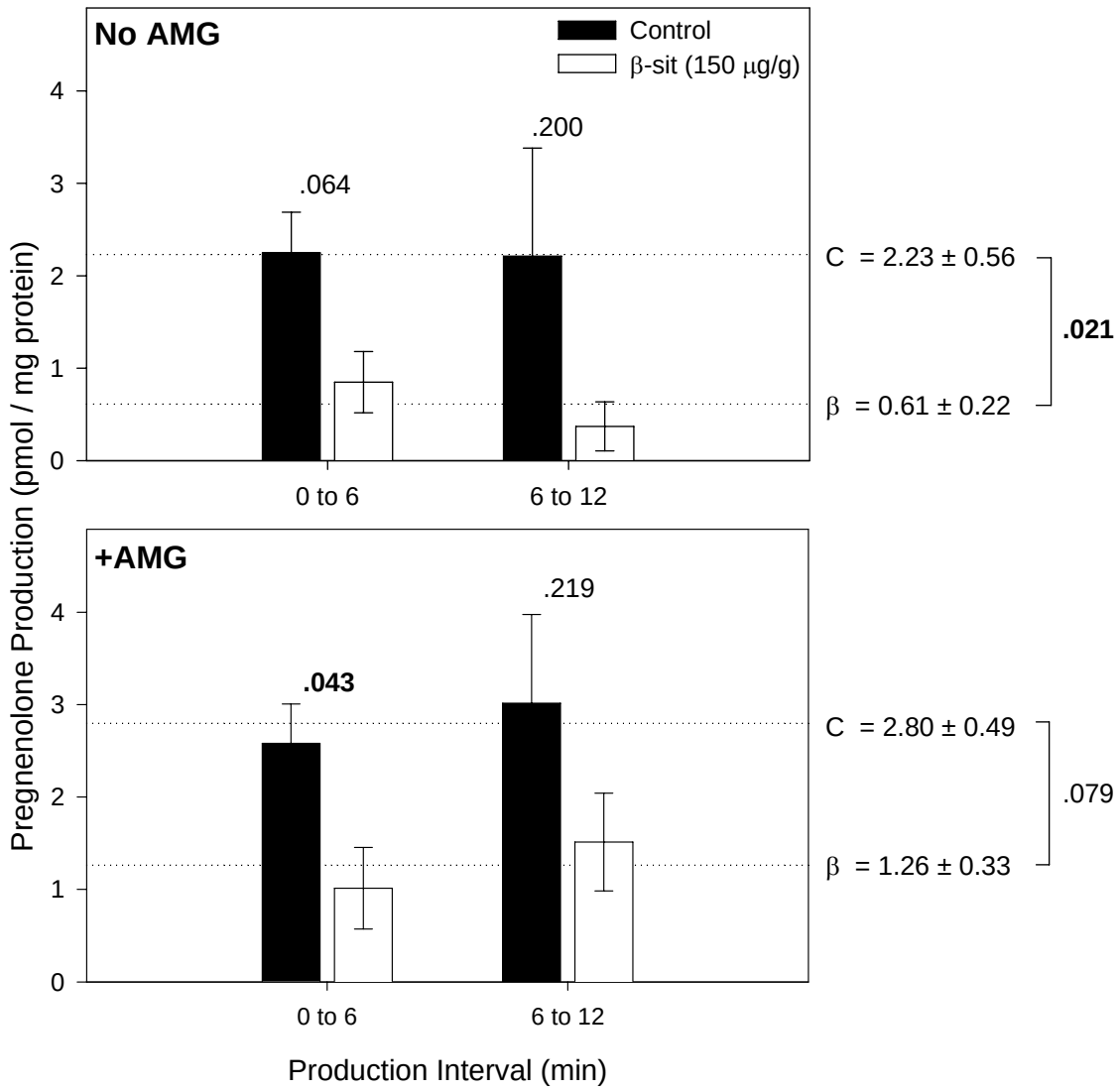

Figure 20: Experiment 3 **trial 2** – P5 production over time (by interval). Error bars represent ±1SE. Numbers above set of means are p-values of simple t-tests between control and β-sitosterol at each interval. Numbers at the right are means ± SE for control (C) and β-sitosterol (β) groups. Values at the right of the bracket are p-values for a main effect of treatment on P5 production (n=3 for the no AMG group, n=4 for the +AMG group).

IV. DISCUSSION

1. Significance

This is the first study to show that β-sitosterol can hinder cholesterol translocation across mitochondrial membranes, an essential step of steroidogenesis. This could explain why fish exposed to β-sitosterol have lower plasma steroids (MacLatchy and Van Der Kraak, 1995; MacLatchy *et al.*, 1997; Tremblay and Van Der Kraak, 1998; Tremblay and Van Der Kraak, 1999; Gilman, 2000; this study) and decreased gonadal biosynthetic capacities (MacLatchy and Van Der Kraak, 1995; Gilman, 2000; this study). It also supports the contention that β-sitosterol and other phytosterols could be responsible for at least some of the reproductive dysfunctions observed in fish exposed to BKME.

2. β-Sitosterol affects steroidogenesis downstream of P450scc

β-Sitosterol exposure has been shown to decrease plasma P5 (Tremblay and Van Der Kraak, 1998; Tremblay and Van Der Kraak, 1999) as well as gonadal production of both P5 and T (MacLatchy and Van Der Kraak, 1995; MacLatchy *et al.*, 1997). In this study, β-sitosterol was shown to affect other steroidogenic intermediates.

While there were differences in the level of significance of the disruption at each step between males and females, the general trend was the same: β-sitosterol exposure negatively affects the steroidogenic pathway as far down as DHEA and AD (Figure 21).

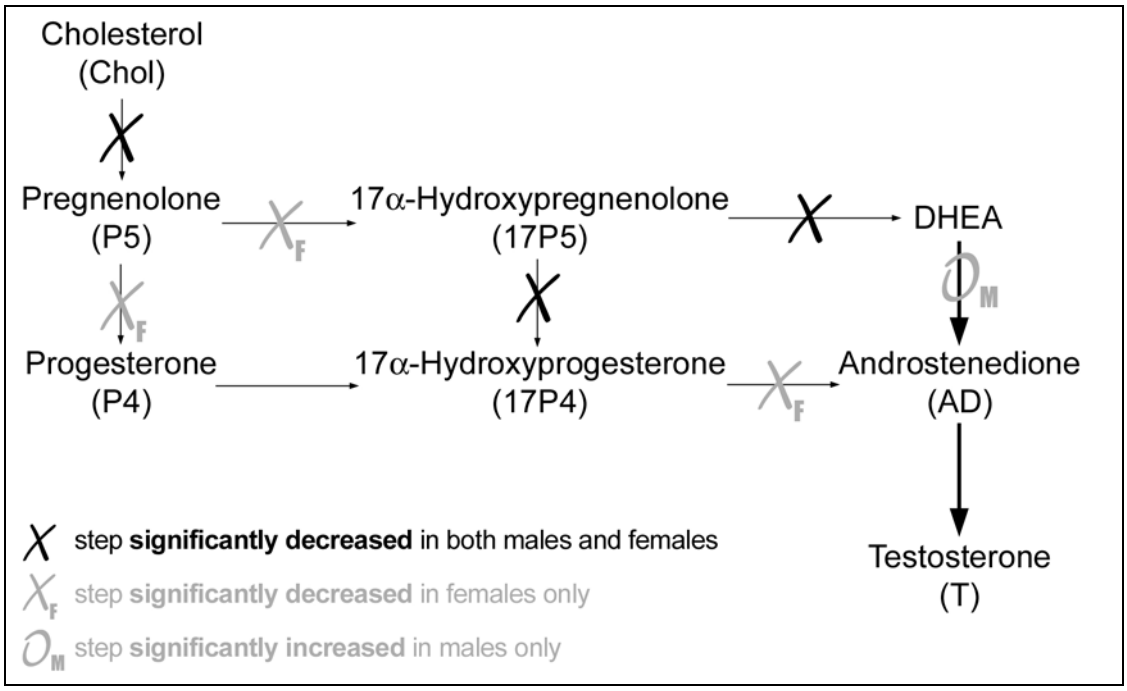

Figure 21: Steps of the steroidogenic pathway affected by β-sitosterol exposure in common goldfish, adapted from results presented in Figures 6 & 7.

