Dietary exposure of 1, 2-dibromo-4-(1,2-dibromoethyl)cyclohexane to juvenile brown trout (*Salmo trutta*): bioaccumulation parameters and effects on circulating plasma sex hormones

By

Bonnie Gemmill

A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Environment and Geography
University of Manitoba
Winnipeg, Manitoba

ABSTRACT

1,2-Dibromo-4-(1,2 dibromoethyl)cyclohexane or tetrabromoethylcyclohexane (TBECH) is an additive bromine based flame retardant used primarily in expandable polystyrene beads that are used mainly to produce thermal insulation for housing. Secondary uses include extrusion into polystyrene foam, adhesive in fabric and vinyl lamination, electrical cable coatings and construction materials. The technical formulation contains two diastereoisomers, α- and β-, which are present in equimolar amounts. Under elevated temperatures two other isomers, γ- and δ-, can be formed. The recent detection of TBECH in the environment and suggestions that all four isomers are androgenic prompted me to examine the bioaccumulation and biochemical effects of one of the isomers, β-, in a controlled laboratory environment. I purposely chose to examine this isomer as it has been detected in biota. Juvenile brown trout (*Salmo trutta*) were exposed to three different amounts of the β-isomer *via* their food for 56 days (uptake phase). This was followed by a depuration phase in which all fish were exposed to unfortified food for 77 days. A fourth group of fish were exposed to unfortified food for the duration of the experiment. On days 0, 7, 14, 35, 49, 56, 63, 77, 91, 105, and 133 eight fish from each treatment group were sacrificed and liver, plasma, thyroid and gonad gland were collected and whole-fish (carcass minus tissues above) were collected. Residues of β-isomer were analyzed in the whole-fish and in liver extracts by gas chromatography mass spectrometry in the electron ionization while estradiol (E2), 11-ketotestosterone (11-KT) and testosterone (T) were extracted from plasma and analysed by liquid chromatography tandem mass spectrometry. The bioaccumulation of β-isomer was similar in fish from all treatment groups with steady-

state occurring before the end of the uptake phase. Depuration of the β-isomer from fish obeyed first order kinetics and there were no statistically significant differences in the depuration half life ($t_{1/2}$) among the treatment groups: 22.5 ± 10.4 (low), 13.5 ± 5.9 (med) and 13.8 ± 2.2 (high) days. Steady-state biomagnification factors were much smaller than 1 for fish in all treatment groups. I was unable to detect debrominated metabolites in liver or whole-fish extracts and I also found no evidence of isomerization of the β-isomer to other isoforms *in vivo*. While there were some differences in E2, T and 11-KT levels in plasma of fish from the treated groups relative to plasma in fish from the control group there were no clear, consistent, discerning trends.

ACKNOWLEDGMENTS

To my entire family, thank you for always showing interest and supporting me in everything that I do! Everyone constantly asked me the question of when I was going to be done and guess what, the time has come! To my mom and dad, thank you for giving me the choice after high school of either getting an education or moving out, I obviously chose the education but I bet you didn't think it was going to take this long!

To my husband, Bryan Hockridge, thank you for putting up with me all these years and being there for me when I needed you the most. You would come in with me on weekends to help me finish my work faster or to just simply keep me company. Thank you!

To my family and friends, for all their support throughout my university career,

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Flame retardants (FRs) are introduced into products because they are heat resistant chemicals that can delay or reduce the ignition or combustion of a product. There are four main chemical groups of flame retardants; inorganic, organo halogens, organophosphorus and nitrogen based compounds (Birnbaum and Staskal 2004). Of the total 175 classified FRs, 75 of them contain the element bromine. By far, the most commonly used FRs are the brominated flame retardants (BFRs) and this is due to their high efficiency, low decomposing temperature and low cost (Alaee and Wenning 2002).

Flame retardants are further subdivided into two chemical types: reactive and additive (De Wit 2002). Reactive FRs are covalently bonded to the polymer while additive ones are added as a component of the dissolved material (De Wit 2002). A good FR should be inexpensive, stable to heat, colorless, easily blended in with the polymer, decompose around the same temperature as the polymer, must not change physical and mechanical properties once it is in the final product and be stable at temperatures under which the intended product is formed (Kesner and de Vos 2001).

In general, additive FRs is more easily released into the environment than reactive FRs because there are no chemical bonds between the FR and the end product. As a result, leaching of additive FRs into the environment after disposal in landfills, for example, is thought to be an important point of entry of these compounds into the environment.

Recently, Tomy *et al*. reported on the detection of a novel additive BFR, 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane or tetrabromoethylcyclohexane (TBECH) in

beluga whales (*Delphinapterus leucas*) from the Canadian Arctic (Tomy et al 2008a). The work described in this thesis attempts to build on this discovery by exploring the chemical and biological behaviour of this chemical in fish under a controlled laboratory environment.

Before describing the objectives of my research and my study design, I will first acquaint the reader with (*i*) the chemical mechanism by which halogenated FR work, (*ii*) the synthesis of technical TBECH (*t*-TBECH), (*iii*) the physical chemical properties and uses of TBECH and, (*iv*) current environmental levels and toxicology of TBECH.

1.1. HOW FLAME RETARDANTS WORK

In order for a fire to occur there is the need for three elements; oxygen, heat and fuel (Figure 1.1). For a FR to work it needs to disrupt one of these elements to delay the combustion of the fire.
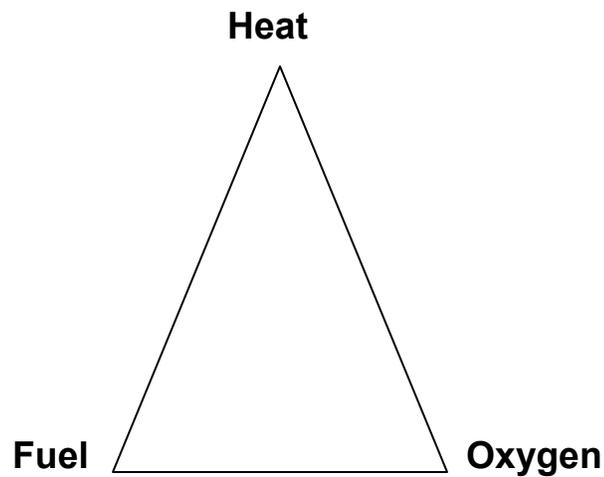
Figure 1.1 Fire triangle demonstrating the three elements needed in order for a fire to occur (Kesner and de Vos 2001).

There are four stages during a fire: ignition, propagation, combustion and termination (Figure 1.2). When a FR is present it can interfere with one of these stages reducing the time of the fire and sometimes eliminate one of the stages (Kesner and de Vos 2001).
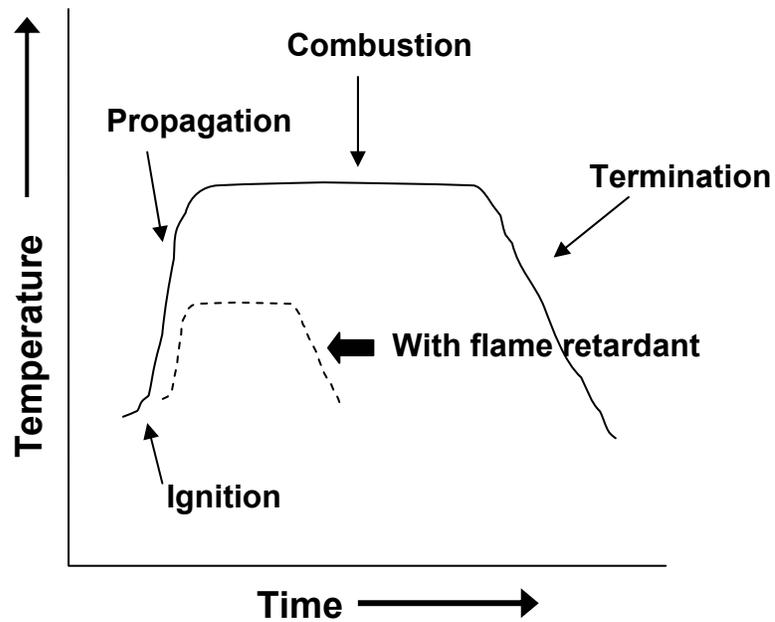
Figure 1.2 Stages of a fire and the effects of a flame retardant (Kesner and de Vos 2001)

When BFR's absorb the required amount of energy, the carbon-bromine bond breaks, liberating bromine radicals. The bromine radical reacts with the hydrogen atoms in the polymer or captures free hydrogen atoms to form HBr, where the RH is the polymer (Kesner and de Vos 2001).

$$RH + Br• \rightarrow HBr + R• \tag{1-1}$$

$$•H + Br• \rightarrow HBr \tag{1-2}$$

HBr slows down the chain reactions that take place during the burning process. For example, HBR can deactivate hydroxyl radicals; (Kesner and de Vos 2001).

$$·OH + HBr \rightarrow H_2O + ·Br \tag{1-3}$$

The bromine atom withdraws energy from the combustion-propagation stage of the fire, in turn slowing the burning process (Kesner and de Vos 2001).

1.2. INDUSTRIAL SYNTHESIS OF TBECH

t-TBECH is produced by the Albermarle Corporation and is marketed as Saytex BCL-462 (Tomy et al 2008a). The industrial synthesis involves the bromination of vinylcyclohexane (Figure 1.3).
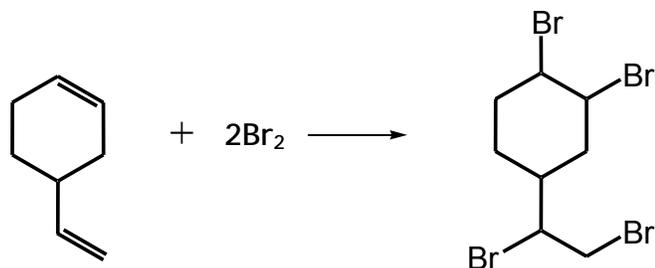
Figure 1.3 Industrial synthesis of *t*-TBECH

Because of the four chiral centers generated, the technical formulation can contain four diastereomers (Figure 1.4). In general, however, only two of the four diastereomers, α- and β-, are detectable in the technical mixture at 45% each (Arsenault et al 2008). The other two possible isomers, δ- and γ-, are not detectable in the technical mixture but because of their thermal instability and under heat-driven manufacturing processes interconversion of diastereomers can arise resulting in their formation (*see* Figure 1.5).
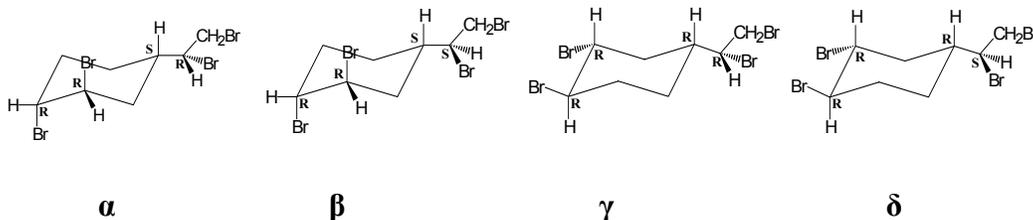
Figure 1.4 Structures of the four possible TBECH diastereomers (Arsenault et al 2008).

The thermally-driven interconversion of diastereomers has been studied by Arsenault *et al*. 2008 (Arsenault et al 2008). At 125ºC interconversion of the isomers can occur. For example, the α-isomer can isomerize into the β- isomer by inversion of CHBr on the ethyl group and vice versa (*see* Figure 1.5). The β- isomer can in turn isomerize to the γ-isomer by inversion of the CHBr group on the ring.

5
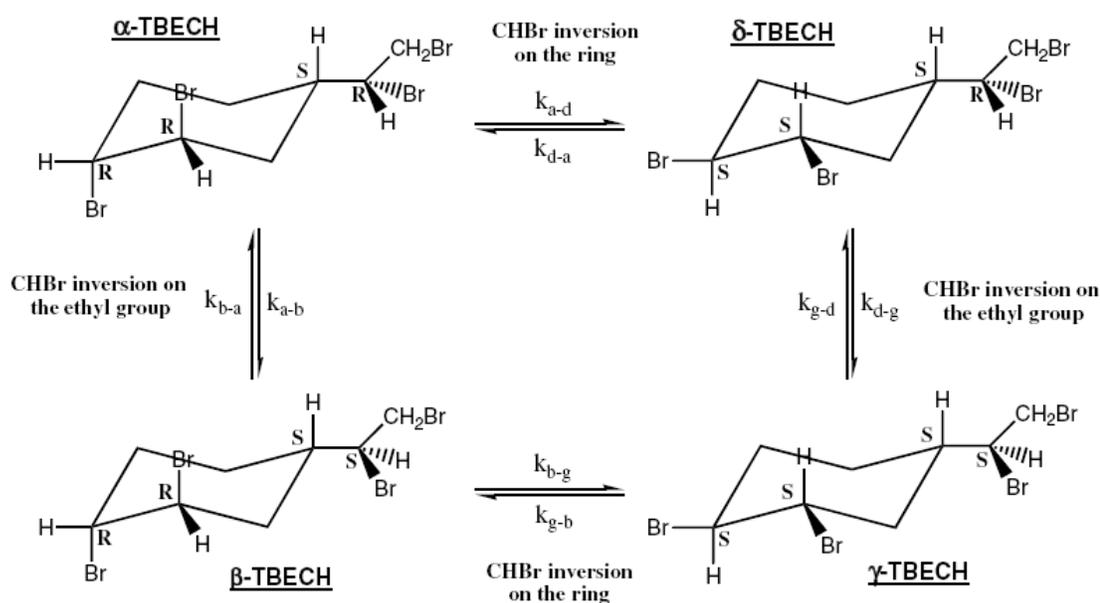
Figure 1.5 Pathways to interconversion between the four TBECH isomers
(Arsenault et al 2008).

1.3. PHYSICAL AND CHEMICAL PROPERTIES

t-TBECH is a solid, white powder at room temperature with near equimolar
amounts of α- and β-isomers (Arsenault et al 2008). The physiochemical properties of
α/β TBECH are as follows: molecular weight of 427.80; melting point ranges from 70-
97ºC; sublimation starting at *ca.* 59ºC; vapour pressure of 0.00011 mm Hg; water
solubility of 0.069 mg/L and the log octanol water partition coefficient (log K_{ow}) of 5.2
(Arsenault et al 2008). The experimental measured melting point sublimation of the β-
isomers are 100.0-101.5ºC and *ca* 97ºC (Arsenault et al 2008).

1.4. USES AND PRODUCTION ESTIMATES

t-TBECH is primarily used in expandable polystyrene beads mainly used to produce thermal insulation for housing (Tomy et al 2008a). The amount of *t*-TBECH used in this application is *ca*. 1% w/w. Secondary uses of *t*-TBECH include extrusion into polystyrene foam, adhesive in fabric and vinyl lamination, electrical cable coatings and construction materials (Tomy et al 2008a).

Production estimates for *t*-TBECH are sparse: one report lists production for the years 1986, 1990, 1994, 1998, and 2002 between 4.5 and 226 tons per year (U.S. EPA 1985, U.S EPA 1984). Older information reports production at less than 453 tons in 1983 (U.S. EPA 1985, U.S EPA 1984).

1.5. ENVIRONMENTAL LEVELS AND TOXICOLOGY

Isomers in the technical mixture are suspected endocrine disruptor chemicals *e.g.*, a chemical substance that interferes with, or has adverse effects on the production, distribution, or function of hormones (Society of Environmental Toxicology and Chemistry 2000). Through a molecular modeling exercise, Larsson *et al*. demonstrated that the structure of TBECH (non isomer specific) is able to bind and activate human androgen receptors (hAR) (Larsson et al 2006). By definition, an AR is any compound that will bind to the androgen binding site and activate it. Based on the theoretical modeling exercise, the authors concluded that TBECH is a strong hAR agonist with a potential of causing endocrine disruption (Larsson et al 2006).

Building on the earlier work of Larsson *et al.*, Khalaf *et al.* used a similar computational modeling exercise to investigate the interaction energies between the 4 isomers of TBECH and the hAR (Khalaf et al 2009). The authors found that the γ- and δ-isomers were more potent activators of hAR than the α- and β-isomers. In the same study, the authors report that all four isomers can induce prostrate specific antigen (a glycoprotein widely used as a marker for prostate cancer) at 100 nM (Khalaf et al 2009).

Nyholm *et al.* recently demonstrated that *t*-TBECH bioaccumulates in zebrafish (Nyholm et al 2009). An uptake and depuration study was carried out through dietary exposure to zebrafish (*Danio rerio*). The authors reported that the uptake efficiency of *t*-TBECH was among the highest of all the BFRs studied. Elimination of *t*-TBECH occurred rapidly from zebrafish with a half life ($t_{1/2}$) of less than 2 days (Nyholm et al 2009).

Nyholm *et al.* carried out another study that measured *t*-TBECH in zebrafish fish eggs after parental exposure for 42 days through diet (Nyhom et al 2008). Two doses were administered through the zebrafish diet at 10 and 100 nmol. After 34-36 days, significant concentrations of *t*-TBECH were measurable in zebrafish eggs: 1 nmol/g lipid weight and 10 nmol/g lipid weight for low and high dose treatments, respectively, suggesting that *t*-TBECH can be depurated to its offspring.

The biodegradation kinetics of *t*-TBECH and other BFRs in aerobic and anaerobic soils has recently been reported (Nyholm et al 2010). Half-lives of *t*-TBECH were similar under both aerobic and anaerobic conditions, 21 and 23 days, respectively. Compared to other BFRs, the $t_{1/2}$'s of *t*-TBECH was greater than 2,4,6-tribromophenol (aerobic: 8 days, anaerobic: 7 days) and similar to hexabromobenzene under aerobic conditions (22

days) but much smaller than that of 2,4,4-tribromodiphenyl ether (210 days) and decabromodiphenyl ether (> 400 days).

Tomy *et al*. measured the β-isomer in Canadian arctic beluga whales (Tomy et al 2008a). Concentrations were reported to range from 1.1 to 9.3 ng/g of lipid weight. The detection of only the β-isomer was of particular interest as it is known that the α- and β-isomers are formed at the same equimolar amounts in the technical mixture. It was hypothesized that differences in the physical-chemical properties such as log K_{ow} and differences in biological processes such as uptake and metabolism might contribute to this observation.