The mechanism by which β-sitosterol disrupts steroidogenesis downstream of P5 is unknown but is probably due to interference with enzyme activity (either directly or by preventing the formation of the enzyme-substrate complex) or with substrate mobilization. For example, rats fed a diet rich in phytosterols (mainly β-sitosterol) showed a significant decrease in prostate P450 aromatase activity (Awad *et al.*, 1998).

It appears that production in the last steps of the pathway is increased by β-sitosterol exposure, which could illustrate a physiological attempt to compensate for the deficiencies of the earlier steps. Yet, even with this effect, fish of both sexes exposed

to β-sitosterol had lower levels of plasma T (Figure 5) and decreased gonadal T production (Figures 6 & 7). How the disruptions of the later steps of steroidogenesis compare to the disruption of the first step (conversion of cholesterol to P5) is not clear, but it is likely that the latter is the critical one, as that first step is the rate-limiting step of steroidogenesis.

3. Technical considerations

The other objective of this study was to determine the mechanism of action of β-sitosterol on that first, crucial rate-limiting step in steroidogenesis. To that end, several technical difficulties had to be overcome.

(a) Purity of the mitochondrial isolation

Gonadal mitochondria were isolated using the protocol developed by Gilman (2000). While the protocol still needed refinement, it was proven to be effective at isolating mitochondria (Gilman, 2000). It is still not known how pure the final fraction is, and it is conceivable that pieces of broken endoplasmic reticulum are present, in which case enzymes that convert P5 down the steroidogenic pathway (3β-HSD and P450c17) would also be present. The addition of enzyme inhibitors (trilostane and SU-10603) to the incubation medium is intended to prevent such conversions.

(b) Providing energy to P450scc

Cytochrome P450scc requires NADPH as an energy source to convert cholesterol to P5 (Hall, 1998). In this study, in preparations where the mitochondrial membranes were broken, this was simply done by adding NADPH to the incubation medium. But when P450scc inside intact mitochondria had to be stimulated, an NADPH-generating system had to be supplied. NADPH-generating systems include Krebs cycle intermediates (Kim *et al.*, 1997).

Isocitrate is the most appropriate reducing precursor in rat adrenal mitochondria preparations (McNamara and Jefcoate, 1990), but Gilman (2000) suggested it might not be optimal for fish gonadal mitochondria preparations. In this study, a concentration of 10 mM malate was found to be at least as effective as the same concentration of isocitrate at inducing P5 production from intact mitochondrial preparations (Figure 8). It is not entirely clear why the higher 50 mM concentrations were not as efficient (Figure 8), but it may be due to the partial insolubility of the reducing precursors at concentrations higher than 20-25 mM.

Malate and isocitrate were equally effective (Figure 8), and malate was used as a reaction initiator in all mitochondrial production experiments. Addition of 25-hydroxycholesterol, which freely diffuses across the mitochondrial membrane, to intact mitochondrial incubations should have resulted in an increase in P5 production which did not happen (Figures 9 & 10, top right). This suggests that NADPH, not cholesterol, could be limiting P5 production in these preparations.

(c) Condition of the mitochondria

There is a fine line between too much homogenization, which would break the mitochondria, and too little homogenization, which would result in a poor yield (Ballantyne, 1994). The final fraction was not examined under a microscope to determine the condition of the isolated mitochondria, but data indicate that both intact and broken mitochondria were present. There was significant P5 production when mitochondrial preparations were supplied with malate alone, which shows the presence of intact mitochondria (malate would provide NAPDH to P450scc in intact mitochondria only) (Figure 9 & 10, top right). When supplied with both NADPH and malate, P5 production was significantly enhanced (Figure 9 & 10, top left), indicating that there was also a good proportion of broken mitochondria in the final preparation. It was possible to restrict P5 production to P450scc in intact mitochondria only by simply adding malate and no NADPH. In contrast, it was possible to have only broken mitochondria by sonicating the final preparation (hence disrupting the mitochondrial membranes) and then adding NADPH.

P5 production from intact mitochondria as reported in this study is underestimated, because P5 production is reported per mg of protein (*i.e.* protein from both intact and broken mitochondria in the final preparation). It is not known whether the proportion of intact vs. broken mitochondria varies from one isolation to the next. Strict adherence to the homogenization protocol for each isolation would most likely result in similar final proportions of broken to intact mitochondria, hence standardizing the error and allowing comparisons.

(d) "Biochemical magnifying glasses"

The ultimate goal of this study was to determine if β-sitosterol had any effect on cholesterol translocation. To this end, it was useful to artificially enhance the steroid-producing arsenal of gonadal cells, causing as a sort of biochemical "magnifying glass". In male goldfish, Ovaprim injections not only increased plasma T by a factor of ten (Figure 11), but also significantly increased gonadal T production (Figure 12). It is not clear why females seemed to be much less responsive to Ovaprim stimulation (Figure 11 & 12), but differences in reproductive state between males and females could be the cause. It is also possible that T conversion to E_2 (the dominant steroid in females) was significantly increased by Ovaprim, resulting in lower measurable T levels.

With the same goal in mind, it was also useful to artificially enhance the reactive pool of cholesterol inside the mitochondria. By preventing conversion of cholesterol to P5 *in vivo*, the pool of reactive cholesterol could be artificially increased, making potential differences between treatment groups even more significant. In this study, a dose of 50 μg of AMG per g of fish was found to be effective at increasing the reactive pool of cholesterol in male fish, as reflected by relatively high mitochondrial P5 production in the early moments of the reaction (first 10 min) (Figure 13).

Although these biochemical tools artificially enhance differences between treatment groups, it is important to recognize that they do not create them. Qualitative conclusions based on experiments that make use of these tools are, therefore, still valid predictors of mechanisms of action of bioactive compounds.

4. Mechanisms of action of β-sitosterol on teleost steroidogenesis

(a) β-Sitosterol does not inhibit P450scc activity

In this study, *in vivo* exposure to a mixture of phytosterols rich in β-sitosterol did not inhibit the activity of P450scc *in vitro*: when supplied with NADPH, sonicated mitochondria from both control and β-sitosterol-treated fish produced P5 in an almost identical response pattern (*i.e.* similar production rates and similar scales) (Figures 9 & 10, bottom left). Gilman (2000) found no effect of β-sitosterol on P450scc activity in regressed male brook trout. The combination of these two studies strongly suggests that P450scc activity is not affected by β-sitosterol, and that another mechanism is responsible for the observed decrease in P5 levels in fish exposed to β-sitosterol (MacLatchy *et al.*, 1997; Tremblay and Van Der Kraak, 1998; Tremblay and Van Der Kraak, 1999).

How β-sitosterol affects P450scc activity in ovaries has not been investigated. P450scc in follicles should be measured to determine if β-sitosterol has the same effect on female gonads.

(b) β-Sitosterol decreases the size of reactive pool of cholesterol

Changes in P5 content in the incubation medium over time were significantly affected by β-sitosterol exposure (Figure 19), and mitochondria isolated from control fish produced significantly more P5 in the first 10-12 min of the reaction (Figure 20). This clearly indicates that the reactive pool of cholesterol in control mitochondria is larger

than in mitochondria from β-sitosterol-exposed fish, *i.e.* that β-sitosterol exposure reduces the size of the reactive pools of cholesterol. This, in turn, suggests that β-sitosterol is interfering with the transfer of cholesterol across the mitochondrial membrane, as is the case in rats treated with TCDD (Moore *et al.*, 1991) or cholesterol sulfate (Lambeth *et al.*, 1987).

After the first 10-12 minutes, it is likely that the majority of the reactive pool of cholesterol has been utilized, and differences in P5 production between control and β-sitosterol groups are not as marked.

Injection of AMG did not have the predicted effect. It was hypothesized that AMG would further enhance differences between control and β-sitosterol groups, as it would artificially build up the reactive pool of cholesterol. Injection of AMG increased P5 production more considerably in the β-sitosterol group than in the control group. In fact, P5 production in the control group was almost unchanged by AMG injection. It is possible that P5 production in the control group is already at maximum capacity before AMG injection, which would explain why artificial build-up of the reactive pool of cholesterol has no effect on P5 production (Figures 16 & 20).

The purity of the β-sitosterol mixture did not seem to affect the results significantly, as both trials of experiment 3 (trial 1 with 55% pure β-sitosterol, trial 2 with 96% pure β-sitosterol) showed similar results: decreased amplitude of change in P5 levels and lower production of P5 levels compared to controls. The results were, however, more significant with the purer preparation (Figures 16 & 20), which implies that

β-sitosterol is the most potent of all phytosterols tested, or that structurally-similar phytosterols all have analogous effects on cholesterol transfer. This similarity in effects of pure β-sitosterol compared to a phytosterol mixture rich in β-sitosterol was also recognized by Tremblay and Van Der Kraak (1999) in their comparison study of β-sitosterol formulations on rainbow trout reproductive endocrine status.

(c) Mechanisms of action of β-sitosterol on cholesterol translocation

Privalle *et al.* (1983) showed that the true limiting step of steroidogenesis was the translocation of cholesterol from the outer to the inner mitochondrial membrane. The main barrier to cholesterol transfer is the aqueous space between membranes, and a protein called steroidogenic acute regulatory protein (StAR – described in 1994 by Clark *et al.*) has been shown to be essential to that translocation in rats (Clark and Stocco, 1996). Mutations in the StAR gene in humans lead to a disease called lipoid congenital adrenal hyperplasia, a condition where steroid synthesis is severely impaired (Lin *et al.*, 1995; Miller, 1995; King *et al.*, 2000; Stocco, 2000). Bauer *et al.* (2000) showed that StAR was also present in non-mammalian vertebrates (including fish), and suggested that StAR had the same function in all vertebrates. Though the mechanism of action of the StAR protein is not yet fully understood, it seems that StAR does not need to cross the mitochondria membrane, but instead creates a bridge by which cholesterol can cross the intermembrane space (Arakane *et al.*, 1996; Stocco and Clark, 1997; Kallen *et al.*, 1998a; Stocco, 2000).

The mechanism of action of β-sitosterol on cholesterol translocation is unknown, but it is hypothesized that β-sitosterol is interfering with the proper function of the StAR protein, possibly by simple competition with cholesterol for translocation. The discovery that StAR is not cholesterol specific, but can also transport other sterols (including β-sitosterol) with the same efficiency (Kallen *et al.*, 1998b) only makes this hypothesis more probable.

Other mechanisms for intramitochondrial cholesterol transport have also been suggested, such as the steroidogenic activator peptide (SAP) (Jefcoate *et al.*, 1992), the sterol carrier protein$_2$ (SCP_2) (Woodford *et al.*, 1995; Gallegos *et al.*, 2000) and the peripheral-type benzodiazenpine receptors (PBR) (Kim *et al.*, 1997; Papadopoulos *et al.*, 1997; Culty *et al.*, 1999). It is also possible that β-sitosterol acts on these proteins to modulate cholesterol mobilization to the inner mitochondrial membrane.

5. Similarities between BKME and β-sitosterol

It is not known how a dose of 150 μg/g of β-sitosterol via Silastic® pellet implant compares to waterborne exposure in the field, but concentrations as high as 1200 μg/L after primary treatment and 280 μg/L after secondary treatment have been reported in BKME (MacLatchy *et al.*, 1997). If one compares the magnitude of the effects on plasma T and gonadal T production in this study to those reported by MacLatchy *et al.* (1997) after exposing male goldfish to waterborne β-sitosterol, it would seem that a dose of 150 μg/g of β-sitosterol via Silastic® implant produces effects similar to a waterborne

dose of 300-600 μg/L. This would have to be established through experimentation, as differences in reproductive states between the two studies may have had an impact on the magnitude of the observed effects.

Tremblay and Van Der Kraak (1999) showed that waterborne exposure to β-sitosterol at concentrations of 75 and 150 μg/L and BKME containing 124 and 208 μg/L of β-sitosterol resulted in similar effects on plasma P5, plasma T and plasma Vtg levels. Likewise, in this study, exposure to β-sitosterol (150 μg/g, Silastic® implants) for a period of 20 to 30 days in the lab resulted in decreased plasma T, decreased gonadal T production, and increased plasma Vtg in males. Fish exposed to BKME in the field also exhibited decreased plasma steroid levels and decreased biosynthetic capacity (McMaster *et al.*, 1991; Munkittrick *et al.*, 1992; Van Der Kraak *et al.*, 1992a; McMaster *et al.*, 1995b; McMaster *et al.*, 1996; Munkittrick *et al.*, 1998; Munkittrick, 2001) and had higher levels of Vtg mRNA in the liver, which indicates that the Vtg gene is induced by exposure to BKME (Mellanen *et al.*, 1999).

The similarities between the effects exhibited by fish exposed to β-sitosterol and fish exposed to BKME suggest that at least some of the reproductive dysfunctions seen in fish exposed to BKME could be due to the presence of β-sitosterol and other phytosterols in the final effluent. If this is true, then process changes aiming at lowering the high content of plant sterols in BKME are justified. It has been suggested that better washing efficiency, adequate black liquor recovery and better prevention of black liquor spills could significantly decrease the amount of phytosterols found in the final effluent (Folke

et al., 1993). More work remains to be done to determine if implementing new technologies aimed at decreasing or altering sterol concentration and/or makeup in final effluent could restore reproductive status of fish downstream of pulp mill effluent discharges to natural levels.

V. CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, this study showed that β-sitosterol decreased P5 production not by inhibiting the activity of P450scc but by interfering with mobilization of cholesterol to the inside of the mitochondria, where P450scc resides. This work identifies a mechanism by which β-sitosterol and other phytosterols in BKME could be affecting the endocrine status in fish downstream of pulp mills. It is possible that process changes and/or fine-tuning of current technologies to prevent such high concentrations of phytosterols in the final effluent could potentially improve the quality of the final effluent.

The entire range of possible mechanisms by which β-sitosterol could be affecting P5 production has not yet been fully investigated. It remains to be tested whether β-sitosterol interferes with HMG-CoA reductase, the enzyme responsible for *de novo* synthesis of cholesterol from acetate. As well, the mechanism by which cholesterol translocation is affected deserves further study.

β-Sitosterol has fascinating effects on cholesterol metabolism, and further research into the interaction of β-sitosterol and cholesterol is currently under way in Dr. MacLatchy's laboratory. One idea would be to look at what happens to the cholesterol that must be accumulating on the external mitochondrial membrane or in the cytoplasm, as β-sitosterol prevents its translocation to the inner mitochondrial membrane.

There is some concern that EDSs are most potent during crucial period of embryonic development (Colborn *et al.*, 1996; Crisp *et al.*, 1998). Exposure of adult fish

to BKME results in temporary reproductive dysfunctions (Munkittrick *et al.*, 1992; Munkittrick *et al.*, 1998), but exposure of eggs can result in much more serious and permanent effects (Lehtinen *et al.*, 1999; Johnsen *et al.*, 2000). More life-cycle studies looking at the effects of β-sitosterol exposure at different stages of development and with multiple generations would provide insights about particularly sensitive stages of development, as well as the degree of dysfunction caused by β-sitosterol exposure, and its overall impact in term of populations dynamics.

Finally, this study focused on the effect of β-sitosterol on male goldfish steroidogenesis. Further studies are necessary to evaluate the effects of β-sitosterol in females as well as in other species of fish, particularly sentinel species that are ecologically relevant in relation to BKME exposure.

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APPENDIX I

1. *In vitro* Incubations

Based on McMaster *et al.* (1995a). All chemicals are from Sigma-Aldrich Canada Ltd., Oakville, ON. All supplies are from Fisher Scientific Ltd. Nepean, ON.

(a) Cortland's buffer

- Make solution A: 72.5g sodium chloride (NaCl); 2.3g calcium chloride, dihydrate ($CaCl_2$-$2H_2O$); 3.8g potassium chloride (KCl); 4.1g sodium phosphate, monobasic, monohydrate (NaH_2PO_4-H_2O); 2.0g magnesium chloride, hexahydrate ($MgCl_2$-$6H_2O$); 2.3g magnesium sulfate, heptahydrate ($MgSO_4$-$7H_2O$) in 1L of double-distilled water.
- Make solution B: 10g sodium bicarbonate ($NaHCO_3$) in 1L double-distilled water.
- Solutions A and B may be stored as stock solutions and used as needed to make the final Cortland's buffer.
- Complete Cortland's buffer: 100mL solution A; 100mL solution B; 1.0g glucose, anhydrous ($_D$-(+)-glucose); 1.0g bovine serum albumin (BSA); 0.1g streptomycin (streptomycin sulfate). Add solutions A and B to 700mL double-distilled water. Add glucose and streptomycin and stir. Adjust pH to 7.5. Make up to final volume of 1L. Add BSA. Stir slightly and refrigerate so cool when used.
- Cortland's buffer must be used within 24h once the BSA has been added.