Gauthier *et al.* measured all four isomers of TBECH in herring gull (*Larus argentatus*) eggs from the Laurentian Great Lakes of North America (Gauthier et al 2009). The greatest concentrations of TBECH were found in 2006 at 0.54 ng/g wet weight in one colony with an average of <0.20 ng/g wet weight in the other six colonies studied. The study period was between 1982 and 2006 and demonstrated no real trends in the concentration of TBECH over these years.

1.6. OBJECTIVES OF PRESENT RESEARCH

It is clear that isomers of TBECH are present in the environment. While one study has clearly shown TBECH to be an endocrine disrupting chemical in humans, it is not known if TBECH can have the same impact on fish such as the androgenic effects and at what concentrations this impact would be observed.

To address this knowledge gap, I designed a fish exposure study in which juvenile brown trout (*Salmo trutta*) were exposed to three concentrations of the β-isomer *via* their diet. The primary object of my research was to test the hypothesis that TBECH would affect circulating concentrations of sex hormones in fish plasma. I chose to conduct my study on a single isomer so as not to confound the interpretation of my results. My experiment design allowed me to examine the uptake and clearance kinetics of the β-isomer in fish.

CHAPTER 2

EXPERIMENTAL

2.1. CHEMICALS

The chemicals used in this study were supplied by a number of sources. *Rac–*(1*R*,2*R*)–1,2-dibromo–(4*S*)–4–((1*S*)–1,2–dibromoethyl)cyclohexane, *1* *(see Appendix A)*, (β–TBECH, $C_8H_{12}Br_4$, MW=427.1 g/mol) 2,3',4',6–tetrabromodiphenylether, *2*, (BDE–71, $C_{12}H_6Br_4O$, MW = 481.72 g/mol) and 3,3',4,4',5–pentabromodiphenyl ether, *3*, (BDE–126, $C_{12}H_5Br_5O$ MW= 564.69 g/mol) all >99.5% stated purity and a crude synthetic metabolite solution containing debrominated forms of TBECH were all obtained from the Wellington Laboratories (Guelph, ON, Canada). HPLC optima grade methanol and water were obtained from Fisher Scientific (Nepean, ON, Canada). Distilled in glass hexane, dichloromethane (DCM), acetone and ethyl acetate were obtained from Caledon Laboratories (Georgetown, ON). Corn oil, gelatine, and tricaine methanesulfonate (MS 222) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Ottawa sand (20–30 mesh) was obtained from Fisher Scientific (ON, Canada). Individual solutions of testosterone, *4*, (T, $C_{19}H_{28}O_2$, MW= 288.4 g/mol), 11–ketotestosterone, *5*, (11–KT, $C_{19}H_{26}O_3$, MW= 302.4 g/mol), estradiol, *6*, (E2, $C_{18}H_{24}O_2$, MW= 272.38 g/mol), carbon mass labelled estradiol, *7*, ($^{13}C_3{}^{13}C_4$–E2, $^{13}C_2{}^{12}C_{16}H_{24}O_2$, MW = 274.38 g/mol) and deuterium mass labelled progesterone, *8*, ($d_{2,2,6,6,17,21,21,21}$–P, $C_{21}H_{22}D_8O_2$ MW=322.51) in methanol were obtained Cambridge Isotope Laboratories (Cambridge Isotope Laboratories, Andover, MA). (Oakville, ON, Canada).

2.2. FOOD PREPARATION

Commercial starter fish food (1.8 kg of Silver Cod; Martin's Feed Mills, Elmira, ON, Canada) was added to a pre-cleaned (soap and water, then rinsed with methanol) Hobart blender. Diets were formulated by adding a known amount of β–TBECH to 20 mLs of corn oil (Sigma Aldrich, St.Louis, MO) at three levels: low (500 ng), medium (5400 ng) and high (54,000 ng). The range of β-isomer was determined by measuring fish from Lake Winnipeg [mean (n=8): 6 pmoles/g, (lw) [G.Tomy, *personal communication*]. A fourth solution containing only the corn oil was used in preparing the control food. Each of the corn oil solutions were added to four separate batches of food and mixed at low speed for *ca*. 20 minutes to ensure the food was well mixed. A gelatine binder was prepared by vigorously stirring 40 g of gelatine (Sigma-Aldrich; Oakville, ON, Canada) to 1.8 L of Milli-Q water (heated to 30-37ºC). The aqueous gelatine was slowly added into the food and mixed until the food had a firm consistency (*ca*. 15 minutes). Food was then air dried for 1 hour, extruded (4 mm diameter) through a die using a meat grinder and the noodles dried at 25ºC for 2-3 days. Noodles were manually broken into pellets and stored in plastic bags at –20ºC. The mean lipid content of the food was determined to be 10.6 ± 0.2 % and measured concentration of the β-isomer in the food was (pmoles/g, lipid basis): 2.02 ± 0.36 (low), 14.7 ± 0.9 (medium) and 118.4 ± 3.1 (high). There were statistically significant differences in the amount of the β–isomer in all three batches of the food ($p<0.001$) (ANOVA).

2.3. EXPOSURE EXPERIMENT

Four hundred juvenile brown trout (*Salmo trutta*) male and female (mean weight *ca.* 55 g) obtained from the Whiteshell Fish Hatchery (West Hawk Lake, Manitoba) were randomly separated into four 800 L fibreglass aquaria in groups. Juvenile brown trout were used in this study because they are an environmentally relevant species. Each tank was a one pass flow through system and received water at a constant water flow of 1.5L/min of UV and carbon-dechlorinated Winnipeg city tap water, at a temperature of 12–15º C. The water pH was between 7.6 and 9.4, measured once a week using a pH meter and probe (Fisher Scientific, Pittsburgh, PA). The dissolved oxygen was always at level of >90% saturation, measured once a week using an YSI model 58 dissolved oxygen meter (Yellow Springs, Ohio, USA). A 12-h light and 12-h dark photoperiod was maintained throughout the experiment. Fish were acclimatized in their respective tanks for 7 days prior to the start of the experiment and fed a diet of control *i.e.*, unfortified, food. Fish in three of the tanks were exposed to the β–isomer by sprinkling a known amount of fortified food at the surface of the water. Fish in the fourth tank were exposed to unfortified food for the duration of the experiment. Fish were fed a ration diet of 1% of their body weight three times per week. My experiment consisted of two phases: (*i*) an uptake phase lasting 56 days where fish were in three tanks were fed fortified food; fish in the fourth tank were fed unfortified food and, (*ii*) a depuration phase lasting 77 days where all fish were fed unfortified food. Eight fish from each tank were sacrificed on days 7, 14, 21, 35, 49 and 56 of the uptake phase and days 63, 77, 91, 105 and 133 of the depuration phase. Fish were sacrificed 48 hours after the previous feeding by an overdose of a pH buffered solution of MS-222 (0.5 g/L). Once operculum movement

ceased (<5 min), fork lengths and weights were recorded and 1–3 mL of blood was removed *via* the caudal vein with a heparinised syringe. Liver, gall bladder, gonads and thyroid were also dissected. Whole blood was centrifuged at 6000rpm for 10 minutes to separate plasma from red blood cells. Plasma along with other portions of the fish were stored at –80°C. Whole fish (excluding the liver, gonads, gall bladder and thyroid) was used for calculating bioaccumulation parameters while circulating levels of the studied hormones were measured in the plasma.

2.4. EXTRACTION

2.4.1. Whole-fish

Figure 2.1 shows a schematic of my approach to extracting β–TBECH from whole-fish (Law et al 2006). Whole fish samples were dry ice homogenized and weighed prior to extraction. Extraction of β–TBECH from whole-fish was achieved using a Dionex accelerated solvent extractor (ASE-300, Dionex Canada Ltd., Oakville, ON, Canada). Weighed samples (*ca.* 15 g) were mixed with heat-treated (600ºC for 6h) pelleted diatomaceous earth and added to the 100 mL cell. Cells were spiked with a recovery internal standard (RIS) of 10 ng of BDE–71 (10 μL of a 1 ng/μL solution). Heat treated (600ºC for 6h) Ottawa sand was added to fill any voids. The ASE parameters used were as follows: solvent 50:50 DCM:hexane; temperature 125ºC; pressure 1500 psi; heat-up time 6 min; static time 5 min; flush volume 60%; purge time 120s and 2 static cycles. Obtained extracts (*ca*. 50 mL) had water removed by heat treated (600ºC for 6h) anhydrous sodium sulphate (10–60mesh size), reduced in volume to 6 mL by rotoevaporation (Heidolph, Fisher Scientific, Pittsburgh, PA) and filtered using 1 μm

polytetrafluoroethylene syringe filters (Gelman Laboratory, MI). Lipids were determined gravimetrically on a 1 mL portion of the extract. Lipids were removed from the remaining extract (5 mL) using an automated gel permeation chromatograph (J2 Scientific, Columbia, Missouri, US) on a column (29.5 mm i.d x 400 mm) packed with 60 g (dry weight) of 200–400 mesh S-X3 Envirobeads (ABC Instruments, MO). Lipid-free extracts were then reduced in volume (1 mL) and applied to a column (300 mm x 10.5 mm i.d) of reagent-grade Florisil (1.2% deactivated (w/w), 8g, 60-100 mesh size, Fisher Scientific). β–TBECH was eluted using 48 mL of hexane followed by 20 mL of 50:50 hexane:DCM. The purified fraction was reduced in volume to 100 μL by a gentle stream of ultra high purity N_2 and spiked with 10 ng of BDE–126 (10 μL of 1 ng/μL solution) prior to gas chromatography mass spectrometry (GC/MS) analysis.
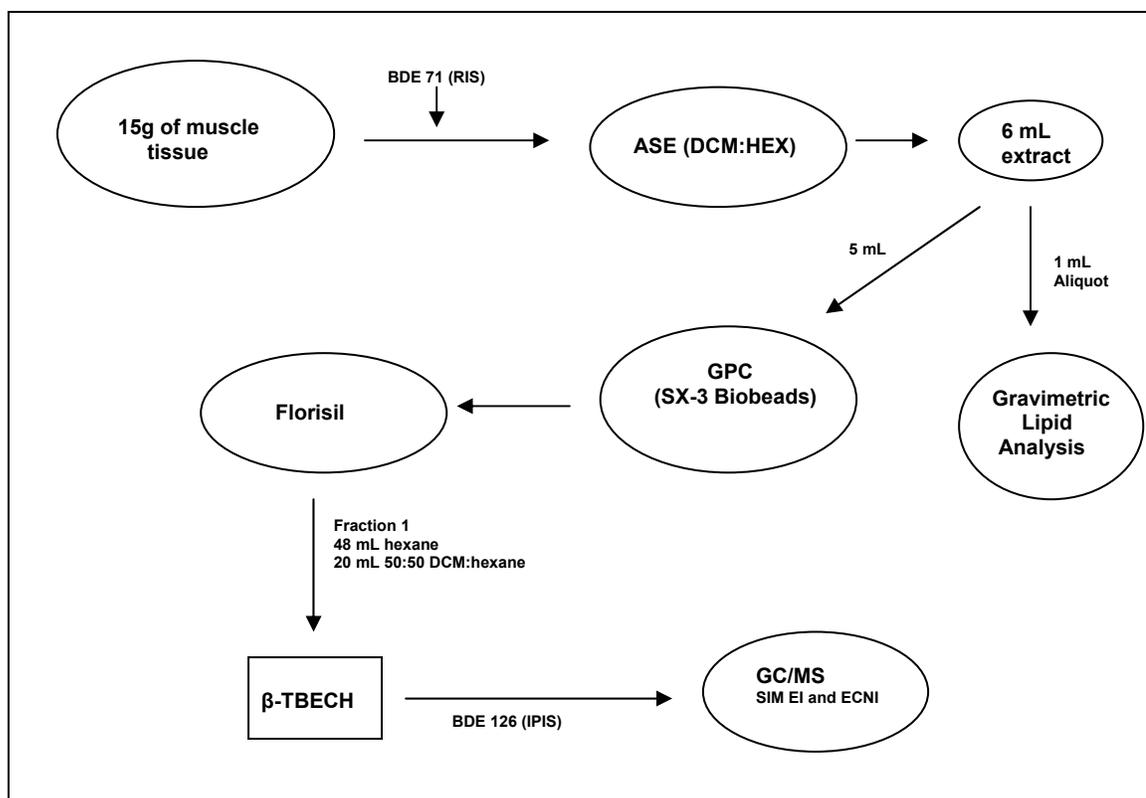
Figure 2.1 Schematic of β–TBECH extraction from whole fish.

2.4.2. Liver

For each sampling time point, livers of fish from the control group were pooled according to their sexes. Fish from the high group were also pooled into their corresponding sex for each sampling point. Fish from the low and medium treatment groups were not analyzed because if metabolites were formed they would likely be readily detectable in the high dose group. Weighed samples were added to a 45 mL Falcon test–tube (Fisher Scientific, Pittsburgh, PA), spiked with 10 ng of BDE–71 (10 μL of a 1 ng/μL solution) and homogenized with a Polytron homogenizer (Fisher Scientific, Pittsburgh, PA) for 5 minutes in 5 mL of the tertiary solvent 45:45:10 hexane:DCM:acetone. Extracts were then vortexed for 1 minute, sonicated in a water-bath for 5 minutes and centrifuged for 5 minutes at 4000*rpm*. The supernatant was

16

removed, transferred to a clean test-tube and the extraction process repeated on the remaining extract. Sample extracts were then evaporated to 2 mLs using a gentle stream of ultra high purity N_2. Lipids were determined gravimetrically as above and the 1 mL extract was purified on a column containing Florisil. The same elution sequence for the whole-fish was used. The collected fraction was reduced in volume to 100 μL by a gentle stream of ultra purity N_2 and spiked with 10 ng of BDE–126 (10 μL of 1 ng/μL solution) prior to GC/MS analysis.

2.4.3. Plasma

Figure 2.2 shows a schematic of my approach to extracting the suite of studied hormones from plasma. Plasma samples were first thawed on ice and 300 μL pipetted into a 15 mL glass disposable tube and spiked with 5 ng of $^{13}C_2$–E2 and d_8–P (10 μL of 0.5 ng/μL solution). A mixture of 3 mL of 3:2 hexane:ethyl acetate was first added to the samples, vortexed for 30 seconds, centrifuged for 5 minutes at 4000*rpm* and then frozen for 5 minutes at –80 ºC. The top layer was removed and transferred to a clean glass tube. The steps were repeated using 9:1 hexane:ethyl acetate as the solvent system and the upper layer removed and combined with that from the first extraction sequence. The combined fractions were blown down to dryness using N_2 and reconstituted in 1 mL of methanol. Samples were again blown to dryness and reconstituted in 50 μL of methanol, vortexed for 30 seconds and then transferred to a glass vial with a 200 μL glass-insert.
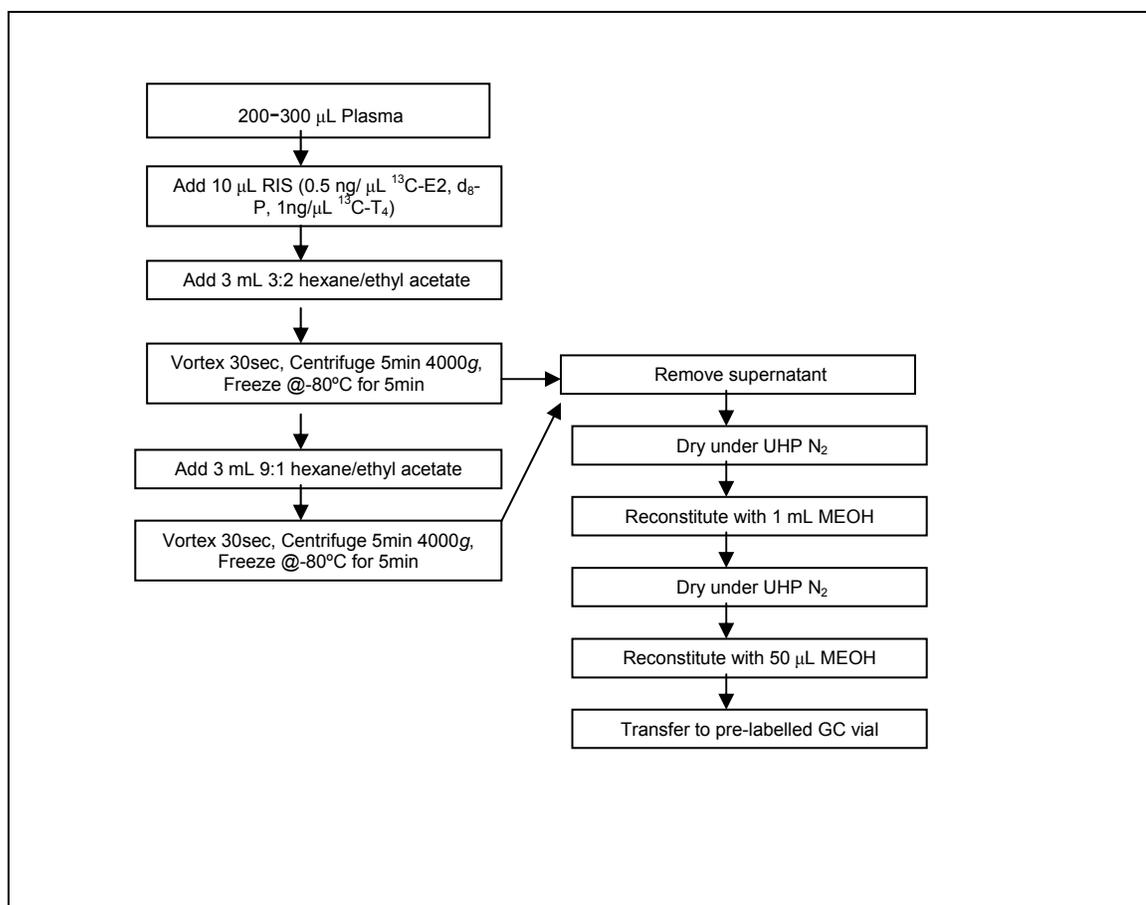
Figure 2.2 Schematic of extraction of sex hormones from plasma.