(b) Protocol for male gonads

1. Label incubation test tubes (12 x 75mm borosilicate tubes).
2. Take weights of fish and testes for calculation of GSI (GSI = gonad weight x 100 / fish weight)
3. Rinse testes with Cortland's buffer in test tube. Keep testes on ice throughout the procedure.
4. Cut up testes into pieces in a glass petri dish (clean dish after each testes). Two pieces, with a total weight of 18-23mg will go into each incubation tube. Places testes pieces into tubes.
5. Add 0.5mL of Cortland's buffer in each tube using repeating pipetter.
6. Repeat steps 4&5 with testes from additional fish.
7. Immediately prior to beginning the incubation, withdraw the Cortland's buffer from all tubes with a Pasteur pipet and replace it quickly with 1mL of test solution (*e.g.* simply 1mL of Cortland's buffer for basal incubations, 1mL of Cortland's buffer with 10IU of hCG / mL for hCG-stimulated incubations, etc).
8. Place tubes in incubator at 18ºC for 18h. During the incubation, label storage tubes (1.5mL polypropylene microcentrifuge tubes with snap cap).
9. After 18h incubation, spin the test tubes at 4ºC for 10 minutes at 1,900 *g*.
10. Pipet off 900µL of the solution and place in storage tubes. Freeze at –20ºC for later steroid quantification.

(c) Protocol for female gonads

1. Label incubation wells (polystyrene multiple-well tissue culture plate).
2. Take weights of fish and gonads for calculation of GSI (GSI = gonad weight x 100 / fish weight)
3. Rinse gonads with Cortland's buffer in test tube. Keep gonads on ice throughout the procedure.
4. Gently loosen follicles apart. Separate out the vitellogenic and pre-vitellogenic follicles and place in a glass petri dish (with a dark underside) held on ice (clean dish after each gonad). Place 20 pre-vitellogenic follicles in each well.

   Note: In the case of underdeveloped follicles, whole tissue totalling 18-23mg was used in each well.
5. Add 0.5mL of Cortland's buffer in well using repeating pipetter.
6. Repeat steps 4&5 with testes from additional fish.
7. Immediately prior to beginning the incubation, withdraw the Cortland's buffer from all wells with a Pasteur pipet and replace it quickly with 1mL of test solution (e.g. simply 1mL of Cortland's buffer for basal incubations, 1mL of Cortland's buffer with 10IU of hCG / mL for hCG-stimulated incubations, etc).
8. Place well plates in incubator at 18ºC for 18h. During the incubation, label storage tubes (1.5mL polypropylene microcentrifuge tubes with snap cap).
9. At the end of the incubation period, pipet off 900μL of the solution and place in storage tubes. Freeze at –20ºC for later steroid quantification.

2. Plasma Extraction Procedure

Based on McMaster *et al.* (1992). All chemicals are from Sigma-Aldrich Canada Ltd., Oakville, ON. All supplies are from Fisher Scientific Ltd. Nepean, ON.

(a) Phosgel Buffer

- For 1L of solution, add 5.75g sodium phosphate, dibasic, anhydrous (NaH_2PO_4), 1.28g sodium phosphate, monobasic, monohydrate (NaH_2PO_4-H_2O), 1g Gelatin (Type A: Porcine), 0.1g Thimerosal to 800mL double-distilled water.
- Heat to 45-50ºC for 15 minutes to dissolve the gelatin. Adjust to 1L with double-distilled water. Adjust the pH to 7.6 if necessary. Store at 4ºC for up to one week.

(b) Protocol

1. Make phosgel buffer.
2. Take plasma samples out of freezer to thaw.
3. Label large test tubes (borosilicate glass, 16 x 150mm) for plasma samples.
4. Label small glass scintillation vials (borosilicate glass, 7mL).
5. Pipette into test tubes equal volumes of plasma (i.e. 200µL, or 100µL, or 50µL etc).
6. Add 500µL of double-distilled water to each tube.

Do the following steps under the fumehood, six (6) tubes at a time.

7. Add 5mL of ether to each tube.
8. Vortex each of the six tubes for 20 seconds, let settle for 5 minutes.

9. Vortex again, let settle for 5 minutes.
10. Set up acetone / dry ice bath. Place dry ice in tray, add acetone, and tip tray.
11. Slowly place tubes in acetone to freeze the aqueous phase. Do all six tubes (one or two at a time) then go back and, one at a time, thaw edges of test tubes at solid phase level with your hand to ensure all solid phase goes to bottom.
12. Refreeze tube just thawed.
13. Decant ether phase into scintillation vial.
14. When a collection of tubes is done (all or some), place scintillation vials in warm water bath to evaporate ether. Alternatively, vials may be left in air (in fumehood) to evaporate overnight.
15. Once scintillation vials are dry, add 1mL of phosgel buffer.
16. Freeze for later steroid quantification.

3. Standard Steroid Assay – Radioimmunoassay (RIA)

Based on McMaster *et al.* (1992). All chemicals are from Sigma-Aldrich Canada Ltd., Oakville, ON, unless otherwise indicated. All supplies are from Fisher Scientific Ltd. Nepean, ON.

(a) Steroids and antibodies

Testosterone was purchased from Sigma-Aldrich Canada Ltd. Radiolabelled T (T*; [1,2,6,7-^{3}H]Testosterone) was purchased from Amersham International, Buckinghamshire, UK. Testosterone antibody (TAb) was purchased from Medicorp, Montreal, QC. Cross-reactivity for TAb was 100% with T, 35% with dihydrotestosterone, and less than 0.1% with any other steroid or steroid intermediate.

Pregnenolone was purchased from Sigma-Aldrich Canada Ltd. Radiolabelled P5 (P5*; [7-^{3}H(N)]Pregnenolone) was purchased from Amersham International. Pregnenolone antibody (P5Ab) was purchased from Immunocorp (now Medicorp). Cross reactivity for P5Ab was 100% with P5 and less than 0.1% with any other steroid of steroid intermediate.

(b) Solutions

Phosgel buffer (1L)

- Add 5.75g sodium phosphate, dibasic, anhydrous (NaH_2PO_4); 1.28g sodium phosphate, monobasic, monohydrate (NaH_2PO_4-H_2O); 1g gelatin (Type A: Porcine); 0.1g thimerosal to 800mL double-distilled water.

- Heat to 45-50ºC for 15 minutes to dissolve the gelatin. Adjust to 1L with double-distilled water. Adjust the pH to 7.6 if necessary. Store at 4ºC for up to one week.

Charcoal solution (100mL)

- Add 0.5g activated charcoal, 0.05g Dextran T-70 to 100mL phosgel.
- Stir. May be stored at 4ºC for two to three days.
- Up to 500 tubes can be processed with 100mL of charcoal solution (200µL per sample).
- *Charcoal should be stirred when used, to prevent charcoal from settling at the bottom.*

(c) Protocol

1. Make phosgel buffer the day before you plan to do the assay so it is cold when you need it.

2. Label tubes (borosilicate, 12 x 75mm) required for assay.

TUBE NUMBER	WILL CONTAIN
1, 2, 3	NSB
4, 5, 6	0 standard
7, 8, 9	1.56 standard
10, 11, 12	3.125 standard
13, 14, 15	6.25 standard
16, 17, 18	12.5 standard
19, 20, 21	25 standard
22, 23, 24	50 standard
25, 26, 27	100 standard
28, 29, 30	200 standard
31, 32, 33	400 standard
34, 35, 36	800 standard
37, 38	Unknown #1
--, --	Unknowns
145, 146 (max)	Unknown #55 (max)
147, 148	Interassay sample

3. Thaw and vortex your unknowns and your interassay samples.

4. Make up your standards.

Dilute 100µL of stock steroid (1,000ng / mL) with 24.9mL of phosgel buffer to produce standard steroid concentration of 800pg / tube. Dilute as follows to prepare other standards (400, 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56pg / tube).

[STEROID] OF PRIOR SOLUTION MADE	VOLUME OF PRIOR SOLUTION ADDED	VOLUME OF PHOSGEL ADDED	FINAL [STEROID]
Stock (100ng / mL)	100µL	24.9mL	800pg / tube
800pg / tube	1mL	1mL	400pg / tube
400pg / tube	1mL	1mL	200pg / tube
200pg / tube	1mL	1mL	100pg / tube
100pg / tube	1mL	1mL	50pg / tube
50pg / tube	1mL	1mL	25pg / tube
25pg / tube	1mL	1mL	12.5pg / tube
12.5pg / tube	1mL	1mL	6.25pg / tube
6.25pg / tube	1mL	1mL	3.125pg / tube
3.125pg / tube	1mL	1mL	1.56pg / tube

5. Pipette into your assay tubes the following:

TUBE	CONTENTS	PHOSGEL (µL)	STANDARD (µL)	UNKNOWN (µL)
1, 2, 3	NSB[1]	400	---	---
4, 5, 6	0 standard	200	---	---
7, 8, 9	1.56 standard	---	200	---
10, 11, 12	3.125 standard	---	200	---
13, 14, 15	6.25 standard	---	200	---
16, 17, 18	12.5 standard	---	200	---
19, 20, 21	25 standard	---	200	---
22, 23, 24	50 standard	---	200	---
25, 26, 27	100 standard	---	200	---
28, 29, 30	200 standard	---	200	---
31, 32, 33	400 standard	---	200	---
34, 35, 36	800 standard	---	200	---
37, 38	Unknown #1	---	---	200
--, --	Unknowns	---	---	200
145, 146	Unknown #55	---	---	200
147, 148	Interassay	---	---	IA 200[2]

[1] NSB = Non Specific Binding.
[2] IA = Interassay (known stock standard).

6. Make up antibody solution in a plastic jar. Antibody dilution will vary. It is calculated so as to bind 50% of added radiolabelled steroid in the absence of a competitor.

7. Make up tracer solution in a plastic jar. Radiolabelled steroid must be diluted with phosgel buffer so as to contain 5,000CPM (counts per minute) in 200µL.

8. Check that tracer dilution is correct by running two TCR tubes (200µL tracer, 600µL phosgel and 5mL scintillation cocktail) in the counter (should be at 5,000CPM).

9. Add 200µL of tracer to every assay tube.

10. Add 200µL of antibody solution to every assay tube except 1, 2 and 3 (NSB).

11. Make up your three TCR tubes (200µL tracer, 600µL phosgel) in scintillation tubes (7mL polyethylene).

12. Places tubes and TCR tubes in incubator at 18ºC for 4-24h (most effective compromise between time and binding is 5-6h).
13. Make up charcoal.
14. Label scintillation tubes.
15. At the end of the incubation period, place the tubes in ice cold water for 10 minutes.
16. After 10 minutes in ice cold water, add 200µL of charcoal to each assay tube (but not to the TCR tubes).*Note: Time from addition of charcoal to LAST assay tube till beginning of centrifugation (step 18) should be 10 minutes. In other words, step 17 should take maximum 10 minutes.*
17. Wipe, vortex and load tubes into centrifuge cooled at 4ºC (balance buckets).
18. Spin tubes at 4ºC for 12 minutes at 1,900 *g*.
19. Decant liquid phase from tubes into scintillation (counting) tubes (7mL polyethylene).
20. Add 5mL of scintillation cocktail to each tube (including TCR tubes).
21. Cap and vortex counting tubes.
22. Load tubes into liquid scintillation counter.

(d) Summary of tube contents

Note that the total volume added to every tube is 800µL.

TUBE	CONTENTS	PHOSGEL (µL)	STANDARD (µL)	SAMPLE (µL)	TRACER (µL)	AB (µL)	CHARCOAL (µL)
a,b,c	TCR	600	---	---	200	---	---
1,2,3	NSB	400	---	---	200	---	200
4,5,6	0	200	---	---	200	200	200
7,8,9	1.56	---	200	---	200	200	200
10,11,12	3.125	---	200	---	200	200	200
13,14,15	6.25	---	200	---	200	200	200
16,17,18	12.5	---	200	---	200	200	200
19,20,21	25	---	200	---	200	200	200
22,23,24	50	---	200	---	200	200	200
25,26,27	100	---	200	---	200	200	200
28,29,30	200	---	200	---	200	200	200
31,32,33	400	---	200	---	200	200	200
34,35,36	800	---	200	---	200	200	200
37,38	Sample 1	---	---	200	200	200	200
--,--	Samples	---	---	200	200	200	200
145,146	Sample 55	---	---	200	200	200	200
147,148	IA	---	---	IA200	200	200	200

4. Fish Mitochondria Isolation Protocol

Based on Gilman (2000), Ballantyne (1994) and Moore *et al.* (1991). All chemicals are from Sigma-Aldrich Canada Ltd., Oakville, ON. All supplies are from Fisher Scientific Ltd. Nepean, ON.

(a) Solutions

Isolation buffer (500mL)

- Add 42.8g sucrose; 3.64g trizma hydrochloride (Tris-HCl); 0.24g trizma base (Tris-Base); to 500mL double-distilled water.
- Adjust pH to 7.4 if necessary. Keep at 4ºC for up to a week.
- Up to 20 gonads can be processed with 500mL of isolation buffer.

Incubation media (500mL)

- Add 42.8g sucrose (250mM); 0.75g potassium chloride (KCl; 20mM), 1.4g triethanolamine hydrochloride (15mM), 0.68g potassium phosphate monobasic (KH_2PO_4; 10mM) and 0.51g magnesium chloride hexahydrate ($MgCl_2$-$6H_2O$; 5mM); to 500mL double-distilled water.
- Adjust pH to 7.2 if necessary. Keep at 4ºC for up to a week.
- Each incubation sample uses about 2mL of media (4mL if incubated in duplicates).

(b) Protocol

Note: All glassware should be rinsed in dilute acid (100mM HCl) and then rinsed with isolation buffer. All solutions must be ice cold (4ºC).

1. Dissect out testes and place in test tube (13 x 100mm borosilicate) on ice with 1.5mL of isolation buffer (ice cold). More than 800mg of gonadal tissue per fish is needed, or else gonads from different fish will have to be pooled.
2. Weigh out 750-850mg of testicular tissue and place on cutting board (food grade polyethylene) on ice.
3. Finely chop testes in 1mm cubes, using a new razor blade for each testis. Clean cutting board after each testis.
4. Transfer to tissue grinder (30mL borosilicate glass grinder tube) and add 8mL of ice-cold isolation buffer.
5. Homogenize (keep tube in ice bath) for a total of four strokes at about 300RPM. A home-made homogeniser consisting of a Makita multiple-speed drill with a PTFE pestle on a stainless-steel rod.
6. Transfer to normal centrifuge tubes (borosilicate), and place tubes in refrigerated centrifuge (4ºC). Add minimal volumes of isolation buffer as needed to balance rotor.
7. Centrifuge at 4ºC for 10 minutes at 475 *g*.
8. Pour supernatant into ultracentrifuge tubes (10mL Oak Ridge polypropylene copolymer tubes with screw caps) on ice and discard pellet.
9. Weigh each tube carefully and balance by adding isolation buffer.
10. Centrifuge ultracentrifuge tubes at 4ºC for 10 minutes at 14,600 *g*.

11. Discard supernatant and gently resuspend pellet in 8mL isolation buffer with Pasteur pipet.
12. Re-centrifuge tubes at 4ºC for 10 minutes at 14,600 *g* (purify the sample).
13. Discard supernatant and resuspend final pellet in 2.4mL of isolation buffer in normal test tube (12 x 75mm borosilicate) on ice.
14. Take 0.4mL sample for protein quantification (put into microcentrifuge tube and freeze at –20ºC until quantification can be done).
15. The 2mL “final mitochondria preparation” must be kept on ice and used within 4-6h.

5. Protein Quantification Protocol

Based on Bradfod (1976). All chemicals (except Bio-Rad Protein Assay) are from Sigma-Aldrich Canada Ltd., Oakville, ON. All supplies are from Fisher Scientific Ltd. Nepean, ON.

(a) Isolation buffer (250mL)

- Add 21.4g sucrose; 1.82g trizma hydrochloride (Tris-HCl); 0.12g trizma base (Tris-Base); to 250mL double-distilled water.
- Adjust pH to 7.4 if necessary. Keep at 4ºC for up to a week.

(b) Protocol

1. Add 10mg of bovine serum albumin (BSA) in 500mL of isolation buffer (final protein concentration is 20µg/mL). Perform serial dilutions to obtain protein standards for standard curve as indicated below (in large 13 x 100mm borosilicate tubes).