2.5. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (HPLC/MS/MS) FOR SEX HORMONE ANALYSIS

HPLC separations were done on an Agilent 1100 series HPLC system (Agilent Technologies, Palo, CA, USA) equipped with a vacuum degasser, binary pump and an autosampler. A 0.2 μm in-line filter (Agilent Technologies) was connected before the C_{18} analytical column (50 mm x 2.1 mm i.d., 4 μm particle size; Genesis, Chromatographic Specialities, Brockville ON, Canada). A binary mobile phase of optima grade water and optima grade methanol was used at a flow-rate of 300 μL/min. For the targeted steroids, an initial composition of 80:20 water/MeOH (v/v), held for 1 minute and ramped linearly

to 100% MeOH in 9 minutes. This was held for 6 min and then returned to starting conditions in 2 minutes. The column was allowed to equilibrate for 7 minutes between runs. A Sciex 2000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) was used in the electrospray ion mode. Native and labelled estradiol (E2 and $^{13}C_2$–E2) were analyzed in the negative (–ve) ion mode, while the androgens, T, 11–KT and d_8-P were monitored in the positive (+ve) ion mode. Detection was achieved by multiple reaction monitoring mode using the transitions listed in Table 2.1. Optimized MS/MS parameters in ESI –ve and +ve ion MRM mode are shown in Table 2.2 and 2.3, respectively.

Table 2.1 MRM and ions monitored

Compound	Parent ion (*m/z*)	Daughter ion 1 (*m/z*)	Daughter ion 2 (*m/z*)
E2	271.200	145.100	183.100
$^{13}C_2$-E2	273.200	147.000	185.000
T	289.240	97.100	109.000
11-KT	303.267	121.000	259.200
d_8-P	323.376	100.000	113.000

Table 2.2 Parameters for negative ion mode of MS/MS

Parameter	Abbreviation	E2	$^{13}C_2$–E2
Curtain gas	CUR (a.u.)	20	20
Sheath gas	GS1 (a.u.)	30	30
Turbo gas	GS2 (a.u.)	45	45
Ionspray voltage	IS (V)	-4400	-4400
Turbo-gas temperature	TEM (ºC)	550	550
Declustering potential	DP (V)	-86	-91
Focusing potential	FP (V)	-330	-340
Entrance potential	EP (V)	-11.5	-6.5
Quad 1 offset	IQ1 (V)		
Prefilter (stubbies)	ST (V)		
Collision gas	CAD (a.u.)	4	4
Collision cell entrance potential	CEP (V)	-16	-20
Collision energy	CE (V)	-52	-48
Collision cell exit potential	CXP (V)	-12	-14

Table 2.3 Parameters for positive ion mode of MS/MS

Parameter	Abbreviation	T	11–KT	d_8–P
Curtain gas	CUR (a.u.)	30	30	30
Sheath gas	GS1 (a.u.)	20	20	20
Turbo gas	GS2 (a.u.)	30	30	30
Ionspray voltage	IS (V)	4600	4600	4600
Turbo-gas temperature	TEM (ºC)	550	550	550
Declustering potential	DP (V)	26	31	26
Focusing potential	FP (V)	340	370	270
Entrance potential	EP (V)	12	12	12
Quad 1 offset	IQ1 (V)			
Prefilter (stubbies)	ST (V)			
Collision gas	CAD (a.u.)	4	4	4
Collision cell entrance potential	CEP (V)	26	26	14
Collision energy	CE (V)	33	33	33
Collision cell exit potential	CXP (V)	10	8	10

2.6. GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS)

All analyses were performed on an Agilent 5973 GC-mass selective detector. Splitless injections of 2 μL were made by a 7683 Agilent autosampler onto a 15 m × 0.25 mm id Rtx-1614 column capillary column (0.10 μm film thickness, Restek, Brockville, ON, Canada). UHP helium was used as the carrier gas at a flow rate of 0.75 mL/min and the injector was set to the oven tracking mode. The temperature program used: initial oven temperature 90ºC with no hold time, ramped at 20ºC/min to 310ºC, and held for 5 min. Both electron ionization (EI) and electron capture negative ionization (ECNI) were employed in my study. In EI the ion source temperature was set at 250°C while in ECNI the ion source temperature was 150°C. Quadrupole temperature was 150°C in both ionization modes. BDE–71 and –126 were analyzed in ECNI, with methane as the buffering gas, in the selected ion mode (SIM) by monitoring the two intense fragment ions of Br^{-}, m/z 79/81. β–TBECH was analyzed in EI-SIM by monitoring the [M–HBr–Br]$^{+}$ m/z 265/267. TBECH metabolites were also monitored in the EI-SIM mode monitoring m/z 79, 107 and 189. Targeted compounds were all quantified in my samples using external standards. (Tomy et al 2008)

2.7. QUALITY ASSURANCE/QUALITY CONTROL

Procedural blanks for GC/MS analysis consisted of ASE extracting heat-treated (600ºC for 6h) pelleted diatomaceous earth and heat treated (600ºC for 6h) Ottawa sand with every 11 samples. No detectable amounts of the β-isomer or my recovery internal standard (BDE-71) could be measured in my procedural blanks or in unfortified fish from my control treatment group. The β-isomer was also intentionally added to 6 additional

procedural blanks and extracted using ASE. For plasma, procedural blanks consisted of extractions of HPLC grade water and were employed with every 10 samples. Injections of methanol (3 μL) after every 10 samples were used as instrument injection blanks. None of the endogenous hormones or the internal standards intentionally added to the samples could be detected in our blanks.

Mean recoveries (arithmetic mean ± SE) of BDE-71 in my samples (*n=352*) and in my procedural blanks (*n=32*) were 45.5 ± 0.9 and 51.0 ± 3.6 %, respectively. Similar recoveries of the β-isomer (46.3 ± 3.6 %, *n=6*) were observed in my fortified procedural blanks. Measured amounts of d_8-P used as my internal standard for plasma and added to account for losses during extraction and possible ion suppression or enhancement were 54.7 ± 0.8 % of the expected value. Fortification studies using mass labelled ^{13}C-E2 into plasma gave recoveries of 86.8 ± 1.8% (*n=12*).

Analytical and method detection limits for all the compounds of interest are listed in Table 2.4. Analytical detection limits (ADLs) were determined by injecting known amounts of material and mathematically suppressing the signal to noise ratio that was determined electronically by the software Analyst 1.5 to a value of 5:1. Because undetectable amounts of the compounds monitored were present in my blanks, my method detection limits, which are defined as the mean ± 3 × standard deviation in the blanks, were determined by adding a known amount of each chemical to the blank extract and suppressing the electronically determined signal to noise value to 5:1. I then normalize this value for the mass of sample extracted; for muscle tissue 15 g and plasma 300 μL.

Table 2.4 Analytical (pmole/g) and method detection limits (pmole/μL)

Compound	ADL	MDL
TBECH	8400	119,000
E2[a]	5300	450
T	3300	120
11-KT	11,000	2500

[a] values based on ^{13}C-E2.

2.8. DATA ANALYSIS

Amount of β-isomer in fish were recovery corrected using BDE-71 and normalized to lipid content. Because no detectable amounts of β-isomer were observed in fish from the control treatment group or in my procedural blanks no correction was applied to the raw data. No correction for growth was needed as fish grew minimally during my experiment. Linear regression analysis, Student *t*-test and ANOVA were done using SigmaStat (Systat Software Inc., Version 11, Chicago, USA). Outliers in my data were determined using the Q-test and is defined as a simple statistical test to determine if a data point that appears to be very different from the rest of the data points in a set may be discarded (Skoog et al 1988).

CHAPTER 3

RESULTS AND DISSCUSION

3.1. GROWTH RATES

Fish growth rates were estimated by plotting the ratio of the average weight of fish at each sampling point (W_t) to the average fish weight at the start of the experiment (W_0= 55 g) versus time (*see* Appendix B). Results of this analysis show that fish grew minimally over our study period for all treatment groups. This result was not unexpected as fish were purposely fed a maintenance diet to limit growth; growth dilution is a variable that can confound the interpretation of bioaccumulation parameters. Not surprisingly, the lipid percent of fish did not change significantly over the experiment period in any of the treatment groups (see Appendix C).

The Spearman rank analysis was used to test for correlations amongst basic morphmetric parameters including fish length, liver weight, gonad weight and % lipid (*see* Appendix D). Of these, strong positive correlations were observed amongst fish length, liver and gonad weight ($p<0.05$). In general, lipid % did not correlate well with the other parameters.

3.2. LIVER AND GONAD SOMATIC INDICES

The liver and gonad somatic indices (LSI and GSI) are good indicators of overall fish health and both have been used as a biomarker for exposure to environmental contaminants (Huuskonen and LindstrømSeppa 1995). For example, LSI values are generally elevated in vertebrates experiencing induction of biotransformation enzymes for detoxification of organic compounds (Huuskonen and LindstrømSeppa 1995).

The indices are calculated simply as percentage ratios of liver or gonad wet weight to total body weight multiplied by 100 (*see* Appendix E). Using this approach, LSI and GSI were calculated for all fish at each sampling time points and tested (ANOVA) whether there were any statistical differences in the magnitude of each index value in any of the treatment groups including the control group. The results of the ANOVA suggest that there were no statistical differences among LSI in any treatment group at any period of the exposure experiment ($p>0.05$). Likewise, there were no statistically significant differences in GSI values in any exposure group at any time period ($p>0.05$).

These results would imply that the β-isomer did not have any adverse overt effects on liver or gonad development during our exposure experiment. This observation could be because the liver and gonad did not have sufficient time to respond to exposure to β-isomer due to (*i*) the short duration of our exposure study and, (*ii*) the fast elimination kinetics of β-isomer (discussed later). More subtle adverse biochemical effects on the gonad *i.e.*, steriodogenesis, were also explored and will be treated later in the thesis.

3.3 BIOACCUMULATION AND DEPURATION

The bioaccumulation and subsequent clearance of the β-isomer in my exposed fish at each of the three doses is shown in Figure 3.1 (*a-c*). The plots are divided into two phases: an uptake and a depuration phase.
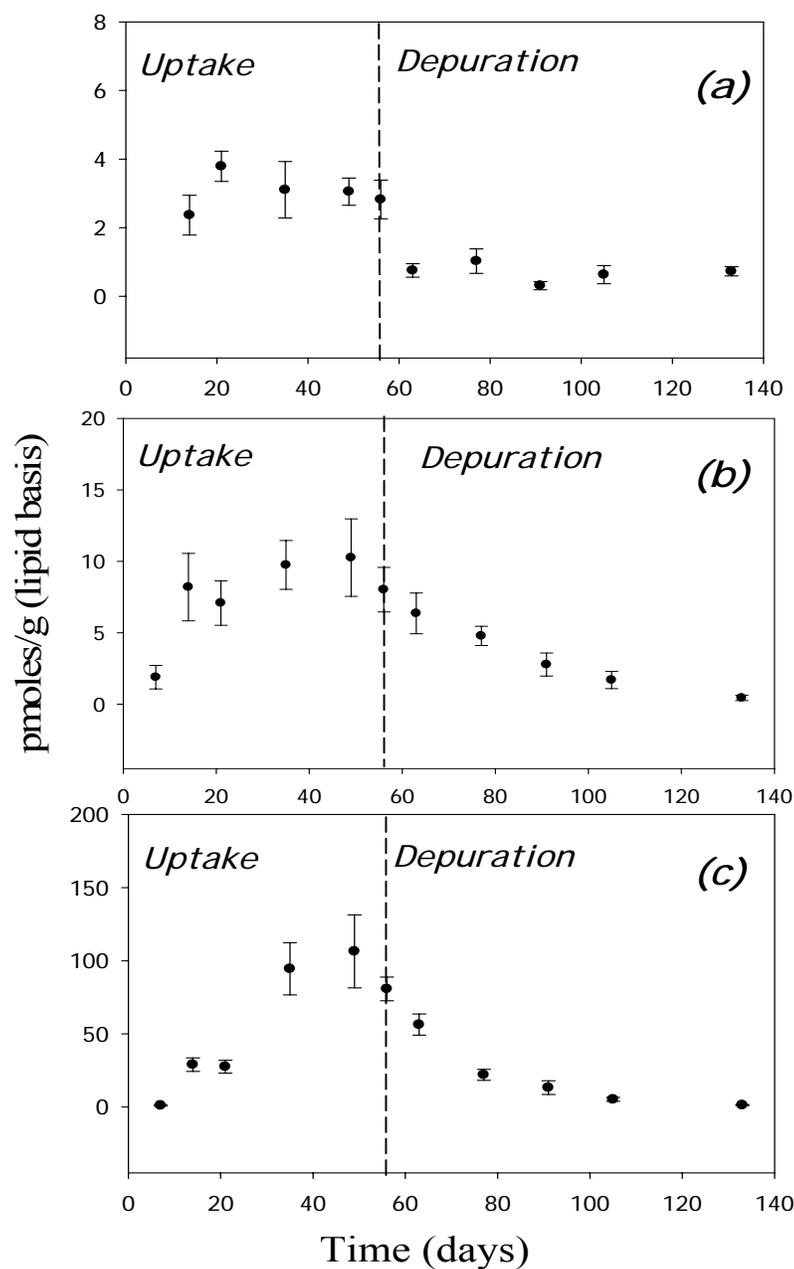
Figure 3.1 Uptake and depuration profiles of β-TBECH in juvenile brown trout carcass from (a) low, (b) medium and (c) high treatment groups. Each point represents the arithmetic mean ± standard error (vertical bars) of eight fish from each group.

Although there was no correlation between lipid % and number of pmoles per g of tissue of β-isomer measured in fish (p>0.05), likely due to the rapid depuration of β-isomer in the organism, I still chose to express the number of pmoles per g of tissue of the β-isomer on a lipid weight basis. However, using units of number of pmoles per g of tissue of β-isomer expressed on a wet weight basis did not change the overall appearance of the plots (data not shown). Male and female fish were grouped together because there were no gender differences in accumulation (p>0.05) at any sampling point and each time point on my graphs represent the arithmetic mean ($n=8$) of the number of pmoles per g of tissue of β-isomer (lw) measured in *ca.* 15 g of the fish carcass. No β-isomer could be detected in control fish at any period of my study.

The β-isomer was detectable after day 14 [arithmetic mean ± 1 × SE: 2.4 ± 0.6 pmoles/g (lw)] and peaked on day 21 (3.8 ± 0.4 pmoles/g (lw)) in fish from the low treatment group. Fish from the medium treatment group had detectable amounts of the β-isomer after just 7 days of exposure [1.89 ± 0.82 pmoles/g (lw)] with a peak occurring on day 49 [10.3 ± 2.7 pmoles/g (lw)]. The range of β-isomer in fish from these two dosing groups brackets those measured in fish from Lake Winnipeg [mean ($n=8$): 6 pmoles/g, (lw), G. Tomy, *personal communication*].

Using ANOVA, I tested to see if there were statistically significant differences in amounts of the β-isomer in fish between sampling points in each of the three treatment groups. For fish from the low group, there were no statistically significant differences (p>0.05) in measured amounts of the β-isomer at any sampling point. In fish from the medium treatment group, the amount of the β-isomer measured in fish sampled on day 7 was significantly smaller (p<0.05) in fish from the other sampling points. In fish from

the high treatment group, there were no statistically significant differences ($p<0.05$) in amounts of β-isomer on days 35, 49 and 56. Likewise, there were no statistically significant differences ($p>0.05$) in the amounts of β-isomer in fish sampling on days 7, 14 and 21. However, there were statistically significant differences ($p<0.05$) in the measured amounts of the β-isomer between the first and last three sampling points.

The appearance of the uptake profiles and results of my statistical analysis implies that fish from all three treatment groups reached steady-state before the end of my uptake phase and that the time to steady state likely occurred after day 21. This is consistent with the fact that the time to steady-state is dependent only on the elimination rate and, as I will show, there were no statistical differences in the measured elimination rate in any of the treatment groups.

The elimination of the β-isomer in fish from all three treatment dose groups was rapid. This is consistent with the work of Nyholm *et al*., who showed that *t*-TBECH is also cleared rapidly in zebrafish (Nyholm et al, 2009). To test if the disappearance of the β-isomer from fish followed first-order depuration kinetics, I plotted the natural log of the amount of the isomer against time [*see* Figure 3.2(a-c)]. By definition, if elimination of a chemical from an organism obeys first-order kinetics then the rate of elimination should be directly proportional or dependent on the number of moles (or concentration) at any given moment (Ballantyne et al 1995).

$$\text{rate} = k_d\,[A] \qquad\qquad [3.1]$$

where k_d is the depuration rate constant (derived from the slope of plot ln [A] vs time)
and [A] is the concentration of the chemical.

Because the amounts of the β-isomer in fish from my last two sampling time
points in the low treatment group remained relatively unchanged (Student *t-test*, p>0.05) I
omitted data from my final sampling point (day 133) in my first-order kinetic test.

The elimination of the β-isomer from fish from each of the treatment groups does
in fact follow a first-order rate process (p<0.05, in all cases). The depuration rate
constants (k_d's) derived from the slope of each of the three plots, were 0.0308 ± 0.0142
(low), 0.0515 ± 0.0225 (medium) and 0.0502 ± 0.0079 (high) days[1]. There were no
significant differences in the slopes of the regression lines in the three plots (p>0.05)
which agrees with the theory that k_d is independent of the concentration of the chemical.

The k_d is inversely related to the depuration half-life ($t_{1/2}$) which is perhaps the
most important bioaccumulation parameter in defining chemical persistence. By
definition, the depuration $t_{1/2}$ is the time it takes for a chemical to reach half its initial
concentration (or amount). The smaller the $t_{1/2}$ the less persistent that specific chemical is
to that species.
Using equation [3.2]:

$$t_{1/2} = 0.693 \,/\, k_d \qquad\qquad [3.2]$$

Half-lives of 22.5 ± 10.4, 13.5 ± 5.9 and 13.8 ± 2.2 days, for β-isomer were calculated in fish from the low, medium and high treatment groups, respectively. Like the k_d, there were no differences in the $t_{1/2}$ values between treatment groups.