[PROTEIN] OF PRIOR SOLUTION MADE	VOLUME OF PRIOR SOLUTION ADDED	VOLUME OF ISOL. BUFFER ADDED	FINAL [PROTEIN]
20µg/mL	5mL	5mL	10µg/mL
10µg/mL	3mL	0.75mL	8µg/mL
10µg/mL	3mL	3mL	5µg/mL
5µg/mL	3mL	3mL	2.5µg/mL
2.5µg/mL	3mL	3mL	1.25µg/mL

2. Pipet 100µL of sample to be assayed and add to 1.99mL of isolation buffer (200x dilution) into a clean, dry test tube (12 x 75mm borosilicate) and vortex.

3. Pipet 800μL of each standard and sample solution into spectrophotometer cuvet (1.5mL UV grade methacrylate semimicro cuvet), add 200μL of concentrated Bio-Rad protein assay dye reagent (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON) and vortex. Do in triplicate for each standard and sample.
4. Incubate at room temperature for 5-20 minutes. Absorbance will increase over time.
5. Measure absorbance at 595nm.

6. Goldfish Vitellogenin ELISA Protocol

Based on Denslow (1995). All chemicals are from Sigma-Aldrich Canada Ltd., Oakville, ON. All supplies are from Fisher Scientific Ltd. Nepean, ON.

Antibodies and proteins were gifts of Dr. Glen Van Der Kraak (Zoology, University of Guelph, ON, Canada).

(a) Solutions

Sodium bicarbonate buffer (SBB)

- 4.20g of NaHCO3, 5.0mg of Gentamycin
- Add 1L of double-distilled water (ddH_2O)
- Adjust pH to 9.6
- Can be stored for up to 1 year at 4ºC

Tris buffer (TBS-T) (Tris buffered saline-Tween)

- 1.211g of Tris-HCl, 8.766g of NaCl, 1.0mL of Tween (20) (1.0%), 5.0mg of Gentamycin
- Add 1L of double-distilled water (ddH_2O)
- Adjust pH to 7.5
- Can be stored for up at 1 year at 4ºC

TBS-T-BSA

- 0.5g of BSA

- Add 100mL of TBS-T
- Adjust pH to 7.5
- Can be stored for up to 3 weeks at 4ºC (watch for flocculants)

Ammonium acetate-citric acid (AACA) solution

- Prepare ammonium acetate solution (1) by adding 0.385g of ammonium acetate to 100mL of double-distilled water (ddH_2O), adjust pH to 6.68
- Prepare citric acid solution (2) by adding 0.525g of citric acid to 50mL of double-distilled water (ddH_2O), adjust pH to 2.13
- Adjust pH of solution (1) to pH 5.0 with solution (2)
- Store for up to 4 months in a amber bottle at 4ºC

OPD solution (for one plate, 96 wells)

- Take 16mL of AACA solution + 8mL of 30% Hydrogen Peroxide + 8mg of OPD powder
- Mix well. Prepare immediately before use (DO NOT make it up beforehand and store it!).

(b) Protocol

1. Add 150mL of Vtg coating solution (see part c, special steps) to each well with a repeater pipette. Incubate plate for 3 hours at 37ºC (oven in IH 5) under plastic wrap.

While plate(s) is (are) incubating, prepare the samples, standards and primary antibody (see part c, special steps).

2. After the 3h incubation, remove the plate(s) from the incubator. Shake off excess Vtg solution into the sink. Add TBS-T going up and down the plate with multitip repeater pipette (over 150mL/well total), and then shake off solution into the sink. Do this five times.
3. Add 200mL of TBS-T-BSA to every well.
4. Incubate for 30 minutes at 37ºC under plastic wrap. This time is crucial, so that the BSA builds up but doesn't coat over the Vtg. Afterwards, discard solution (do not wash).
5. Now it's time to add the primary antibody, standards, samples, and interassay to the plate.

   i.) Buffer-only wells: add 150mL of TBS-T-BSA

   ii.) Antibody-only wells: add 50mL of TBS-T-BSA, plus 100mL antiserum

   iii.) Standards and samples: add 50mL to each well, plus 100mL antiserum

   iv.) Vtg Inter-assay: add 50mL to each well, plus 100mL antiserum.
6. Cover the plate with plastic wrap and incubate on desktop overnight.
7. After the plate has incubated overnight, turn on the incubator and rinse plate 5 times with TBS-T buffer (as in step 2).
8. Add 150mL of prepared secondary antibody (see part c, special steps) to every well (even the antibody-only and buffer-only wells).
9. Incubate the plate under plastic wrap for 2 hours at 37ºC.

10. Remove the plate from the incubator and rinse 5 times with TBS-T (as in step 2).
11. Add 150mL of OPD solution to each well with repeater pipette, then cover with plastic wrap.
12. Incubate at room temperature in a dark drawer for 30 minutes. Turn on spectrophotometer at 490nm.
13. Remove plate from the drawer. DO NOT WASH!
14. Add 50mL of 5M H_2SO_4 to each well (to denature the proteins and stop the enzymatic production of colour).
15. Place on shaker plate for 10 minutes at 100rpm.
16. Read plate on spectrophotometer at 490nm.
17. Neutralize the acid with $NaHCO_3$. Dispose of plate.

(c) Special steps

Vtg coating of the wells

- The plates must be first pre-coated with Vtg. We want 16.5ng/well of Vtg. We have 1.3912mg/mL (=1.3912mg/mL) Vtg solution. Since we are going to add 150mL of solution to each well: x=16.5ng/150mL = 0.11ng/mL solution must be made.
- Need 18.8mL (or 18800mL) of solution for one plate, so: (y*1.3912mg/mL)/18800mL=0.11ng/mL
- Rearranging and watching units: y= 1.5mL
- So for one plate: 1.5mL of 1.3912mg/mL solution in 18.8mL of SBB = coating solution

Plasma sample preparation

- The best dilution of goldfish plasma is 10,000x. This can be accomplished in many ways. I like the following: 2mL of plasma in 1000mL TBS-T-BSA (500x dilution).
- Vortex.
- Take 50mL of this solution and mix with 950mL TBS-T-BSA (20x dilution).
- This gives the desired 10,000x dilution.

Standards preparation

- The following curve needs to be constructed:
    - 75ng/50mL
    - 37.5ng/50mL
    - 18.75ng/50mL
    - 9.4ng/50mL
    - 4.69ng/50mL
    - 2.34ng/50mL
    - 1.17ng/50mL
    - 0.59ng/50mL
    - 0.29ng/50mL
    - 0.15ng/50mL

- Take 2.2mL of the 1.3912mg/mL (=1.3912mg/mL) stock Vtg solution and pipette it into 2000mL of TBS-T-BSA. This is the 75ng/50mL dilution.Serial dilute this (1:2) with TBS-T-BSA to get the above dilutions.

Primary antibody preparation

- The best dilution of the primary antibody (antiserum) is 165000x . Use the formula: (volume of diluted sample we need)/(dilution factor) = volume of antiserum we want the final volume.
- This means that we use 5.22mL of antiserum in 18.0mL of TBS-T-BSA.

Secondary antibody preparation

- The best dilution for the secondary antibody (antiserum) is 2000x. Using the formula above, for one plate: 18000mL/2000 = 9mL.
- Note that our secondary antiserum is already diluted 10x. So take: 90mL of secondary antiserum in 18mL of TBS-T-BSA.

APPENDIX II

1. Experiment 2: Repeated-measures ANOVA table for P5 content (all factors)

Reminder: Experiment 2 focused on the effect of β-sitosterol on P450scc activity.