The mean β-TBECH $t_{1/2}$ values determined in this study (*ca*. 17 days) are *ca*. 15× greater than those measured by Nyholm *et al*. in zebrafish (Nyholm et al 2009). Interspecies difference in uptake, storage, elimination and metabolic capability likely explain this discrepancy. In addition, zebrafish are multiple spawners and have greater potential to depurate TBECH to their eggs. Figure 3.3 shows the $t_{1/2}$'s of β-TBECH and other FRs measured in my laboratory on rainbow trout (*Oncorhynchus mykiss*) (Tomy et al 2004, Tomy et al 2007, Tomy et al 2008b). In general, the measured $t_{1/2}$ of the β-isomer derived from this study is smaller than those of other common FRs. For example, the $t_{1/2}$ of the β-isomer for both the medium and high dose group is *ca*. 10 and 4 times smaller than that of γ-HBCD and the *syn*-isomer of Dechlorane Plus, respectively (*see* Figure 3.3). And relative to BDE-209, the $t_{1/2}$ of β-TBECH is *ca*. 2 times smaller for that of the medium and high dose group $t_{1/2}$. The $t_{1/2}$ of β-TBECH for the low dose group is *ca*. 6 times smaller than that of γ-HBCD (*see* Figure 3.3).
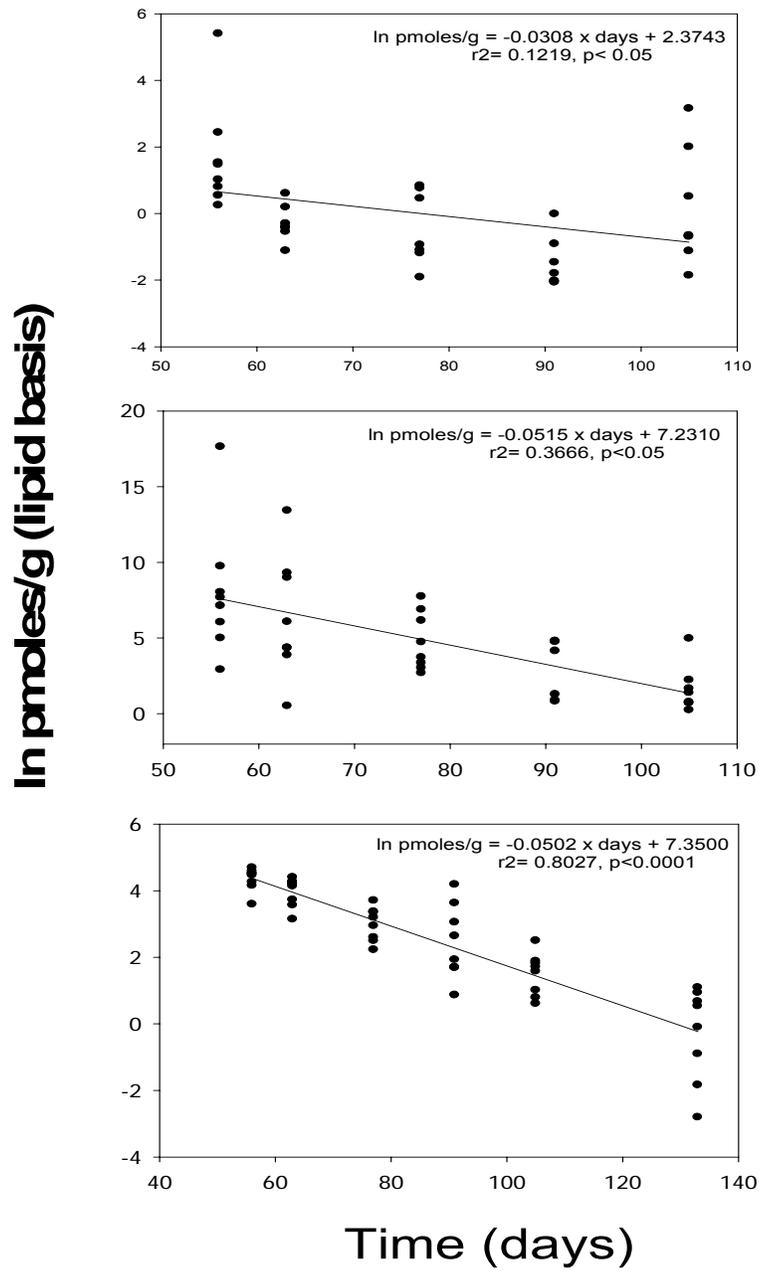
Figure 3.2 Plots of the natural log of [pmoles/g (lw)] in fish from (a) low, (b) medium and (c) high treatment groups versus time (days). Individual data points are for female and male fish. Results of the regression analysis are given in each panel.
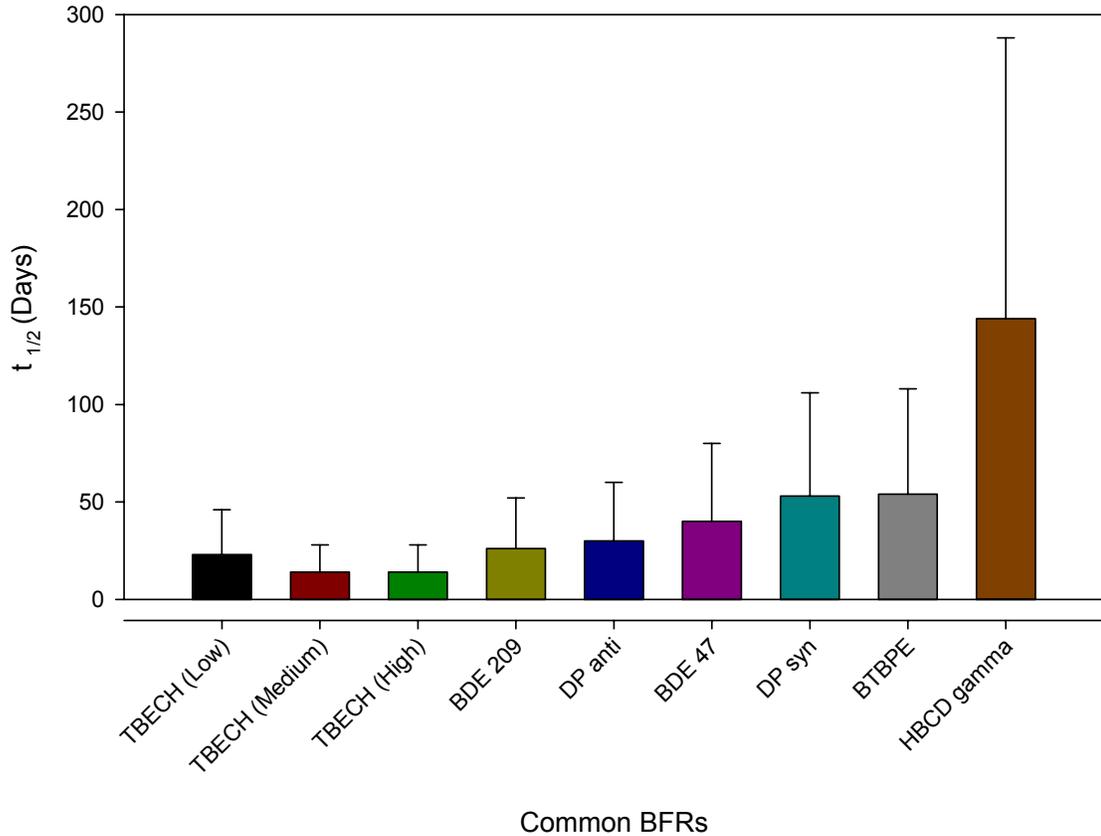
Figure 3.3 Comparison of $t_{1/2}$ for low medium and high dose group of β-TBECH and other FRs.

The assimilation efficiency (AE) is defined as the number of moles of the β-isomer measured in the tissue relative to the theoretical number of moles expected based on the amount of the β-isomer administered *via* the food (Ballantyne et al 1995). The assimilation efficiency (AE) was calculated as the ratio of the number of moles of the β-isomer measured in the tissue relative to the theoretical number of moles expected based on the amount of the β-isomer administered *via* the food. The AE is an important

parameter in calculating laboratory based biomagnification factors (BMFs) and later can be calculated using equation 3.3:

$$BMF = AE \times F_d / k_d \qquad\qquad \text{...3.3}$$

where F_d is the feeding rate.

Assimilation efficiencies calculated for each treatment group were >100% (low), 87% (medium) and 99% (high). Using these numerical values BMF_{ss} of 0.32 ± 0.15, 0.17 ± 0.07 and 0.19 ± 0.03 were calculated for fish from low, medium and high treatment groups, respectively. Even at these large AE's calculated BMF_{ss} for β-TBECH is still much smaller than 1 indicating low potential to biomagnify in aquatic food webs. The short $t_{1/2}$ of the β-TBECH (*i.e.*, large k_d) is certainly a major driver of an observed BMF < 1.

3.4. METABOLITE FORMATION AND BIOISOMERIZATION

Like other BFRs, β-TBECH has the ability to debrominate or dehydrobrominate *via* loss of Br_2 and HBr, respectively. I focused my attention on metabolites formed *via* loss of Br_2 as I felt that because the two Br atoms are vicinal to one another and due to the facile nature of the C-Br bond (bond energies of C-Br and C-H are 276 and 413 kJ/mol, respectively) that this loss would be more favoured as compared to loss of HBr (Ballantyne et al 1995).

I was aided in my endeavours because of a synthetic solution prepared for me by Wellington Laboratories that contained debrominated products of TBECH synthesized by

(*i*) loss of the two Br atoms on the side-chain of TBECH (compound *I* in Figure 3.4) and

(*ii*) loss of two Br atoms on the ring (compound *II* in Figure 3.4).
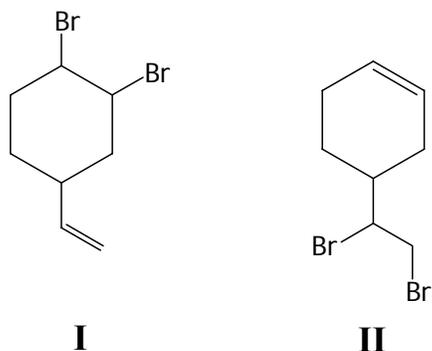
Figure 3.4 Structures of the debrominated metabolites of TBECH

The metabolite with the double bond on the side-chain (*I*) has a steric energy of 90.0 kJ/mol, while the metabolite with the double bond on the ring (*II*) has a steric energy of 822.4 kJ/mol. The steric energy for *I* is *ca.* 10 times smaller than *II* which suggests that *I* is thermodynamically more favoured and therefore more likely to be formed. While steric energy is primarily used for comparing energies of conformers I think it is a useful good first approximation of products likely to be formed in this case.
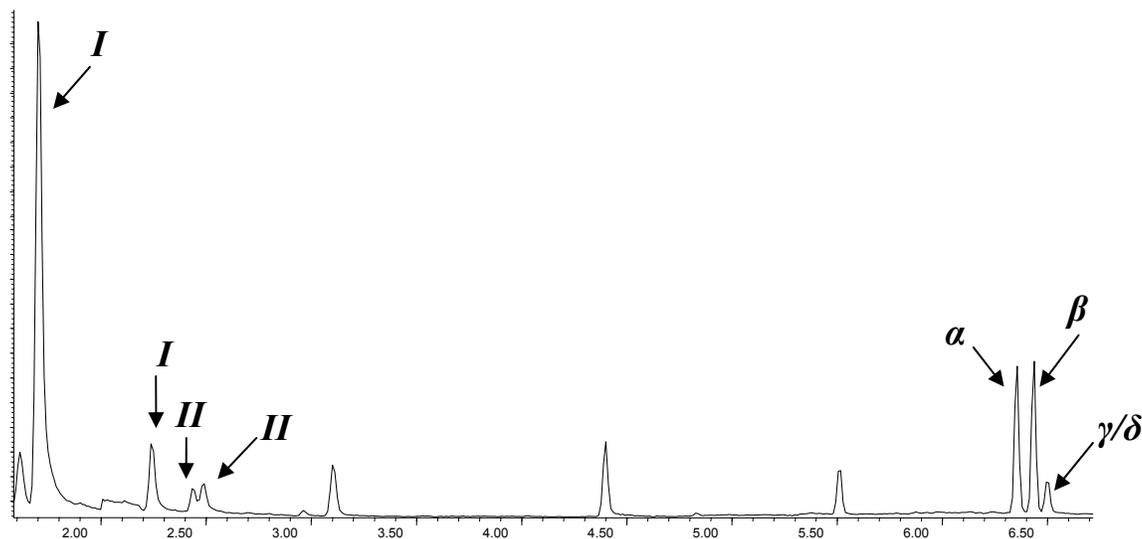
Figure 3.5 Full scan ion chromatogram of the synthetic TBECH metabolite solution.

The appearance of the full-scan ECNI ion chromatogram (*m/z* 50-450) of the synthetic solution is shown in Figure 3.5. For compound *I* that has the two ring bromines (1, 2-dibromo-4-vinylcyclohexane) two diastereomers are possible and hence we observe two peaks (r_t: 1.8 and 2.4 mins). Likewise, for compound *II*, [4-(1, 2-dibromoethyl)cyclohexane] two diastereomers are possible and hence two peaks are present in the synthetic mixture (r_t = 2.5 and 2.6 mins).

Surprisingly, none of the four peaks were detected in whole fish muscle samples or in my composite liver samples. Because the β-isomer disappeared so rapidly from the fish, the facile nature of the C-Br bond and because I have evidence that debromination occurs with other BFRs, I initially hypothesized that metabolites of β-isomer were likely (Law et al 2006). If however the β-isomer is being excreted rapidly or if metabolites are

being formed but are eliminated before sequestration in tissue compartments then this might explain why I did not observe any metabolites in my samples.

I showed by fortification experiments that the extraction efficiency of the β-isomer was *ca*. 50% (see QA/QC section). If I assume that 1% of the TBECH is converted to its debromo-metabolite and that the metabolites are recovered similarly to the parent compound, the amount of TBECH metabolite present would still be an order of magnitude greater than my analytical detection limits would allow. So, smaller than optimum extraction recoveries can not explain the non-detection of TBECH metabolites.

Bioisomerization *i.e.*, conversion of one diastereomer into another, has been shown to occur for HBCD in juvenile rainbow trout (*Oncorhynchus mykiss*) (Law et al 2006). Based on this earlier observation and the interconversion pathway shown in Figure 1.5, I hypothesized that the β-isomer could bioisomerize to either of the α- or γ-isomers in my exposed fish. Because conversion to the δ-isomer was a two step process I felt that this conversion was less likely to occur.

No detectable amounts of any other isomer of TBECH other than the β-isomer were observed in my whole fish muscle samples or in my composite liver samples. Either the depletion of the β-isomer is too fast to induce the enzymes responsible for bioisomerization and/or bioisomerization of β-isomer is not possible in my fish species.

3.5. BIOCHEMICAL EFFECTS

Steroid hormones play a major role in the endocrine system of fish, regulating mineral and water balance, immune response, and various parts of reproduction (Pankurst 2008). Steroids are synthesized from the base molecule cholesterol (Figure 3.6). The

primary reproductive steroids in male fish are the nineteen carbon (C_{19}) androgens testosterone (T) and 11-ketotestosterone (11-KT). Testosterone is produced in the testes and then converted by the cytochrome P450 11β enzyme to 11-KT, which is responsible for gonadal growth, initiation of spermatogenesis, and the development of secondary sex characteristics such as colour displays. Oocytes in the female gonad also produce T and then convert it to eighteen carbon (C_{18}) estrogen, 17β-estradiol (E2), by P450 aromatase (Pankurst 2008). E2 is involved in the development of secondary sex characteristics in females, however, its primary role is to stimulate the hepatic synthesis of the egg yolk protein-precursor vitellogenin and to regulate its deposition into the maturing oocytes (Pankurst 2008).

Circulating steroid hormones in wild fish has been used as a biomarker of exposure to pollutants and other environmental stressors. Other environmental variables can confound interpretations of hormone levels in wild fish and their relationship to contaminant exposure. Laboratory based exposure experiments, where many of the confounding variables are controlled for, are ideal in establishing cause and effect relationships between contaminant exposure and any observed changes in hormone levels.