(a) Factors

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 level at 0 min
2	P5 level at 15 min
3	P5 level at 30 min

Between subject factors (TRE = treatment; INC = incubation; SUB = substrate, TRI = trial):

Factors	Levels	N
TRE	C (Control)	48
	β (β-Sitosterol)	48
INC	1 (Malate + NADPH)	24
	2 (Malate only)	24
	3 (NADPH only)	24
	4 (No stimulation)	24
SUB	0 (No cholesterol added)	48
	1 (25-Hydroxycholesterol added)	48
TRI	1 (Trial 1)	48
	2 (Trial 2)	48

(b) Test of effects

Within-subject effects:

Effect	Value (Wilk's λ)	F	Hyp. df	Error df	Sig.	Obs. Pow.[a]
TIM * TRE * INC	.963	.404	6	126	.875	.164
TIM * TRE * SUB	.972	.917	2	63	.405	.202
TIM * INC * SUB	.764	3.030	6	126	**.008**	.898
TIM * TRE * TRI	.898	3.561	2	63	**.034**	.641
TIM * INC * TRI	.924	.847	6	126	.536	.325
TIM * SUB * TRI	.993	.215	2	63	.807	.082
TIM * TRE * INC * SUB	.970	.323	6	126	.924	.137
TIM * TRE * INC * TRI	.939	.671	6	126	.674	.259
TIM * TRE * SUB * TRI	.985	.467	2	63	.629	.123
TIM * INC * SUB * TRI	.889	1.276	6	126	.273	.487
TIM * TRE * INC * SUB * TRI	.961	.417	6	126	.867	.168

[a] – Observed power (β) based on $\alpha = 0.05$

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TRE * INC * SUB	5.848	3	1.949	.122	.947	.071
TRE * INC * TRI	12.109	3	4.036	.252	.860	.095
TRE * SUB * TRI	2.730	1	2.730	.170	.681	.069
INC * SUB * TRI	88.488	3	29.496	1.839	.149	.456
TRE * INC * SUB * TRI	17.796	3	5.932	.370	.775	.119
Error	1026.306	64	16.036			

[a] – Observed power (β) based on $\alpha = 0.05$

2. Experiment 2: Repeated-measures ANOVA table for P5 content (malate + NADPH incubation)

(a) Factors

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 level at 0 min
2	P5 level at 15 min
3	P5 level at 30 min

Between subject factors (TRE = treatment; SUB = substrate):

Factors	Levels	N
TRE	C (Control)	6
	β (β-Sitosterol)	6
SUB	0 (No cholesterol added)	6
	1 (25-Hydroxycholesterol added)	6

(b) Test of effects, TRIAL 1

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	749.801	2	374.901	31.483	**.000**	1.000
TIM * TRE	15.702	2	7.851	.659	.531	.141
TIM * SUB	142.363	2	71.181	5.978	**.012**	.809
TIM * TRE * SUB	16.722	2	8.361	.702	.510	.148
Error (TIM)	190.530	16	11.908			

[a] – Observed power (β) based on $\alpha = 0.05$

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	7822.550	1	7822.550	201.787	**.000**	1.000
TRE	3.944	1	3.944	.102	.758	.059
SUB	475.243	1	475.243	12.259	**.008**	.864
TRE * SUB	1.257E-05	1	1.257E-05	.000	1.000	.050
Error	310.131	8	38.766			

[a] – Observed power (β) based on α = 0.05

(c) Test of effects, TRIAL 2

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	653.887	2	326.944	41.463	**.000**	1.000
TIM * TRE	18.482	2	9.241	1.172	.335	.221
TIM * SUB	66.993	2	33.497	4.248	**.033**	.657
TIM * TRE * SUB	1.967	2	.984	.125	.884	.066
Error (TIM)	126.163	16	7.885			

[a] – Observed power (β) based on α = 0.05

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	6068.723	1	6068.723	583.828	**.000**	1.000
TRE	13.767	1	13.767	1.324	.283	.433
SUB	113.410	1	113.410	10.910	**.011**	.967
TRE * SUB	.220	1	.220	.021	.888	.052
Error	83.158	8	10.395			

[a] – Observed power (β) based on α = 0.05

3. Experiment 2: Repeated-measures ANOVA table for P5 content (malate only)

(a) Factors

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 level at 0 min
2	P5 level at 15 min
3	P5 level at 30 min

Between subject factors (TRE = treatment; SUB = substrate):

Factors	Levels	N
TRE	C (Control)	6
	β (β-Sitosterol)	6
SUB	0 (No cholesterol added)	6
	1 (25-Hydroxycholesterol added)	6

(b) Test of effects, TRIAL 1

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	150.756	2	75.378	50.312	**.000**	1.000
TIM * TRE	1.298	2	.649	.433	.656	.108
TIM * SUB	4.253	2	2.127	1.419	.271	.260
TIM * TRE * SUB	2.729	2	1.365	.911	.422	.180
Error (TIM)	23.971	16	1.498			

[a] – Observed power (β) based on $\alpha = 0.05$

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	4217.076	1	4217.076	395.591	**.000**	1.000
TRE	5.494	1	5.494	.515	.493	.097
SUB	5.538	1	5.538	.519	.492	.098
TRE * SUB	5.074	1	5.074	.476	.510	.094
Error	85.282	8	10.660			

[a] – Observed power (β) based on α = 0.05

(c) Test of effects, TRIAL 2

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	218.630	2	109.315	18.341	**.000**	1.000
TIM * TRE	5.504	2	2.752	.462	.638	.112
TIM * SUB	11.053	2	5.526	.927	.416	.182
TIM * TRE * SUB	9.406	2	4.703	.789	.471	.161
Error (TIM)	95.362	16	5.960			

[a] – Observed power (β) based on α = 0.05

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	4360.784	1	4360.784	854.841	**.000**	1.000
TRE	1.596	1	1.596	.313	.591	.079
SUB	36.098	1	36.098	7.076	**.029**	.646
TRE * SUB	15.783	1	15.783	3.094	.117	.341
Error	40.810	8	5.101			

[a] – Observed power (β) based on α = 0.05

4. Experiment 2: Repeated-measures ANOVA table for P5 content (NADPH only)

(a) Factors

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 level at 0 min
2	P5 level at 15 min
3	P5 level at 30 min

Between subject factors (TRE = treatment; SUB = substrate):

Factors	Levels	N
TRE	C (Control)	6
	β (β-Sitosterol)	6
SUB	0 (No cholesterol added)	6
	1 (25-Hydroxycholesterol added)	6

(b) Test of effects, TRIAL 1

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	1124.227	2	562.114	39.743	**.000**	1.000
TIM * TRE	12.299	2	6.150	.435	.655	.109
TIM * SUB	95.907	2	47.954	3.390	.059	.554
TIM * TRE * SUB	1.994	2	.997	.070	.932	.059
Error (TIM)	226.301	16	14.144			

[a] – Observed power (β) based on $\alpha = 0.05$

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	7575.982	1	7575.982	214.837	**.000**	1.000
TRE	1.887	1	1.887	.054	.823	.055
SUB	228.067	1	228.067	6.467	**.035**	.608
TRE * SUB	2.169	1	2.169	.062	.810	.056
Error	282.111	8	35.264			

[a] – Observed power (β) based on α = 0.05

(c) Test of effects, TRIAL 2

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	717.331	2	358.666	139.139	**.000**	1.000
TIM * TRE	13.939	2	6.970	2.704	.097	.458
TIM * SUB	26.471	2	13.236	5.135	**.019**	.744
TIM * TRE * SUB	2.366	2	1.183	.459	.640	.112
Error (TIM)	41.244	16	2.578			

[a] – Observed power (β) based on α = 0.05

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	5313.795	1	5313.795	598.067	**.000**	1.000
TRE	2.934	1	2.934	.330	.581	.080
SUB	71.457	1	71.457	8.042	**.022**	.701
TRE * SUB	9.869E-02	1	9.869E-02	.011	.919	.051
Error						

[a] – Observed power (β) based on α = 0.05

5. Experiment 2: Repeated-measures ANOVA table for P5 content (no stimulation)

(a) Factors

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 level at 0 min
2	P5 level at 15 min
3	P5 level at 30 min

Between subject factors (TRE = treatment; SUB = substrate):

Factors	Levels	N
TRE	C (Control)	6
	β (β-Sitosterol)	6
SUB	0 (No cholesterol added)	6
	1 (25-Hydroxycholesterol added)	6

(b) Test of effects, TRIAL 1

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	2.090	2	1.045	.431	.657	.108
TIM * TRE	4.923	2	2.462	1.016	.384	.196
TIM * SUB	7.243	2	3.622	1.494	.254	.272
TIM * TRE * SUB	5.509	2	2.755	1.137	.346	.215
Error (TIM)	38.777	16	2.424			

[a] – Observed power (β) based on $\alpha = 0.05$

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	2656.656	1	2656.656	174.558	**.000**	1.000
TRE	5.322	1	5.322	.350	.571	.082
SUB	2.657	1	2.657	.175	.687	.066
TRE * SUB	.619	1	.619	.041	.845	.054
Error	121.755	8	15.219			

[a] – Observed power (β) based on α = 0.05

(c) Test of effects, TRIAL 2

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	3.035	2	1.518	1.552	.242	.281
TIM * TRE	1.336	2	.668	.683	.519	.145
TIM * SUB	.937	2	.469	.479	.628	.115
TIM * TRE * SUB	2.433E-02	2	1.216E-02	.012	.988	.052
Error (TIM)	15.650	16	.978			

[a] – Observed power (β) based on α = 0.05

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	1924.232	1	19.24.232	481.361	**.000**	1.000
TRE	11.370	1	11.370	2.844	.130	.318
SUB	7.453	1	7.453	1.865	.209	.226
TRE * SUB	2.709	1	2.709	.678	.434	.113
Error	31.980	8	3.997			

[a] – Observed power (β) based on α = 0.05

6. Experiment 3 (trial 1): Repeated-measures ANOVA table for P5 content (all factors)

Reminder: Experiment 3 focused on the effect of β-sitosterol on the pool of reactive cholesterol. A mixture of phytosterols (55% β-sitosterol) was used in trial 1.