Because androgenic effects have been implicated by molecular modeling and *in vitro* ligand-binding and receptor-activation bioassay I chose to examine whether under my dosing regimens an effect might be observed (Larsson et al 2006, Khalaf et al 2009). Androgenic effects by a chemical can occur through androgen receptors (AR)-ligand interactions or through exogenous substances that interfere with aromatase activity which is responsible for the conversion of androgens T to E2 (Olsson et al 2008). In two

separate studies, female mosquito fish developed male-like gonadopodium when exposed to 11-KT and pulp mill effluent with androgen receptor agonist activity (Angus et al 2001, Parks et al 2001). Reduced fecundity, altered mating behaviours and aggression have also been reported in fish exposed to androgenic compounds (Olivera et al 2002) (Ankley et al 2003, Miller et al 2004). In females, exposure to aromatase inhibitors can increase levels of circulating androgens (Olsson et al 2008). In males, substances causing anti-androgenic effects may block steroidogenic enzyme activity, stopping the conversion of cholesterol into any of the endogenous androgenic hormones (Olsson et al 2008).
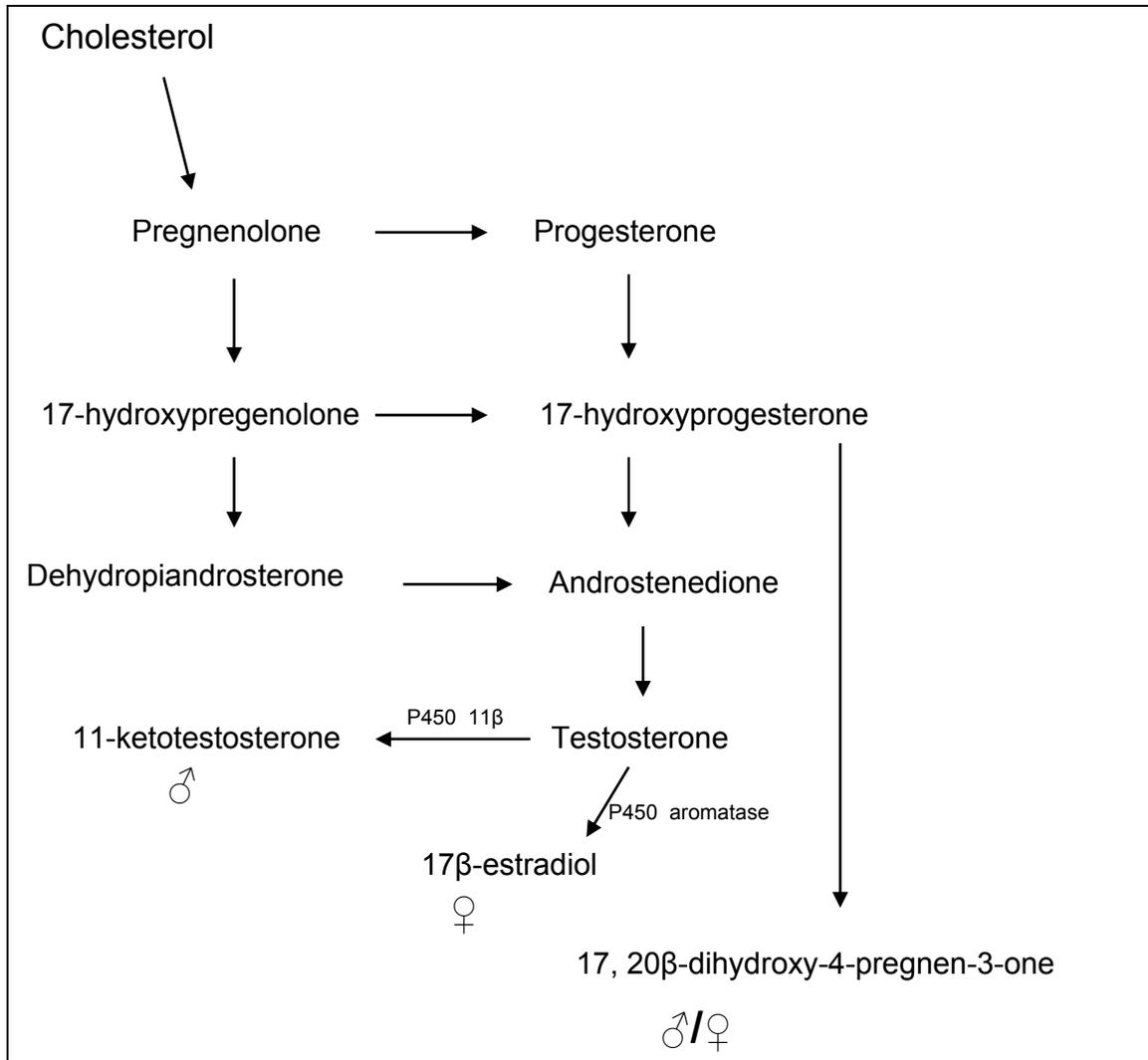
Figure 3.6 Flow diagram of steroid synthesis in teleost fish (Pankurst 2008).

Figures 3.7, 3.8 and 3.9 show the change in pmoles of E2, T and 11-KT (normalised to 300 μL of plasma), respectively, in male and female fish from my three treatment groups in relation to fish from my control group. Because sampling was done on a random basis the number of male and female fish at each time point varied. Nevertheless, each data point on my graphs represents the arithmetic mean of the pmolar amounts of each hormone for male or female fish sampled on each given day.

For male fish, E2 levels remained unchanged throughout the study period. There were no differences in E2 levels between males from the treated groups to those from the control group except at day 91 where fish from the high treatment group had smaller E2 levels than male fish from the control group (*see* Table 3.1).   There were also no differences in E2 levels in male fish within the three treatment groups.

In females, I observed a notable decrease in E2 levels ($p<0.05$, *see* Table 3.2) on day 133 in fish from the high treatment group relative to fish from the control group. Female fish from the low and medium treatment groups did not show a similar result. Reasons for the smaller E2 levels in fish from the high treatment group at such a late stage in the experimental exposure could be signs of effects occurring in the juvenile brown trout from the β-TBECH but still remains unclear.
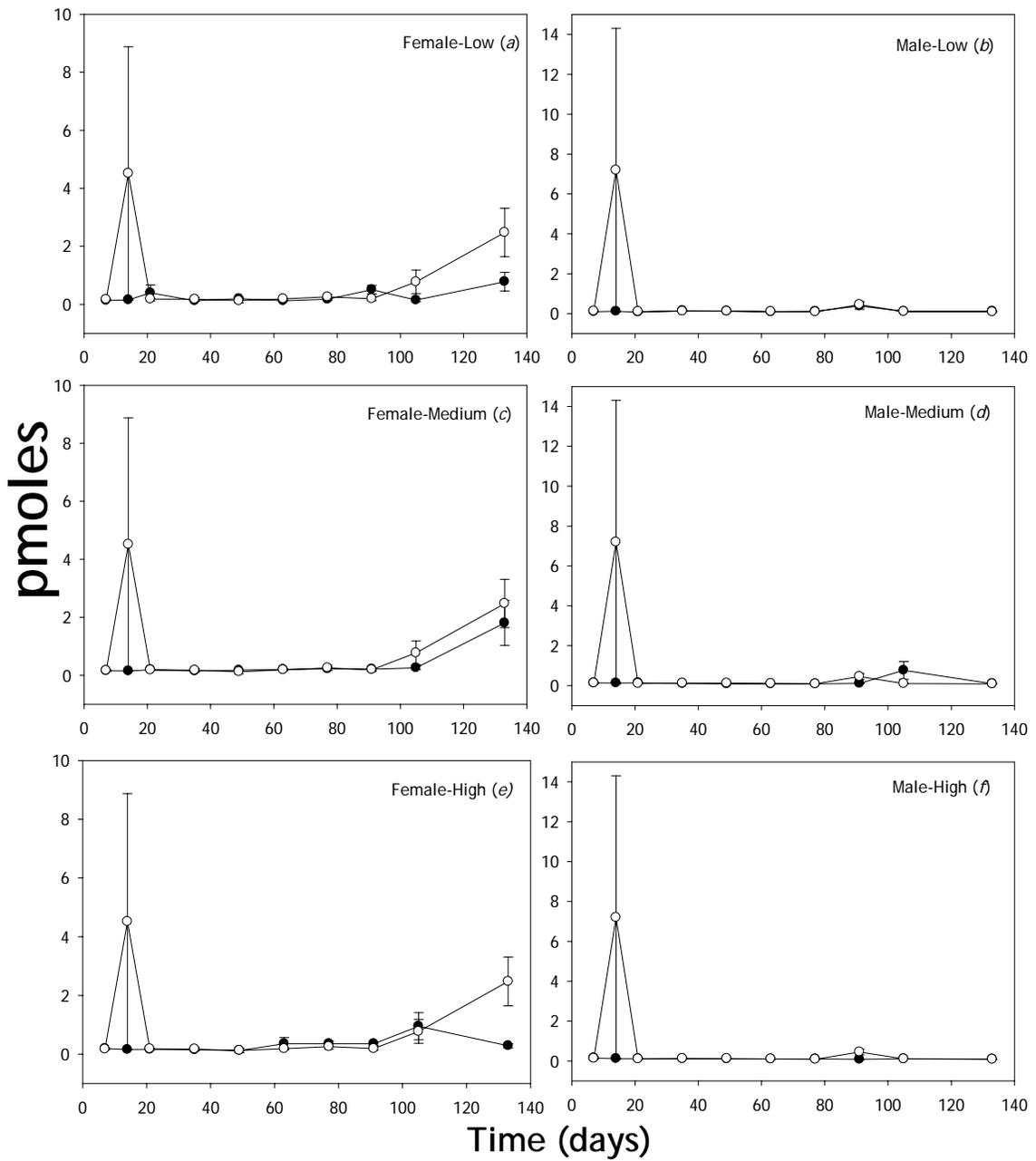
Figure 3.7 Pmoles of E2 in fish plasma from (*a*) low-female (*b*) low-male (*c*) medium-female (*d*) medium-male, (*e*) high-female and (*f*) high-male treatment groups versus time (days). Open and closed circles represent the mean E2 amount in males and females from the control and treatment group, respectively.

41
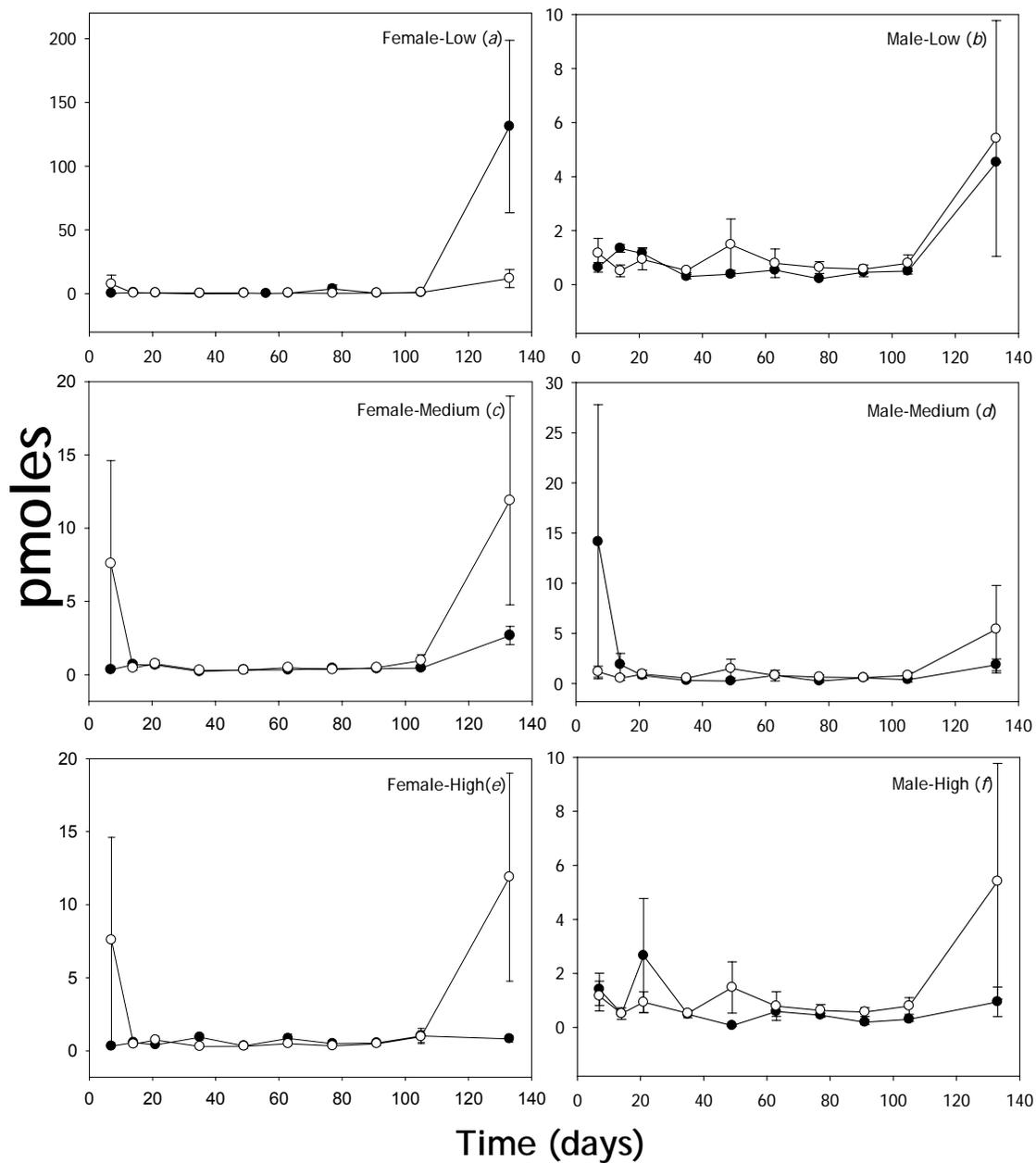
Figure 3.8 Pmoles of T in fish plasma from (*a*) low-female (*b*) low-male (*c*) medium-female (*d*) medium-male, (*e*) high-female and (*f*) high-male treatment groups versus time (days). Open and closed circles represent the mean T amount in males and females from the control and treatment group, respectively.
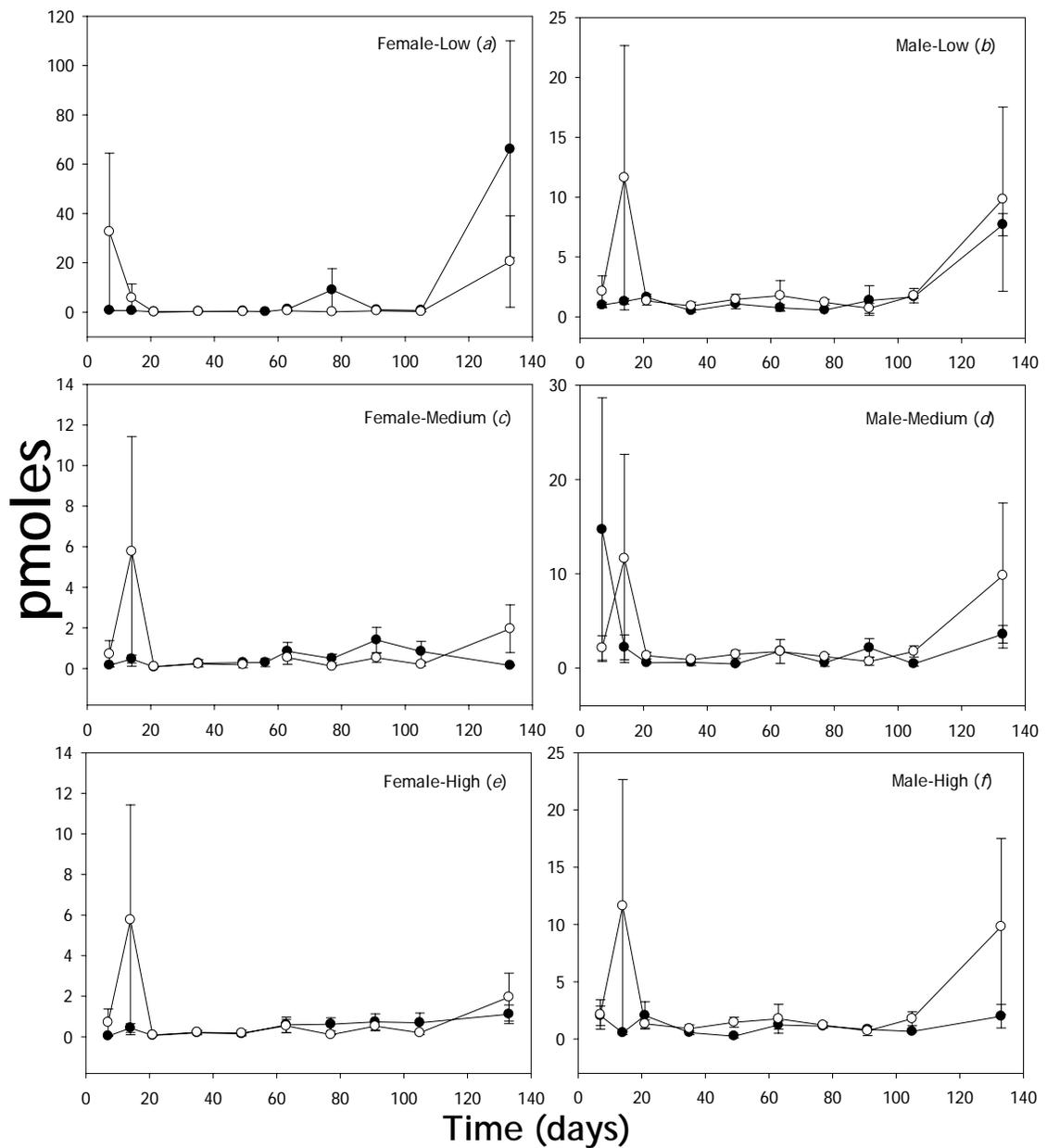
Figure 3.9 Pmoles of 11-KT in fish plasma from (*a*) low-female (*b*) low-male (*c*) medium-female (*d*) medium-male, (*e*) high-female and (*f*) high-male treatment groups versus time (days). Open and closed circles represent the mean 11-KT amount in male and female fish from the control and treatment group, respectively.

The overall trend in T levels in male and female fish from all groups are similar: T levels remain relatively constant throughout the exposure period except for day 133 where in all cases I see an increase in T levels. Only in female fish from the low treatment group do I observe greater T levels relative to control fish although this difference is not significant (p=0.07, *see* Table 3.4). It is likely that the observed increase in T levels at day 133 is due in part to the maturation of the fish during the study period. However, T levels in female fish from the high treatment group at day 133 are significantly smaller than T levels in female fish from the control group (p<0.01, *see* Table 3.4).

Not surprisingly, the trends in 11-KT in fish are similar to trend observed for T. Female fish from the low treatment group show greater amounts of 11-KT relative to the controls, however, this difference was not statistically significant (p=0.4, *see* Table 3.6). The only statistically meaningful difference between 11-KT levels in fish from the treated groups relative to fish from the control group was for female fish from the medium treatment group where on day 133 we observed a significantly smaller 11-KT level in exposed fish relative to fish from control group (p<0.05, *see* Table 3.6).

I also compared hormone levels measured in fish between the treatment groups (*see* Appendix F). While there were a few instances in which hormone levels differed there were no consistent trends.

Table 3.1 Significant values (p-value, Student t-test) for the comparison of the amount of E2 in male fish from each treatment group at each sampling time point to amount of E2 in male fish from the control group.

Day	p-value		
	Low	Medium	High
7	**0.022 (3,3)[a]**	0.306 (3,4)[a]	0.167 (3,6)[a]
14	0.276 (3,3)[a]	0.377 (3,3)[a]	0.376 (3,3)[a]
21	0.128 (5,5)[a]	0.223 (5,4)[a]	0.636 (5,3)[a]
35	0.667 (4,4)[a]	0.332 (4,6)[a]	0.902 (4,5)[a]
49	1.000 (6,4)[a]	0.248 (6,3)[a]	0.602 (6,2)[a]
63	0.667 (2,2)[a]	1.000 (2,2) [a]	0.816 (2,5)[a]
77	N/A	0.784 (3,2)[a]	0.265 (3,3)[a]
91	0.791 (4,2)[a]	0.057 (4,3)[a]	**0.029 (4,4)[a]**
105	0.582 (4,2)[a]	0.114 (4,3)[a]	0.218 (4,2)[a]
133	0.400 (3,2)[a]	0.629 (3,4)[a]	0.669 (3,3)[a]

[a] values in parenthesis is the number of male fish in the treatment and control group, respectively.

Table 3.2 Significant values (p-value, Student *t*-test) for the comparison of the amount of E2 in female fish from each treatment group at each sampling time point to amount of E2 in female fish from the control group.

Day	p-value		
	Low	Medium	High
7	0.181 (3,5)[a]	0.815 (3,4)[a]	0.553 (3,2)[a]
14	0.421 (5,5)[a]	0.548 (5,5)[a]	0.421 (5,5)[a]
21	0.597 (2,3)[a]	0.847 (2,4)[a]	0.381 (2,5)[a]
35	0.533 (2,4)[a]	0.314 (4,2)[a]	0.750 (2,3)[a]
49	0.447 (2,4)[a]	0.453 (2,5)[a]	1.000 (2,6)[a]
63	0.485 (6,6)[a]	0.762 (6,6) [a]	0.282 (6,3)[a]
77	0.214 (5,7)[a]	0.931 (5,6)[a]	0.448 (5,5)[a]
91	0.580 (4,6)[a]	0.782 (4,5)[a]	0.114 (4,4)[a]
105	1.000 (4,6)[a]	0.190 (4,5)[a]	1.000 (4,4)[a]
133	0.262 (5,6)[a]	0.583 (5,4)[a]	**0.031 (5,5)[a]**

[a] values in parenthesis is the number of female fish in the treatment and control group, respectively.

Table 3.3 Significant values (p-value, Student t-test) for the comparison of the amount of T in male fish from each treatment group at each sampling time point to amount of T in male fish from the control group.

Day	p-value		
	Low	Medium	High
7	0.415 (3,3)[a]	0.280 (3,4)[a]	0.804 (3,6)[a]
14	0.100 (3,3)[a]	0.292 (3,3)[a]	0.996 (3,3)[a]
21	0.611 (4,5)[a]	0.777 (4,4)[a]	0.389 (4,3)[a]
35	0.105 (4,4)[a]	0.114 (4,6)[a]	0.840 (4,5)[a]
49	0.486 (4,4)[a]	0.114 (4,3)[a]	0.379 (4,2)[a]
63	1.000 (2,2)[a]	1.000 (2,2) [a]	0.649 (2,5)[a]
77	N/A	0.290 (3,2)[a]	0.484 (3,3)[a]
91	0.533 (4,2)[a]	0.952 (4,3)[a]	0.200 (4,4)[a]
105	0.564 (4,2)[a]	0.348 (4,3)[a]	0.349 (4,2)[a]
133	0.886 (3,2)[a]	0.382 (3,4)[a]	0.367 (3,3)[a]

[a] values in parenthesis is the number of male fish in the treatment and control group, respectively. Values in bold are statistically significant values(p value<0.05).

Table 3.4 Significant values (p-value, Student t-test) for the comparison of the amount of T in female fish from each treatment group at each sampling time point to amount of T in female fish from the control group.

Day	p-value		
	Low	Medium	High
7	0.071 (3,5)[a]	0.057 (3,4)[a]	0.481 (3,2)[a]
14	0.040 (5,5)[a]	0.310 (5,5)[a]	0.605 (5,5)[a]
21	0.703 (2,3)[a]	0.741 (2,4)[a]	0.108 (2,5)[a]
35	0.292 (2,4)[a]	0.667 (2,2)[a]	0.333 (2,2)[a]
49	0.214 (2,4)[a]	0.887 (2,5)[a]	0.792 (2,6)[a]
63	0.799 (6,6)[a]	0.347 (6,6) [a]	0.225 (6,3)[a]
77	0.149 (5,7)[a]	0.668 (5,6)[a]	0.351 (5,5)[a]
91	0.811 (4,6)[a]	0.668 (4,5)[a]	0.745 (4,4)[a]
105	0.883 (4,6)[a]	0.203 (4,5)[a]	0.914 (4,6)[a]
133	0.082 (5,6)[a]	0.111 (5,4)[a]	**0.008 (5,5)[a]**

[a] values in parenthesis is the number of female fish in the treatment and control group, respectively. Values in bold are statistically significant values (p value<0.05).

Table 3.5 Significant values (p-value, Student t-test) for the comparison of the amount of 11KT in male fish from each treatment group at each sampling time point to amount of 11KT in male fish from the control group.

Day	p-value		
	Low	Medium	High
7	0.426 (3,3)[a]	0.857 (3,4)[a]	0.714 (3,6)[a]
14	0.402 (3,3)[a]	0.445 (3,3)[a]	0.373 (3,3)[a]
21	0.486 (4,5)[a]	0.096 (4,4)[a]	0.525 (4,3)[a]
35	0.343 (4,4)[a]	0.171 (4,6)[a]	0.354 (4,5)[a]
49	0.552 (4,4)[a]	0.102 (4,3)[a]	0.136 (4,2)[a]
63	0.667 (2,2)[a]	1.00 (2,2)[a]	0.576 (2,5)[a]
77	N/A	0.236 (3,2)[a]	0.750 (3,3)[a]
91	0.538 (4,2)[a]	0.187 (4,3)[a]	0.343 (4,4)[a]
105	0.912 (4,2)[a]	0.143 (4,3)[a]	0.288 (4,2)[a]
133	0.845 (3,2)[a]	0.385 (3,4)[a]	0.370 (3,3)[a]

[a] values in parenthesis is the number of male fish in the treatment and control group, respectively. Values in bold are statistically significant values (p value<0.05).

Table 3.6 Significant values (p-value, Student t-test) for the comparison of the amount of 11KT in female fish from each treatment group at each sampling time point to amount of 11KT in female fish from the control group.

Day	p-value		
	Low	Medium	High
7	0.571 (3,5)[a]	0.400 (3,4)[a]	0.487 (3,2)[a]
14	0.310 (5,5)[a]	0.310 (5,5)[a]	0.548 (5,5)[a]
21	0.300 (2,3)[a]	0.437 (2,4)[a]	0.436 (2,5)[a]
35	0.816 (2,4)[a]	1.000 (2,2)[a]	0.667 (2,2)[a]
49	0.432 (2,4)[a]	0.857 (2,5)[a]	0.735 (2,6)[a]
63	0.180 (6,6)[a]	0.485 (6,6)[a]	0.905 (6,3)[a]
77	1.000 (5,7)[a]	0.052 (5,6)[a]	0.151 (5,5)[a]
91	0.355 (4,6)[a]	0.556 (4,5)[a]	0.664 (4,4)[a]
105	0.762 (4,6)[a]	0.286 (4,5)[a]	0.476 (4,6)[a]
133	0.329 (5,6)[a]	**0.016 (5,4)[a]**	0.421 (5,5)[a]

[a] values in parenthesis is the number of female fish in the treatment and control group, respectively. Values in bold are statistically significant values (p value<0.05).

Overall, while I did observe a few instances where the measured amount of E2, T and 11-KT differed in fish from the treated groups relative to controls, I did not see a consistent or discernable trend. This implies that at the dosing amounts used in my study, the β-isomer of TBECH has no impact on circulating levels of E2, T and 11-KT in juvenile brown trout.

SUMMARY

The β-isomer was found to accumulate the same in fish from all three treatment groups. The β-isomer reached steady-state before the end of my uptake phase and the time to steady state likely occurred after day 21. The depuration of the β-isomer in fish obeyed first-order kinetics with no significant differences in calculated $t_{1/2}$'s amongst the treatment groups: 22.5 ± 10.4 (low), 13.5 ± 5.9 (med) and 13.8 ± 2.2 (high) days.

There were no detectable amounts of any other isomers of TBECH other than the β-isomer or debrominated metabolites in the whole fish muscle samples or in the composite liver samples. Based on this I hypothesize that (i) metabolism and/or bioisomerization of β-TBECH does not occur in juvenile brown trout or, (ii) the rate of elimination of the β-isomer is much greater than the rate of metabolism and storage of other isoforms or metabolites themselves or (iii) elimination of more polar metabolites, *i.e.*, debrominated forms, is greater than their rate of storage in juvenile brown trout.

While I did observe a few instances where the measured amount of E2, T and 11-KT differed in fish from the treated groups relative to controls, we did not see a consistent or discernable trend. Based on this I hypothesize that at the dosing levels employed β-TBECH does not impact circulating levels of these hormones in juvenile brown trout.

In hindsight, the experimental exposure study could be improved by using fish that were more sexually mature which may have allowed me to tease out any subtle effects on sex hormone levels that might have been missed in my study because of the juvenile nature of fish used.

Findings from my research have provoked further questions: could the β-isomer affect hormone levels in sexually mature fish? Would plasma have been a better compartment in fish to screen for debrominated metabolites or for other TBECH isomers formed by in vivo bioisomerization? These questions could certainly be the basis of future research endeavours on TBECH.

LITERATURE CITED

Alaee M and Wenning R.J. **2002**. The significance of brominated flame retardants in the environment: current understanding, issues and challenges. *Chemosphere*, 46, 579-582.

Ankley, G. T.; Jensen, K. M.; Makynen, E. A.; Kahl, M. D.; Korte, J. J.; Hornung, M. W.; Henry, T. R.; Dennt, J. S.; Leino, R. L.; Wilson, V. S.; Cardon, M. C.; Hartig, P. C.; Gray, L. E. **2003**. Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. *Environmental Toxicology and Chemistry 22*, 1350-1360.

Angus, R. A.; McNatt, H. B.; Howell, W. M.; Peoples, S. D. **2001**. Gonopodium development in normal males and 11-ketotestosterone-treated female mosquitofish (*Gambusia affinis*): a quantitative study using computer image analysis. *General and Comparative Endocrinology 123*, 222-267.

Arsenault G, Lough A, Marvin C, Mcalees A, McCrindle R, Maclnnis G, Pleskach K, Potter D, Riddell N, Sverko E, Tittlemier S and Tomy G. **2008**. Structure characterization and thermal stabilities of the isomers of the brominated flame retardant 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane. *Chemosphere*, 72, 1163-1170.

Ballantyne B, Marris T and Turner P. **1995**. General and Applied Toxicology. Publishers: Stockton Press, New York, USA, ISBN: 1-56159-167-X.

Birnbaum L.S and Staskal D.F. **2004**. Brominated Flame Retardants: Cause for concern? *Environmental Health Perspectives*, 112 (*1*), 9-17.

De Wit C.A. **2002**. An overview of brominated flame retardants in the environment. *Chemosphere*, 46, 583-624.

Gauthier L, Potter D, Hebert C, and Letcher R. **2009.** Temporal trends and spatial distribution of non-polybrominated diphenyl ether flame retardants in the eggs of colonial popoulations of great lakes herring gulls. *Environmental Science and Technology*, 43 (*2*), 312-317.

Huuskonen S and LindstrømSeppa P. **1995**. Hepatic cytochrome P4501A and other biotransformation activities in perch (*Perca fluviatilis*) – the effects of unbleached pulp-mill effluents. *Aquatic Toxicology*, 31, 27-41.

Kesner M and de Vos W. **2001.** Teaching about flame retardants. A joint Israeli-Dutch project. *Journal of Chemical Education*, 78 *(1),* 41-45.

Khalaf H, Larsson A, Berg H, McCrindle R, Arsenault G and Olsson P. **2009**. Diastereomers of the brominated flame retardant 1,2-Dibromo-4-(1,2 dibromoethyl)cyclohexane induce androgen receptor activation in the HepG2 hepatocellular carcinoma cell line and the LNCaP prostate cancer cell line. *Environmental Health Perspectives*, 117 (*2*), 1853-1859.

Larsson A, Eriksson L.A, Andersson P.L, Ivarson P, and Olsson P. **2006**. Identification of the brominated flame retardant 1,2-dibromoethyl)cyclohexane as an androgen agonist. *Journal of Medical Chemistry*, 49, 7366-7372.

Law K, Palace V, Halldorson T, Danell R, Wautier K, Evans B, Alaee M, Marvin C and Tomy G. **2006**. Dietary accumulation of hexabromocyclododecane diastereoisomers in juvenile rainbow trout (*Oncorhynchus mykiss*) I: Bioaccumulation parameters and evidence of bioisomerization. *Environmental Toxicology and Chemistry*, 25 (*7*), 1757-1761.

Miller, D. H.; Ankley, G. T. **2004**. Modeling impacts on populations: fathead minnow (*Pimephales promelas*) exposure to the endocrine disruptor 17β-trenbolone as a case study. *Ecotoxicology and Environmental Safety 59*, 1-9.

Nyholm J, Lundberg C and Andersson P. **2010**. Biodegradation kinetics of selected brominated flame retardants in aerobic and anaerobic soil. *Environmental Pollution*, 1-6.

Nyholm J, Norman A, Norrgren L, Haglund P. and Andersson P. L. **2009**. Uptake and biotransformation of structurally diverse brominated flame retardants in zebrafish (*Danio rerio*) after dietary exposure. *Environmental Toxicology and Chemistry*, 28, 1035-1042.

Nyholm J, Norman A, Norrgren L, Haglund P. and Andersson P. L. **2008**. Maternal transfer of brominated flame retardants in zebrafish (Danio rerio). *Chemosphere*, 73, 203-208.

Olivera, R. F.; Hirschenhauser, K.; Carnerio, L. A.; Canario, V. M. **2002**. Social modulation of androgen levels in male teleost fish. *Comparative Biochemistry and Physiology Part B 132*, 203-215.

Olsson, P. E.; Karlsson, J.; Larsson, A.; Modig, C.; Mayer, I. **2008.** Molecular markers of androgen disruption. In Fish Reproduction, Rocha, F. R. P.; Arukwe, A.; Kapoor, B. G., Eds.; Science Publishers: New Hampshire, USA, pp 515-535.

Pankurst, N. W. **2008.** Gonadal steroids: functions and patterns of change. In Fish reproduction, Rosha, J. M., Arukwe, A., Kapoor, B. G.,; Science Publishers: New Hampshire, USA, 67-111.

Parks, L. G.; Lanbright, C. S.; Orlando, E. F.; Guillette Jr, L. J.; Ankley, G. T. **2001**. Masculinization of female mosquitofish in Kraft mill-effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicological Sciences 62*, 257-267.

Pottinger T. G. **1988**. Seasonal Variation in Specific Plasma- and Target-Tissue Binding of Androgens, Relative to Plasma Steroid Levels, in the Brown Trout, *Salmo trutta* L. *General and Comparitive Endocrinology* 70, 334-344.

Skoog D, West D. and Holler F. **1988**. Fundamentals of analytical chemistry, 5th edition. Publishers: Saunders College. New York, Chicago, San Fransico, Philidelphia, Lodon, Sydney, Tokyo. ISBN: 0-03-14828-6.

Society of Enivronmental Toxicology and Chemistry 2000. SETAC Endocrine disruptors and Modulators. http://www.steac.org/node/100/ (Accessed June 2010).

Tomy G.T, Pleskach K, Arsenault G, Potter D, McCrindle R, Marvine C.H, Sverko E. and Tittlemier S. **2008a**. Identifications of the novel cycloaliphatic brominated flame retardant, 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane in Canadian arctic beluga (*Delphinapterus leucas*). *Environmental Science and Technology*. 42, 543-549.

Tomy G, Thomas C, Zidane T, Murison K, Pleskach K, Hare J, Arsenault G, Marvin C and Sverko E. **2008b.** Bioaccumulation parameters and potential in vivo hepatic metabolites of *syn*- and *anti*-Dechlorane plus isomers in juvenile rainbow trout (*Oncorhynchus mykiss*). *Environmental Science and Technology*, 42 (*15*), 5562-5567.

Tomy G.T, Palace V, Pleskach K, Ismail N, Oswald T, Danell R, Wautier K and Evans B. **2007**. Dietary exposure of juvenile rainbow trout (*Oncorhynchus mykiss*) to 1,2-bis(2,4,5-tribromophenoxy)ethane: bioaccumulation parameters, biochemical parameters, biochemical effects, and metabolism. *Environmental Science and Technology*, 15:41 (*14*), 4913-4918.

Tomy G.T, Palace V.P, Holldorson T, Braekevelt E, Danell R, Wautier K, Evans B, Brinkworth L and Fisk A.T. **2004**. Bioaccumulation, biotransformation and biochemical effects of brominated diphenyl ethers in juvenile lake trout (*Salvelinus namaycush*). *Environmental Science and Technology*, 38, 1496-1504.

U.S. EPA. **1985**. 1,2-Dibromo-4-(1,2-dibromoethyl)cyclohexane (TBEC) test results: Decision no to test. *Federal Register*, U.S. Government Printing Office: Washington, DC, 50 FR 19460.

U.S. EPA. **1984**. 1,2- Dibromo-4-(1,2-dibrmoethyl)cyclohexane (TBEC) test results: ITC designation- Fourteenth report. *Federal Register*; U.S. Government Printing Office: Washington, DC, 49 FR 22389.