(a) Factors

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 level at 0 min
2	P5 level at 5 min
3	P5 level at 10 min
4	P5 level at 15 min
5	P5 level at 20 min

Between-subject factors (TRE = treatment; AMG = AMG injection group):

Factors	Levels	N
TRE	C (Control)	6
	β (β-Sitosterol)	6
AMG	0 (No AMG, vehicle injected)	6
	1 (AMG injected)	6

(b) Test of effects

Within-subject effects:

Effect	Value (Wilk's λ)	F	Hyp. df	Error df	Sig.	Obs. Pow.[a]
TIM	.009	140.632	4	5	**.000**	1.000
TIM * TRE	.118	9.314	4	5	**.015**	.877
TIM * AMG	.244	3.883	4	5	.085	.508
TIM * TRE * AMG	.094	12.061	4	5	**.009**	.945

[a] – Observed power (β) based on α = 0.05

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	10467.151	1	10467.151	378.658	**.000**	1.000
TRE	95.894	1	95.894	3.469	.100	.375
AMG	89.068	1	89.068	3.222	.110	.353
TRE * AMG	348.234	1	348.234	12.598	**.008**	.873
Error	221.151	8	27.643			

[a] – Observed power (β) based on $\alpha = 0.05$

7. Experiment 3 (trial 1): Repeated-measures ANOVA table for P5 content and P5 production (no AMG, vehicle injection group only)

(a) Factors for P5 content (no AMG, vehicle injection group)

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 level at 0 min
2	P5 level at 5 min
3	P5 level at 10 min
4	P5 level at 15 min
5	P5 level at 20 min

Between-subject factor (TRE = treatment):

Factors	Levels	N
TRE	C (Control)	3
	β (β-Sitosterol)	3

(b) Test of effects for P5 content (no AMG, vehicle injection group)

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	375.371	4	93.843	18.686	**.000**	1.000
TIM * TRE	173.830	4	43.458	8.653	**.001**	.991
Error (TIM)	80.355	16	5.022			

[a] – Observed power (β) based on α = 0.05

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	4312.560	1	4312.560	86.051	**.001**	1.000
TRE	404.803	1	404.803	8.077	**.047**	.575
Error	200.465	4	50.116			

[a] – Observed power (β) based on α = 0.05

(c) Factors for P5 production (no AMG, vehicle injection group)

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 production between 0 and 5 min
2	P5 production between 5 and 10 min

Between-subject factor (TRE = treatment):

Factors	Levels	N
TRE	C (Control)	3
	β (β-Sitosterol)	3

(d) Test of effects for P5 production (no AMG, vehicle injection group)

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	39.990	1	39.990	8.552	**.043**	.598
TRE	24.463	1	24.463	5.231	.084	.416
Error	18.705	4	4.676			

[a] – Observed power (β) based on α = 0.05

8. Experiment 3 (trial 1): Repeated-measures ANOVA table for P5 content and P5 production (AMG injected group)

(a) Factors for P5 content (AMG injected group)

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 level at 0 min
2	P5 level at 5 min
3	P5 level at 10 min
4	P5 level at 15 min
5	P5 level at 20 min

Between-subject factor (TRE = treatment):

Factors	Levels	N
TRE	C (Control)	3
	β (β-Sitosterol)	3

(b) Test of effects for P5 content (AMG injected group)

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	908.366	4	227.092	25.631	**.000**	1.000
TIM * TRE	104.671	4	26.168	2.953	.053	.655
Error (TIM)	141.761	16	8.860			

[a] – Observed power (β) based on $\alpha = 0.05$

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	6243.659	1	6243.659	1207.876	**.000**	1.000
TRE	39.325	1	39.325	7.608	.051	.552
Error	20.676	4	5.169			

[a] – Observed power (β) based on α = 0.05

(c) Factors for P5 production (AMG injected group)

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 production between 0 and 5 min
2	P5 production between 5 and 10 min

Between-subject factor (TRE = treatment):

Factors	Levels	N
TRE	C (Control)	3
	β (β-Sitosterol)	3

(d) Test of effects for P5 production (AMG injected group)

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	132.436	1	132.436	17.412	**.014**	.870
TRE	27.177	1	27.177	3.573	.132	.307
Error	30.423	4	7.606			

[a] – Observed power (β) based on α = 0.05

9. Experiment 3 (trial 2): Repeated-measures ANOVA table for P5 content and P5 production (no AMG, vehicle injection group only)

Reminder: Experiment 3 focused on the effect of β-sitosterol on the pool of reactive cholesterol. Pure β-sitosterol (95.6%) was used in trial 2.

(a) Factors for P5 content (no AMG, vehicle injection group)

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 level at 0 min
2	P5 level at 6 min
3	P5 level at 12 min
4	P5 level at 18 min
5	P5 level at 24 min

Between-subject factor (TRE = treatment):

Factors	Levels	N
TRE	C (Control)	3
	β (β-Sitosterol)	3

(b) Test of effects for P5 content (no AMG, vehicle injection group)

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	57.785	4	14.446	15.296	**.000**	1.000
TIM * TRE	14.686	4	3.672	3.887	**.022**	.787
Error (TIM)	15.112	16	.944			

[a] – Observed power (β) based on $\alpha = 0.05$

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	3474.616	1	3474.616	1115.366	**.000**	1.000
TRE	1.122	1	1.122	.360	.581	.076
Error	12.461	4	3.115			

[a] – Observed power (β) based on α = 0.05

(c) Factors for P5 production (no AMG, vehicle injection group)

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 production between 0 and 6 min
2	P5 production between 6 and 12 min

Between-subject factor (TRE = treatment):

Factors	Levels	N
TRE	C (Control)	3
	β (β-Sitosterol)	3

(d) Test of effects for P5 production (no AMG, vehicle injection group)

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	24.216	1	24.216	42.169	**.003**	.996
TRE	7.869	1	7.869	13.702	**.021**	.788
Error	2.297	4	.574			

[a] – Observed power (β) based on α = 0.05

10. Experiment 3 (trial 2): Repeated-measures ANOVA table for P5 content and P5 production (AMG injected group)

(a) Factors for P5 content (AMG injected group)

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 level at 0 min
2	P5 level at 6 min
3	P5 level at 12 min
4	P5 level at 18 min
5	P5 level at 24 min

Between-subject factor (TRE = treatment):

Factors	Levels	N
TRE	C (Control)	4
	β (β-Sitosterol)	4

(b) Test of effects for P5 content (AMG injected group)

Within-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
TIM	178.766	4	44.691	24.663	**.000**	1.000
TIM * TRE	21.210	4	5.302	2.926	**.042**	.698
Error (TIM)	43.490	24	1.812			

[a] – Observed power (β) based on $\alpha = 0.05$

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	3837.202	1	3837.202	264.550	**.000**	1.000
TRE	1.857	1	1.857	.128	.733	.061
Error	87.028	6	14.505			

[a] – Observed power (β) based on α = 0.05

(c) Factors for P5 production (AMG injected group)

Within-subject factor (TIM = time):

TIM level	Dependent variable
1	P5 production between 0 and 6 min
2	P5 production between 6 and 12 min

Between-subjects factor (TRE = treatment):

Factors	Levels	N
TRE	C (Control)	4
	β (β-Sitosterol)	4

(d) Test of effects for P5 production (AMG injected group)

Between-subject effects:

Effect	Type III SS	df	MS	F	Sig.	Obs. Pow.[a]
Intercept	65.991	1	65.991	31.335	**.001**	.996
TRE	9.425	1	9.425	4.476	.079	.428
Error	12.636	6	2.106			

[a] – Observed power (β) based on α = 0.05