APPENDIX A Structures of β-TBECH and circulating sex hormones

1

2

3

4

5

6

7

8

9
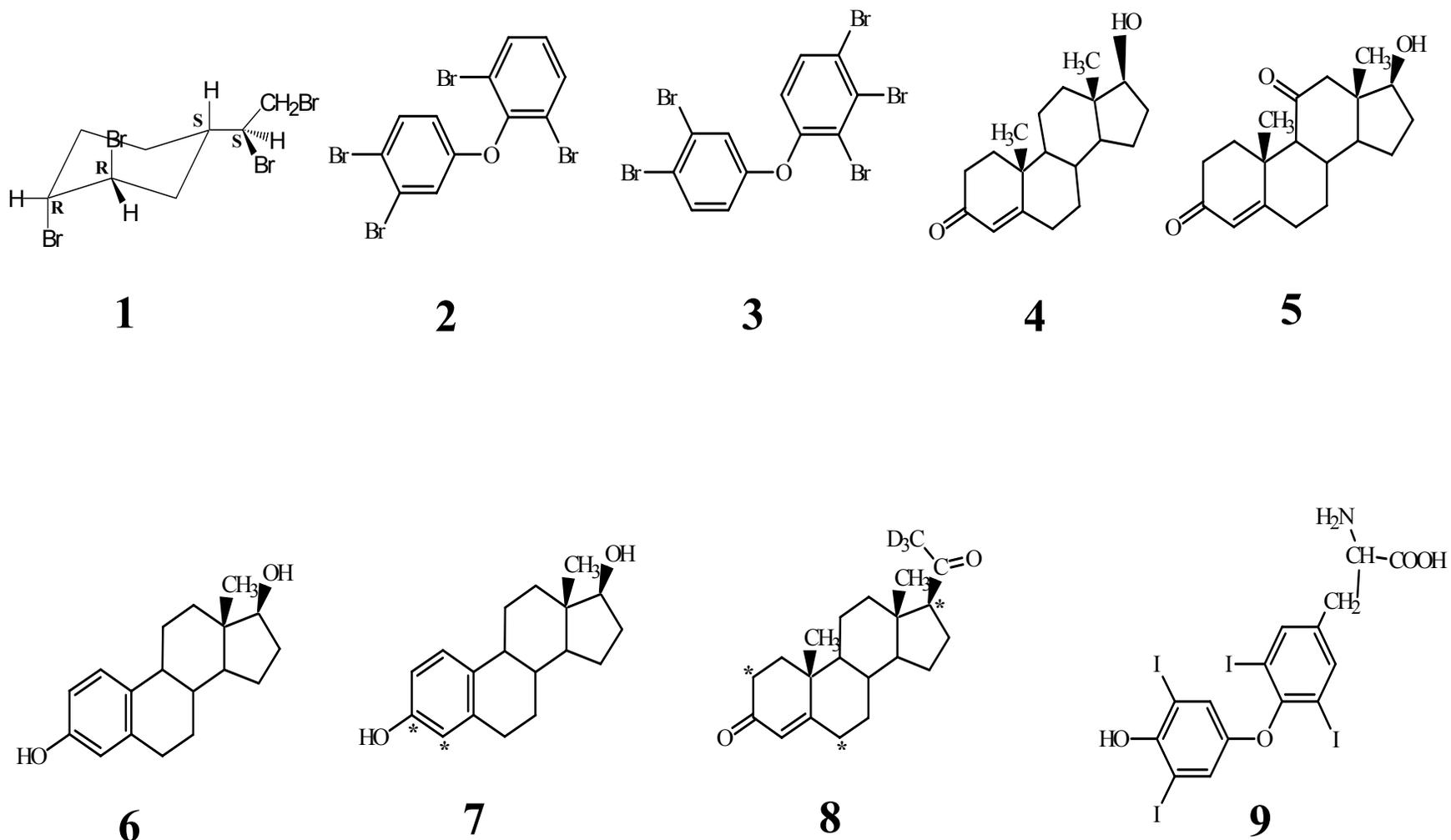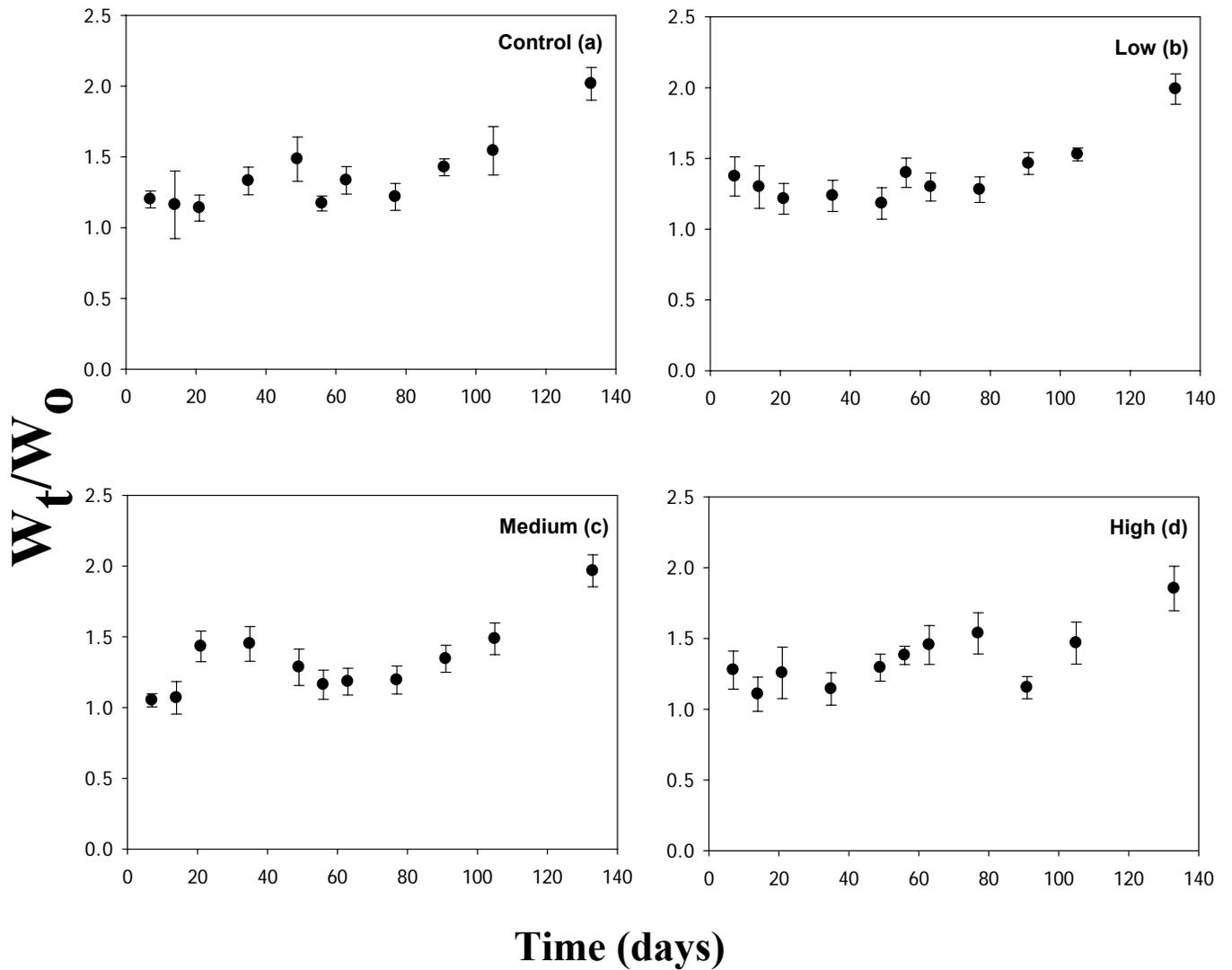
Figure B.1 Growth rates plotted against time for all four groups; control (a), low (b), medium (c) and high (d). Each point represents the arithmetic mean ± standard error (vertical bars) of eight fish from each group. W_t represents the average weight of fish at each sampling point and W_o represents the average fish weight at the start of the experiment.
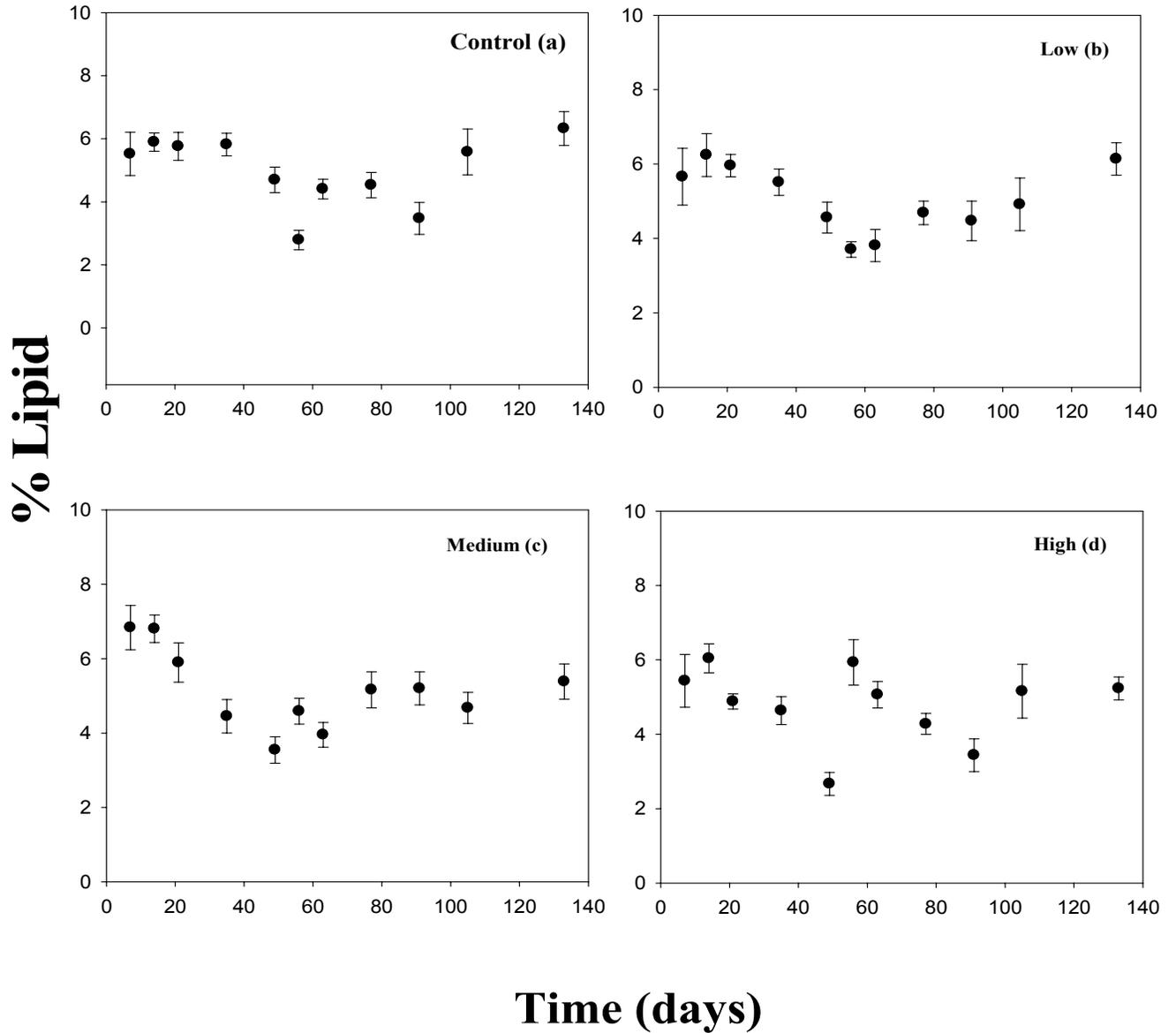
Figure C.1 Lipid percent plotted against time for all four groups; control (a), low (b), medium (c) and high (d). Each point represents the arithmetic mean ± standard error (vertical bars) of eight fish from each group.

Table D.1 Correlation matrix for control fish determined using Spearman Rank Order tests

	Length	Liver weight	Gonad weight	% Lipid	pmoles/g
Weight	0.959	0.865	0.450	0.242	0.177
	(p=<0.05)*	(p= <0.05)*	(p=<0.05)*	(p=<0.05)*	(p=0.0998)
Length		0.812	0.462	0.209	0.153
		(p=<0.05)*	(p=<0.05)*	(p=0.0511)	(p=0.156)
Liver weight			0.449	0.244	0.268
			(p=<0.05)*	(p=<0.05)*	(p=<0.05)*
Gonad weight				0.101	0.181
				(p=0.348)	(p=0.0907)
Gonad weight					-0.122
					(p=0.257)
pmoles/g					

[a]Correlations determined using individual samples. For visual clarity, statistical significant contributions (p<0.05) are presented with an asterisks

Table D.2 Correlation matrix for low fish determined using Spearman Rank Order tests.

	Length	Liver weight	Gonad weight	% Lipid	pmoles/g
Weight	0.959	0.839	0.504	0.315	-0.300
	(p=<0.05)*	(p=<0.05)*	(p=<0.05)*	(p=<0.05)*	(p=<0.05)*
Length		0.783	0.509	0.257	0.300
		(p=<0.05)*	(p=<0.05)*	(p=<0.05)*	(p=<0.05)*
Liver weight			0.500	0.191	-0.337
			(p=<0.05)*	(p=<0.05)*	(p=<0.05)*
Gonad weight				0.172	-0.251
				(p=0.109)	(p=<0.05)*
Gonad weight					0.0777
					(p=0.471)
pmoles/g					

[a]Correlations determined using individual samples. For visual clarity, statistical significant contributions (p<0.05) are presented with an asterisks.

Table D.3 Correlation matrix for medium fish determined using Spearman Rank Order tests.

	Length	Liver weight	Gonad weight	% Lipid	pmoles/g
Weight	0.956 (p=<0.05)*	0.784 (p= <0.05)*	0.375 (p=<0.05)*	-0.0929 (p=0.388)	-0.297 (p=<0.05)*
Length		0.715 (p=<0.05)*	0.355 (p=<0.05)*	-0.101 (p=0.348)	-0.282 (p=<0.05)*
Liver weight			0.251 (p=<0.05)*	0.0203 (p=0.850)	-0.323 (p=<0.05)*
Gonad weight				0.0267 (p=0.805)	-0.230 (p=<0.05)*
Gonad weight					-0.271 (p=<0.05)*
pmoles/g					

[a]Correlations determined using individual samples. For visual clarity, statistical significant contributions (p<0.05) are presented with an asterisks.

Table D.4 Correlation matrix for high fish determined using Spearman Rank order tests.

	Length	Liver weight	Gonad weight	% Lipid	pmoles/g
Weight	0.956 (p=<0.05)*	0.916 (p=<0.05)*	0.525 (p=<0.05)*	0.124 (p=0.251)	-0.213 (p=0.0533)
Length		0.860 (p=<0.05)*	0.516 (p=<0.05)*	0.130 (p=0.225)	-0.183 (p=0.0967)
Liver weight			0.454 (p=<0.05)*	0.204 (p=0.0561)	-0.215 (p=0.0507)
Gonad weight				0.106 (p=0.327)	-0.271 (p=<0.05)*
Gonad weight					-0.202 (p=0.0676)
pmoles/g					

[a]Correlations determined using individual samples. For visual clarity, statistical significant contributions (p<0.05) are presented with an asterisks

APPENDIX E

Table E.1 LSI and GSI values for uptake day 7, 14 and 21 for control, low, medium and high treatment groups.

Sample	LSI	GSI	SAMPLE	LSI	GSI	SAMPLE	LSI	GSI
UC7 #1	1.375887	0.156028	UC14 #1	1.467516	0.188535	UC21 #1	1.421652	0.188034
UC7 #2	1.520855	0.090348	UC14 #2	1.524816	0.181383	UC21 #2	1.875584	0.116139
UC7 #3	1.716594	0.168294	UC14 #3	1.271374	0.172153	UC21 #3	1.458111	0.249414
UC7 #4	1.554717	0.196226	UC14 #4	1.395349	0.140195	UC21 #4	1.340881	0.177329
UC7 #5	1.482014	0.115108	UC14 #5	1.553383	0.044157	UC21 #5	1.547919	0.136276
UC7 #6	1.268499	0.063425	UC14 #6	1.662469	0.173524	UC21 #6	1.352634	0.022148
UC7 #7	1.111111	0.15873	UC14 #7	1.288917	1.582261	UC21 #7	1.506159	0.095185
UC7 #8	1.659278	0.075997	UC14 #8	1.446945	0.14791	UC21 #8	1.741032	0.179353
UL7 #1	2.061097	0.220832	UL714#1	1.378303	0.125566	UL21#1	1.15216	0.108015
UL7 #2	1.299886	0.13683	UL714#2	1.554857	0.127533	UL21#2	1.177232	0.318564
UL7 #3	1.448276	0.17931	UL714#3	1.233787	0.13406	UL21#3	1.274991	0.082427
UL7 #4	1.171494	0.178978	UL714#4	0.981932	0.13433	UL21#4	1.394892	0.165357
UL7 #5	1.665631	0.174021	UL714#5	1.276165	0.178933	UL21#5	1.437084	0.152439
UL7 #6	1.1927	0.114959	UL714#6	1.102176	0.043756	UL21#6	1.095348	0.097328
UL7 #7	1.085434	0.122549	UL714#7	1.43617	0.161012	UL21#7	1.293843	0.134451
UL7 #8	1.361335	0.166016	UL714#8	1.47754	0.089216	UL21#8	1.417805	0.467263
UM7 #1	1.488227	0.022212	UM14 #1	1.708683	0.142057	UM21 #1	1.104207	0.088026
UM7 #2	1.393019	0.125215	UM14 #2	1.125609	0.207515	UM21 #2	1.515483	0.227687
UM7 #3	1.513307	0.208732	UM14 #3	1.829116	0.977199	UM21 #3	1.687793	0.09369
UM7 #4	1.305658	0.150653	UM14 #4	1.535497	0.194726	UM21 #4	1.425496	0.191501
UM7 #5	1.912461	0.039432	UM14 #5	1.339156	0.205604	UM21 #5	1.161059	0.14419
UM7 #6	1.117219	0.141869	UM14 #6	1.450864	1.129739	UM21 #6	1.520881	0.100296
UM7 #7	1.552393	0.226391	UM14 #7	2.180434	0.145362	UM21 #7	1.688596	0.159959
UM7 #8	1.289271	0.118554	UM14 #8	1.899301	0.111402	UM21 #8	1.23933	0.216099
UH7 #1	1.4216	0.127307	UH14#1	1.535563	0.029832	UH21#1	1.598191	0.162081
UH7 #2	1.79971	0.130624	UH14#2	1.320628	0.229499	UH21#2	1.474739	0.17217
UH7 #3	1.709402	0.22577	UH14#3	1.456255	0.182388	UH21#3	1.392993	0.151963
UH7 #4	1.413018	0.16215	UH14#4	1.572082	0.045739	UH21#4	1.583871	0.164712
UH7 #5	1.535608	0.061837	UH14#5	1.274092	0.077976	UH21#5	1.455674	0.138298
UH7 #6	1.486129	0.858653	UH14#6	1.57314	0.130502	UH21#6	0.900238	4.21127
UH7 #7	1.328761	0.996571	UH14#7	1.424419	0.225291	UH21#7	1.323053	0.05711
UH7 #8	1.307555	0.07823	UH14#8	1.401822	0.113866	UH21#8	1.413915	0.102597

Table E.2 LSI and GSI values for uptake day 35, 49 and 56 for control, low, medium and high treatment groups.

SAMPLE	LSI	GSI	SAMPLE	LSI	GSI	SAMPLE	LSI	GSI
UC35 #1	1.263867	0.158479	UC49 #1	1.080836	0.078754	UC56 #1	1.524593	0.107544
UC35 #2	1.604971	0.066818	UC49 #2	1.408205	2.12894	UC56 #2	1.316746	0.091866
UC35 #3	1.15235	0.084454	UC49 #3	1.56825	0.553059	UC56 #3	1.370857	0.523144
UC35 #4	1.127758	0.128416	UC49 #4	1.421986	0.140662	UC56 #4	1.534019	0.253557
UC35 #5	1.434385	0.130795	UC49 #5	1.521863	0.097171	UC56 #5	1.425393	0.200222
UC35 #6	1.318669	0.101773	UC49 #6	1.6706	0.109575	UC56 #6	1.611687	0.164266
UC35 #7	1.26162	0.095175	UC49 #7	1.412088	0.141026	UC56 #7	1.680237	0.083272
UC35 #8	1.191235	0.073895	UC49 #8	1.699222	0.328436	UC56 #8	1.360607	0.127073
UL35#1	1.257143	0.097297	UL49#1	1.247612	0.054452	UL56#1	1.3979	0.082847
UL35#2	1.40664	0.085291	UL49#2	0.933808	0.197865	UL56#2	1.400356	0.182655
UL35#3	1.644514	0.195747	UL49#3	1.164371	0.167126	UL56#3	1.161431	0.149599
UL35#4	1.638458	0.178655	UL49#4	1.466444	0.059325	UL56#4	1.200199	0.162941
UL35#5	0.783352	0.183579	UL49#5	1.404097	0.134228	UL56#5	1.317622	0.121895
UL35#6	1.145477	0.078427	UL49#6	1.840278	0.13696	UL56#6	1.224232	0.225775
UL35#7	1.090251	0.136066	UL49#7	1.20505	0.126259	UL56#7	1.348579	0.217995
UL35#8	1.201708	0.063034	UL49#8	1.020128	0.254651	UL56#8	1.240876	0.071796
UM35#1	1.372998	0.085812	UM49#1	1.590093	0.069993	UM56#1	1.579494	0.288501
UM35#2	1.306509	0.054373	UM49#2	1.59798	0.084704	UM56#2	1.571816	0.094851
UM35#3	1.414048	0.097043	UM49#3	1.582235	0.205588	UM56#3	1.468467	0.201013
UM35#4	1.468545	0.263941	UM49#4	1.531414	0.12902	UM56#4	1.197428	0.109489
UM35#5	1.186387	0.079517	UM49#5	1.343009	0.214236	UM56#5	1.252018	0.061542
UM35#6	1.444995	0.224301	UM49#6	1.231834	0.176965	UM56#6	2.171632	0.170955
UM35#7	1.192402	0.930655	UM49#7	1.541916	0.125998	UM56#7	1.195274	0.219597
UM35#8	1.353941	0.084407	UM49#8	1.319326	0.124603	UM56#8	1.65496	0.051396
UH35#1	1.490295	0.194093	UH49#1	1.285971	0.19964	UH56#1	1.468357	0.240009
UH35#2	1.416099	0.141994	UH49#2	1.210443	0.187896	UH56#2	1.339431	0.196194
UH35#3	1.31189	0.227683	UH49#3	1.203263	0.140041	UH56#3	1.592629	0.100073
UH35#4	1.582174	2.310453	UH49#4	1.319314	0.145677	UH56#4	1.53272	0.093734
UH35#5	1.800214	0.065883	UH49#5	1.403195	0.187172	UH56#5	1.323654	0.166338
UH35#6	1.938484	0.132332	UH49#6	1.700485	0.048522	UH56#6	1.068426	0.177209
UH35#7	1.26814	0.200938	UH49#7	1.580059	0.049289	UH56#7	1.345255	0.115708
UH35#8	1.272517	0.084284	UH49#8	1.277174	0.183424	UH56#8	1.622074	0.209673

Table E.3 LSI and GSI values for depuration day 63, 77 and 91 for control, low, medium and high treatment groups.

SAMPLE	LSI	GSI	SAMPLE	LSI	GSI	SAMPLE	LSI	GSI
DC63 #1	1.579007	0.198796	DC77 #1	1.37027	0.264655	DC91 #1	1.457516	0.222764
DC63 #2	1.117433	0.221274	DC77 #2	1.408988	0.065008	DC91 #2	1.623013	0.077214
DC63 #3	1.398271	0.044957	DC77 #3	1.489008	0.304219	DC91 #3	1.363794	0.373832
DC63 #4	1.328721	0.321943	DC77 #4	1.251891	0.076904	DC91 #4	1.597299	0.217751
DC63 #5	1.48847	0.124174	DC77 #5	1.238338	0.028273	DC91 #5	1.852165	0.083474
DC63 #6	1.416021	0.088889	DC77 #6	1.215839	0.399068	DC91 #6	1.676042	0.416847
DC63 #7	1.311943	0.165775	DC77 #7	1.464613	0.239012	DC91 #7	1.434202	0.082645
DC63 #8	1.453194	0.110689	DC77 #8	2.042477	0.301707	DC91 #8	1.270047	0.029747
DL63 #1	1.526459	0.147897	DL77 #1	1.509394	0.168883	DL91 #1	1.293416	0.311042
DL63 #2	1.537135	0.197849	DL77 #2	1.065672	0.044503	DL91 #2	1.192162	0.296394
DL63 #3	1.489209	0.179856	DL77 #3	1.523728	0.275711	DL91 #3	1.501832	0.250305
DL63 #4	1.295846	0.087932	DL77 #4	1.516003	0.206089	DL91 #4	1.417207	0.074654
DL63 #5	1.458589	0.118098	DL77 #5	1.520925	0.307928	DL91 #5	1.448996	0.265517
DL63 #6	1.321457	0.263228	DL77 #6	1.452347	0.260637	DL91 #6	1.997165	0.085041
DL63 #7	1.441313	0.153903	DL77 #7	1.284258	0.177413	DL91 #7	1.12449	0.43892
DL63 #8	1.486486	0.225225	DL77 #8	1.082463	0.371422	DL91 #8	1.27152	0.277891
DM63 #1	1.363636	0.087041	DM77 #1	1.82937	0.233625	DM91 #1	1.53636	0.107975
DM63 #2	1.258015	0.254256	DM77 #2	1.878347	0.131982	DM91 #2	1.436861	0.228334
DM63 #3	1.506102	0.109063	DM77 #3	1.51653	0.204653	DM91 #3	1.257391	0.097228
DM63 #4	1.1785	0.177955	DM77 #4	1.320599	0.261097	DM91 #4	1.592132	0.248963
DM63 #5	1.721571	0.171048	DM77 #5	1.19671	0.238335	DM91 #5	1.785924	0.264654
DM63 #6	0.348677	0.171149	DM77 #6	1.830544	0.285415	DM91 #6	1.626378	0.432218
DM63 #7	1.290645	0.256795	DM77 #7	1.339461	0.083652	DM91 #7	4.168682	0.054046
DM63 #8	1.565775	0.245581	DM77 #8	1.546892	0.384295	DM91 #8	1.767415	0.169833
DH63 #1	1.47167	0.042678	DH77 #1	1.46906	0.331601	DH91 #1	1.327326	0.039854
DH63 #2	1.285456	0.066385	DH77 #2	1.216652	0.313185	DH91 #2	1.313753	0.041675
DH63 #3	1.668601	0.375798	DH77 #3	0.998778	0.178104	DH91 #3	1.346477	0.35934
DH63 #4	1.491384	0.130719	DH77 #4	1.382953	0.29354	DH91 #4	1.58552	0.409939
DH63 #5	1.548221	0.209386	DH77 #5	1.329389	0.166313	DH91 #5	1.30369	0.381125
DH63 #6	1.497553	0.189178	DH77 #6	1.3939	0.13529	DH91 #6	1.324449	0.065811
DH63 #7	1.287251	0.259214	DH77 #7	1.503842	0.245152	DH91 #7	1.230564	0.273212
DH63 #8	1.431695	0.138473	DH77 #8	1.742762	0.125873	DH91 #8	1.465043	0.119141

Table E.4 LSI and GSI values for depuration day 105 and 133 for control, low, medium and high treatment groups.

SAMPLE	LSI	GSI	SAMPLE	LSI	GSI
DC105 #1	1.408394	0.519236	DC133 #1	1.373626	0.515222
DC105 #2	1.609307	0.115528	DC133 #2	1.514649	0.245439
DC105 #3	1.678204	0.067128	DC133 #3	12.39789	0.082722
DC105 #4	1.231102	0.101242	DC133 #4	1.609803	0.745584
DC105 #5	1.230518	0.195471	DC133 #5	1.590471	0.163203
DC105 #6	1.248864	0.426047	DC133 #6	1.47276	0.228914
DC105 #7	2.177611	0.108881	DC133 #7	1.853463	0.43363
DC105 #8	1.579567	0.335452	DC133 #8	1.516785	0.09078
DL105 #1	1.606591	0.640577	DL133 #1	1.931691	0.882176
DL105 #2	1.704718	0.086975	DL133 #2	1.572215	0.574176
DL105 #3	1.698637	0.123055	DL133 #3	1.381982	0.034987
DL105 #4	1.302644	0.408913	DL133 #4	1.954397	0.665766
DL105 #5	1.537267	0.459369	DL133 #5	1.402211	0.242347
DL105 #6	1.482373	0.368187	DL133 #6	1.5931	0.200343
DL105 #7	1.619881	0.221169	DL133 #7	1.807062	0.32541
DL105 #8	1.385787	0.18521	DL133 #8	1.380625	0.643593
DM105 #1	1.633394	0.190563	DM133 #1	1.581686	0.098277
DM105 #2	1.86826	0.103937	DM133 #2	1.533954	0.249736
DM105 #3	1.644936	0.059265	DM133 #3	1.890153	0.152821
DM105 #4	1.385234	0.319201	DM133 #4	1.61457	0.686997
DM105 #5	1.329229	0.254218	DM133 #5	1.513359	0.364802
DM105 #6	1.646325	0.289201	DM133 #6	1.601629	0.028414
DM105 #7	1.656655	0.059197	DM133 #7	2.112569	0.525603
DM105 #8	1.6581	0.205597	DM133 #8	1.533194	0.168347
DH105 #1	1.442357	0.448959	DH133 #1	2.044139	0.177897
DH105 #2	1.2939	0.312811	DH133 #2	1.738913	0.119856
DH105 #3	1.226487	0.2141	DH133 #3	1.593476	0.217482
DH105 #4	1.262774	0.787451	DH133 #4	1.473299	0.291149
DH105 #5	1.422644	0.195436	DH133 #5	1.723254	0.285157
DH105 #6	1.269785	0.06758	DH133 #6	1.746589	0.077247
DH105#7	1.289994	0.068354	DH133 #7	1.43339	0.28457
DH105 #8	1.348364	0.496	DH133 #8	1.496002	0.215803

APPENDIX F

Table F.1 Significant values (p-value, Student *t*-test) for the comparison of the amount of estrogen between treatment groups in male fish at each sampling time.

Day	p-value		
	Low/Medium	Low/High	Medium/High
7	0.086 (3,4)[a]	0.548 (3,6)[a]	0.352 (4,6)[a]
14	0.809 (3,3)	0.757 (3,3)	0.931 (3,3)
21	**0.025 (5,4)**	0.393 (5,3)	0.573 (4,3)
35	**0.020 (4,6)**	0.163 (4,5)	0.303 (6,5)
49	0.180 (4,3)	0.533 (4,2)	0.119 (3,2)
56	N/A	N/A	N/A
63	1.000 (2,2)	0.421 (4,2)	0.252 (2,5)
77	N/A	N/A	0.339 (2,3)
91	0.200 (2,3)	0.095 (2,5)	0.193 (3,5)
105	0.327 (2,3)	0.473 (2,3)	0.202 (3,2)
133	0.056 (2,4)	0.169 (2,3)	0.901 (4,3)

[a]values in parenthesis are number of male fish in respective treatment groups that are being compared. Values in bold are statistically significant values (p value<0.05).

Table F.2 Significant values (p-value, Student t-test) for the comparison of the amount of estrogen between treatment groups in female fish at each sampling time.

Day	p-value		
	Low/Medium	Low/High	Medium/High
7	0.395 (5,4)[a]	0.322 (5,2)[a]	0.505 (4,2)[a]
14	0.757 (5,5)	0.981 (5,5)	0.779 (5,5)
21	0.426 (3,4)	0.303 (3,5)	0.508 (4,5)
35	0.308 (4,2)	0.857 (4,3)	1.000 (2,3)
49	0.721 (4,5)	0.115 (4,6)	0.118 (5,6)
56	N/A	N/A	N/A
63	0.611 (6,6)	0.265 (6,3)	0.337 (6,3)
77	0.234 (7,6)	0.455 (7,5)	0.246 (6,5)
91	0.341 (6,5)	**0.035 (6,4)**	0.070 (5,4)
105	0.175 (6,5)	0.818 (6,6)	0.247 (5,6)
133	0.164 (6,4)	**0.015 (6,5)**	0.063 (4,5)

[a]values in parenthesis are number of female fish in respective treatment groups that are being compared. Values in bold are statistically significant values (p value<0.05).

Table F.3 Significant values (p-value, Student t-test) for the comparison of the amount of testosterone between treatment groups in male fish at each sampling time.

Day	p-value		
	Low/Medium	Low/High	Medium/High
7	1.000 (3,4)[a]	0.415 (3,6)[a]	0.914 (4,6)[a]
14	0.653 (3,3)	**0.005 (3,3)**	0.284 (3,3)
21	0.286 (5,4)	0.376 (5,3)	0.348 (4,3)
35	0.257 (4,6)	0.295 (4,5)	0.291 (6,5)
49	0.141 (4,3)	**0.038 (4,2)**	0.102 (3,2)
56	N/A	N/A	N/A
63	0.333 (2,2)	0.857 (2,5)	0.544 (2,5)
77	N/A	N/A	0.249 (2,3)
91	0.746 (2,3)	0.533 (2,4)	0.229 (3,4)
105	0.691 (2,3)	0.333 (2,2)	0.831 (3,2)
133	0.038 (2,4)	**0.015 (2,3)**	0.324 (4,3)

[a]values in parenthesis are number of male fish in respective treatment groups that are being compared. Values in bold are statistically significant values (p value<0.05).

Table F.4 Significant values (p-value, Student *t*-test) for the comparison of the amount of testosterone between treatment groups in female fish at each sampling time.

Day	p-value		
	Low/Medium	Low/High	Medium/High
7	0.877 (5,4)[a]	0.785 (5,2)[a]	0.754 (4,2)[a]
14	0.287 (5,5)	0.096 (5,5)	0.548 (5,5)
21	0.720 (3,4)	0.734 (3,5)	0.119 (4,5)
35	0.895 (4,2)	**<0.001(4,2)**	0.333 (2,2)
49	0.112 (4,5)	0.113 (4,2)	0.874 (5,6)
56	N/A	N/A	N/A
63	0.451 (6,6)	0.154 (6,3)	0.067 (6,3)
77	0.181 (7,6)	0.639 (7,5)	0.789 (6,5)
91	0.940 (6,5)	0.605 (6,4)	0.424 (5,4)
105	0.315 (6,5)	0.937 (6,6)	0.537 (5,6)
133	**0.010 (6,4)**	**0.004 (6,5)**	**0.018 (4,5)**

[a]values in parenthesis are number of female fish in respective treatment groups that are being compared. Values in bold are statistically significant values (p value<0.05).

Table F.5 Significant values (p-value, Student t-test) for the comparison of the amount of 11-ketotestosterone between treatment groups in male fish at each sampling time.

Day	p-value		
	Low/Medium	Low/High	Medium/High
7	0.857 (3,4)[a]	0.400 (3,4)[a]	0.914 (4,6)[a]
14	0.525 (3,3)	0.310 (5,5)	0.277 (3,3)
21	**0.016 (5,4)**	0.437 (2,4)	0.400 (4,3)
35	0.352 (4,6)	1.000 (4,5)	1.000 (6,5)
49	0.248 (4,3)	0.225 (4,2)	0.295 (3,2)
56	N/A	N/A	N/A
63	0.333 (2,2)	0.469 (2,5)	0.396 (2,5)
77	N/A	N/A	0.200 (2,3)
91	0.800 (2,3)	1.000 (2,4)	0.629 (3,4)
105	**0.046 (2,3)**	0.333 (2,2)	0.627 (3,2)
133	0.054 (2,4)	**0.031 (2,3)**	0.306 (4,3)

[a]values in parenthesis are number of male fish in respective treatment groups that are being compared. Values in bold are statistically significant values (p value<0.05).

Table F.6 Significant values (p-value, Student t-test) for the comparison of the amount of 11-ketotestosterone between treatment groups in female fish at each sampling time.

Day	p-value		
	Low/Medium	Low/High	Medium/High
7	0.142 (5,4)[a]	0.213 (5,2)[a]	0.316 (4,2)[a]
14	1.000 (5,5)	0.548 (5,5)	1.000 (5,5)
21	0.061 (3,4)	**0.042 (3,5)**	0.979 (4,5)
35	0.969 (4,2)	0.707 (4,2)	1.000 (2,2)
49	0.493 (4,5)	0.257 (4,6)	0.329 (5,6)
56	N/A	N/A	N/A
63	0.818 (6,6)	0.431 (6,3)	0.905 (6,3)
77	0.445 (7,6)	0.639 (7,5)	0.792 (5,5)
91	0.495 (6,5)	0.693 (6,4)	0.425 (5,4)
105	0.537 (6,5)	0.589 (6,6)	0.792 (5,6)
133	**0.038 (6,4)**	0.082 (6,5)	0.063 (4,5)

[a]values in parenthesis are number of female fish in respective treatment groups that are being compared. Values in bold are statistically significant values (p value<0.05).